Enzyme and Metabolic Inhibitors

Volume II

Malonate, Analogs, Dehydroacetate,
Sulfhydryl Reagents, o-Iodosobenzoate, Mercurials

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Malonate, Analogs, Dehydroacetate, Sulfhydryl Reagents, o-Iodosobenzoate, Mercurials

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This volume is dedicated with sincere gratitude to the medical librarians — Vilma, Ruth, Lilian, Clara, Esther, Michelle, Rose, Shari, Nancy, Nahida, and others — who have not only helped me to the limit, but have made each visit to the library a pleasure and often the most delightful experience of the day.

PREFACE

For those rare readers who may feel inclined to pursue their way through Volumes II and III from beginning to end, I have tried to arrange the chapters and sections in a logical and interdependent order. Malonate has been approached first because its actions so well illustrate some of the general principles covered in Volume I, and, indeed, malonate is discussed in greater detail than any other inhibitor in order to suggest how one would like to deal with all inhibitors if one had either the time or space. Inasmuch as malonate is the classic substrate analog, the next chapter takes up various types of analogs and here we are able to obtain some rough idea of the energies involved in the interactions of inhibitors with enzyme surfaces, as well as study some of the factors which determine specificity. Some readers may feel that too much attention has been given to these analogs, but I believe they represent a very important group of inhibitors and illustrate many principles — competitive behavior, group specific interactions, protection and reversal, and even mutual depletion kinetics since some analogs are extremely potent inhibitors — and, in addition, contribute to our understanding of feedback inhibition and metabolic regulation. Most of the remainder of the volumes is devoted to substances considered to react with SH groups, certainly one of the most commonly used and important classes of inhibitors, about which it is surprisingly difficult to find adequate and comprehensive treatment. Certain aspects of inhibition have been treated in detail, not necessarily because of any intrinsic importance, but because of the information which is provided to help us comprehend the general phenomena of inhibition. There are many ways of writing about inhibitors, and I have tried to alter the approach according to what I believe to be the most interesting aspects of each inhibitor. These aspects may not happen to be those which would have been chosen by the reader, but it is impossible to cover any inhibitor completely and present it from all viewpoints. On the other hand, there are certain sections which I have been unable to make very interesting, to organize into a coherent picture, sometimes because the data are insufficient or too heterogeneous, but nevertheless some worthwhile material

can often be included in these areas. One finds much of the subject to be somewhat disconnected and it is seldom possible to present an orderly clear version of any inhibition because of the gaps in our knowledge, but one must consider that these isolated strands may some day be woven into a durable fabric. Each chapter has in general been organized so that the treatment proceeds from the simplest system to the higher levels of organization, since this generates progressive understanding it may be hoped, although one occasionally wishes that the effects on the simpler systems could be appreciated against a background of the actions on tissues and animals. Perhaps to some extent the historical introductions at the beginning of most chapters may serve as provisional backgrounds. As in many fields of science one is confronted with the problem of vertical or horizontal presentations. Efforts, however inadequate, have been made to correlate the results at the different levels, and it is hoped that unlike bacteria under certain conditions this volume does not too much exhibit the phenomenon of accumulation without synthesis, or suffer from an even worse danger, that in the psychosynthesis of concepts and over-all pictures some abnormal or spurious units have been lethally incorporated.

This periplus of the field of enzyme inhibition presents a rather large and often heterogeneous group of information, but everything has been selected for some reason; the reasons may be debatable, since different readers come to a book for different purposes, but occasionally one detects something in a report, perhaps intuitively, which others would not, and hence includes it for reasons difficult to express. The half-life for the general use of a book is, indeed, determined in part by the ability or good fortune of the author to select that which will have the most value or pertinence in the pseudopodal fronts of science. One has no time for justifications, since some decisions have to be made, and only the naïve think they can please or help everyone, but there is perhaps one justification I feel impelled to make. Certainly there will be those who ask why the effects of an inhibitor on the blood pressure or the central nervous system have been presented when there is little or no obvious correlation with any metabolic inhibition, or why I have made up tables of tolerated or lethal doses, and, in general, some may criticize the discussion of inhibitor actions which are likely to be unrelated to enzyme inhibition, or at least for which there is no direct evidence. In defense of this, I can only say that I believe we should not so rigorously categorize the actions of inhibitors. The refusal to consider the nonmetabolic actions has led many investigators to very biased interpretations of their data. If we are interested in the mercurials, we are, I assume, interested in all their possible actions, whether they are

based on metabolic disturbances or not. To be narrow here would be like discussing only the beneficial effects of drugs and omitting the toxic actions. Of course, space limitations make it impossible to treat all these actions equally, and I have tried to emphasize those actions in which a disturbance of metabolism is the most likely mechanism. But we must never ignore the possibilities of other mechanisms with any inhibitor, particularly those reacting with groups on proteins and other cell components. The mercurials offer an especially clear example of enzyme inhibitors producing characteristic effects on many tissues (e.g. kidney, heart, central nervous system, liver, muscle, etc.) and where not a single action can be definitely correlated with a mechanism involving enzyme inhibition. Nevertheless, with further improvements in techniques and more knowledge, it is quite possible that in the future at least some of these actions will be related to effects on enzymes. To be perfectly honest, at the present time we cannot say in the majority of cases just how substances called enzyme inhibitors act to produce their interesting and often clinically or industrially important effects on microorganisms or tissues, and it is necessary to realize our ignorance so that progress in understanding may take place. Inhibitors do produce some very intriguing effects on tissue function or in whole animals, and many of these effects are unknown to those who look upon inhibitors merely as biochemical tools; so by reading of these effects some may be activated to study the ultimate causes in greater detail. Incidentally, since inhibitors will be used more and more frequently in animals, information on dosage ranges to produce various effects may serve a very practical purpose.

I would like to express my gratitude to those who have written to me saying they have found the first volume of interest or of some value to them, who have sent me unpublished manuscripts or difficultly obtainable material, and who have given me encouragement during those periods when I sincerely wished I were in a monastery in Kyoto.

J. Leyden Webb

November, 1965

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INTRODUCTION

Certain principles or prejudices in the approach should be clearly stated since these occasionally heretical opinions have been the basis for much of the organization of this volume.

What is hoped to be pertinent information on the physical and chemical properties of the inhibitors has been given in the belief that the proper use of any inhibitor requires as much knowledge of its properties as possible. Such data are often difficult to find and I am afraid that much important material has been omitted.

The quantitative formulation of inhibitions has been stressed because here, as in every science, progress often depends on accurate recording and reporting of observations. It is, for example, not only not informative but actually misleading to state that aldehyde oxidase is or is not inhibited by *p*-mercuribenzoate. What is meant by " inhibited " — 10%, 50%, or 100%? What is the concentration of the inhibitor? If it is 0.01 m*M* it may mean something, but if it is 10 m*M* probably nothing. What is the source of the enzyme? There are many aldehyde oxidases and they differ quite markedly according to their sources. What substrate and acceptor were used? Is the substrate acetaldehyde, glyceraldehyde, formaldehyde, retinene, or even hypoxanthine, and are electron acceptor dyes used or the O_2 uptake measured? These and other factors must be made explicit. Of course, it is impossible in a book like this to give a complete picture of each inhibition mentioned, but I have tried to state the source of the preparation, the substrate, the inhibitor concentration, and the per cent inhibition in every case, as well as the pH and the incubation times when necessary.

One can understand a phenomenon better when it can be visualized in some manner and much of the recent work on the inhibition of enzymes has been done with the purpose of clarifying the topography of the enzyme surface and the nature of the interactions occurring there. Thus I have tried to emphasize the interpretation of data in terms of an accurate delineation of group orientation and intermolecular forces, although in the present state of our knowledge this can seldom be done satisfactorily.

Metabolism within cells is almost always a matter of multienzyme systems and so the effects of inhibitors on such systems have been discussed fully wherever possible, although this is even more difficult to describe quantitatively than the behavior of single enzymes.

The importance of the specificity of inhibition was sufficiently emphasized in the previous volume and it should be clear that this is a critical problem which has been neglected, ignored, or abused extensively. It is not an easy matter to evaluate the specificity of an inhibitor under various conditions, particularly when the necessary data are lacking, but it is hoped that at least a provisional picture has been presented in some instances.

Certain aspects of metabolism (e.g. glucose utilization, respiration, photosynthesis, protein synthesis, or oxidative phosphorylation) and cellular activity (e.g. active transport, membrane potentials, movement, mitosis, or proliferation) are obviously of general significance, and the effects of inhibitors on these have been emphasized. This is not to say that other pathways or functions are unimportant, and indeed where necessary they have been treated as adequately as possible, but one cannot discuss all the actions of each inhibitor, so that some compromises must be made.

A major use of inhibitors is in the attempt to correlate cellular functions with particular enzymes or metabolic pathways, and for this reason, as well as the fact that this represents one of the most fascinating aspects of inhibitor study, these correlations have been discussed fully if the information has been available, and the effects on certain organisms or processes have often been given in the hope that some correlation will emerge or further work will be stimulated. It is believed that conceiving inhibitor actions in terms of deviations in the energy flow is of some value although an accurate formulation of this must await the development of a new terminology.

It is simpler to restrict the treatment of an inhibitor's action to a particular organism or tissue, but it is felt that a great deal may be learned from comparative inhibitor enzymology. Therefore, in the tables, the effort has been made to present the results from as many sources as possible for a particular enzyme or metabolic pathway since by doing this one is better able to see the great extent of the variability in responses; only a distorted view is obtained if a limited range of action is considered.

Paradoxical actions have been both the despair and delight of scientists in many fields, and it is recognized that some of our finest theories have originated in the observation and study of anomalies. There is an inherent desire in most of us to eliminate anomalies and perhaps devote a good deal

of effort to this, since we feel that an anomaly really is something we would expect if we knew the system or mechanism better, or as Henry Miller has said in the " Tropic of Capricorn," " confusion is a word we have invented for an order which is not understood." I have thus brought up certain so-called anomalies, not only for their interest but again because they often stimulate deeper investigation, although at present they may to some only confuse the picture.

Many of the results have been put into tabular form, first because this is the most efficient way of presenting certain types of data, second because such simple observations are often the sole information on the inhibitors provided in the reports, third because this allows a more convenient comparison of results (e.g., for those interested in possible phylogenetic relationships, for which reason the source organisms have usually been given in the classic taxonomic sequence, or for studying the variability in responses on a comparative basis), fourth because this is the clearest way to provide information from which specificity may be evaluated, and fifth because these tables may serve as reference sources for those interested in the actions of a particular inhibitor on a certain enzyme or organism. There is much more in these tables than anyone can assimilate or understand or interpret today, but it is these data which could possibly contribute to some idea or concept if placed against the proper experience or background. Nothing makes some data look more miserable or incomplete than putting them in tables, but perhaps this is an asset, since it shows what is missing, what should have been done, and what more there is to do. A great deal of information could not be included in the tables, for, although some of them look formidably long, they represent only a fraction of what is available in reports. One tries to include only that which is important, but the definition of this word becomes more difficult as one applies it. There are so many very specialized and unique enzymes being isolated and studied these days that it becomes more of a problem each year to determine which of the enzymes are generally significant. An enzyme which at first sight might seem esoteric, if for no other reason than its gargantuan name, implying a specificity of catalysis incommensurate with anything but a very limited role in metabolism, may well be of great importance in a particular pathway, a pathway perhaps as yet undiscovered. Every enzyme is of some importance to some organism or tissue, or it would not be there. And we often take a limited viewpoint; one of the numerous enzymes in the pathway of steroid biosynthesis is recognized as important in cholesterol or adrenal corticoid formation, but it may be equally important to some microorganism in producing steroids which function

in their membranes, the inhibition of the formation of which could lead to a suppression of growth. In view of the past history of science, anyone is presumptuous to claim they can distinguish what is important from what is not — we have to do this much of the time, of course, but we should realize we are presumptuous. There are probably some errors in the tables, since it is often difficult to determine exactly the conditions used; one is sometimes referred to a previous report, but cannot be certain that all the conditions have been maintained throughout the work. One must often guess a parameter from other work the investigators have done, and sometimes calculate results from heterogeneous data. There has been a good deal of calculation, and recalculation, and averaging, and I take full responsibility for anything right or wrong I may have done. A number of curves have been replotted or data represented in a way that differs from that of the original investigator, and I fully realize that this usually results in nothing but animosity.

CONVENTIONS

The naming of enzymes is not an easy task. On the one hand, there are the more trivial names with their occasional confusions — on the other, there are the official names in the " Report of the Commission on Enzymes " (1961) which are reasonably precise but often unwieldy. I have usually chosen the former because I feel most readers will recognize these more readily, but frequently I have taken an intermediate course which probably will not please anyone. It is much more accurate to write NADH:menadione oxidoreductase than to use the designation NADH oxidase or NADH dehydrogenase, since the former name indicates the substrate and acceptor used. In addition it is cumbersome to use D-xylulose-5-phosphate D-glyceraldehyde-3-phosphate-lyase (phosphate-acetylating) instead of phosphoketolase, yet there is no doubt that this longer term accurately describes the enzyme. There are also preferences in nomenclature, for various reasons. I never cared much for the term invertase; I prefer to call it β-fructofuranosidase, although it is clumsier, but not as much so as β-D-fructofuranoside fructohydrolase. In other instances the older and shorter names are more pleasing to me and I imagine to others. I have tried to use enzyme names which, at least, can be found in the index of the " Report of the Commission on Enzymes," and some cross referencing of names has been included in the index. There are certain instances of inconsistency which I do not particularly regret.

As in the first volume, concentrations have been given as millimolar (mM) except when designated otherwise, and in other matters the conventions given there have been retained.

SYMBOLS

A	absorbance
ADP	adenosinediphosphate
AMP	adenosinemonophosphate
ATP	adenosinetriphosphate
9,10-AQ	9,10-anthraquinone
BAL	dimercaprol
ChE	cholinesterase
CoA	coenzyme A
6-DFG	6-deoxy-6-fluoro-D-glucose
2-DG	2-deoxy-D-glucose
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
DQ	duroquinone
DQH_2	durohydroquinone
E_0	standard oxidation-reduction potential (pH = 0)
E_0'	oxidation-reduction potential at specified pH (usually 7)
ED_x	effective dose or concentration for x per cent
EDTA	ethylenediaminetetraacetate
EI	enzyme-inhibitor complex
EM	Embden-Meyerhof (pathway)

EP	enzyme-product complex
Epi	epinephrine
ES	enzyme-substrate complex
FAD	flavin-adenine dinucleotide
FDP	fructose-1,6-diphosphate
FMN	flavin mononucleotide
GSH	reduced glutathione
GSSG	oxidized glutathione
i	fractional inhibition
i_f	final fractional inhibition
IA	iodoacetate
IAM	iodoacetamide
IC	intracutaneous
IM	intramuscular
IMP	inosinemonophosphate
IP	intraperitoneal
ISBZ	*o*-iodosobenzoate
IV	intravenous
K_a	ionization constant
K_i	inhibitor constant
K_m	Michaelis constant
K_s	substrate constant
LD_x	lethal dose or concentration for x per cent
MD	menadione
MHD_2	menadiol
MLD	minimal lethal dose
MM	methylmercuric ion
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NEM	*N*-ethylmaleimide
1,2-NQ	1,2-naphthoquinone
1,4-NQ	1,4-naphthoquinone
pI	$-\log(I)$
p-MB	*p*-mercuribenzoate ion
p-MPS	*p*-mercuriphenylsulfonate ion
p-Q	*p*-benzoquinone
p-QH_2	*p*-benzohydroquinone (hydroquinone)
pS	$-\log(S)$
p-XQ	*p*-xyloquinone
9,10-PAQ	9,10-phenanthraquinone
3-PGDH	3-phosphoglyceraldehyde dehydrogenase
PM	phenylmercuric ion
Pyr	pyruvate
SC	subcutaneous
SH	sulfhydryl
S/M	slice/medium ratio
S—S	disulfide
TD	tolerated dose or concentration
T/M	tissue/medium ratio
TQ	toluquinone
TQH_2	toluhydroquinone
v	rate
V_m	maximal rate
p-XQ	*p*-xyloquinone
ε	molar extinction coefficient
φ-AsO	phenylarsenoxide
φ-AsO_2	phenylarsonic acid

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Volume II

Malonate, Analogs, Dehydroacetate,
Sulfhydryl Reagents, o-Iodosobenzoate, Mercurials

CHAPTER 1

MALONATE

Malonate is one of the most interesting, specific, useful, and well-known enzyme inhibitors, and for these reasons will be discussed in detail in order to illustrate some of the general principles delineated in the first volume. It will be valuable perhaps to take up one inhibitor to the degree necessary to consider many of the various problems in the application of these principles, and to examine the pitfalls that may appear even in the use of an inhibitor that in several ways approaches what one would want ideally. Much of what will be said concerning malonate may be applied to the other inhibitors. This discussion will emphasize the often overlooked fact that the effect of relatively simple inhibitors in cells may constitute a very complex problem and that their use in elucidating metabolic relationships in tissues or whole organisms should not be undertaken lightly. It is a simple matter to apply an inhibitor such as malonate but it is often very difficult to use it properly and to interpret the results accurately. The treatment of malonate will, furthermore, provide a foundation for the more general discussion of competitive inhibitions produced by analogs in the following chapter.

EARLY HISTORICAL DEVELOPMENT

The first report of the use of malonate in a biological system was made by Heymans (1889) to the Physiological Society in Berlin. The toxicity of oxalate to animals had been known for many years and Heymans believed that an investigation of the higher homologs of the dicarboxylate series might be interesting. Although sodium oxalate was quite poisonous when injected into the frog dorsal lymph sac, the sodium salts of malonate, succinate, and glutarate were essentially without effect, sodium malonate being nonlethal at a dose as high as about 8 g/kg. However, Pohl (1896), working in Prague, found that the urinary excretion of oxalacetate was increased by administering malonate to dogs and, furthermore, that only a small portion of the malonate given could be recovered in the urine, indicating that the dog can metabolize malonate. The first experiments showing the metabolic inhibitory action of malonate were done by Thun-

berg (1909) in Lund. He had observed the inhibition of minced frog muscle respiration by oxalate and decided to study the higher homologs. Thus the inhibitory action of malonate on muscle respiration was demonstrated, whereas succinate instead stimulated the oxygen uptake. Apparently this observation went unnoticed and the inhibitory activity had to be rediscovered later. Rose (1924) at Illinois showed that malonate exhibits no nephrotoxic action, as does glutarate, when given orally to rabbits, although he later (Corley and Rose, 1926) found that the methyl and ethyl derivatives depress renal function. At about the same time, Momose (1925) in Japan, continuing the work of Pohl, observed that malonate when perfused through dog liver, gives rise to acetoacetate, acetone, and aldol. He postulated that these substances arise from malonate after decarboxylation to acetate, but it is more likely from our present knowledge that malonate gives rise to these substances by a disturbance of the metabolism. During the next few years evidence was accumulated that malonate can arise from normal tissue metabolism — occurring in alfalfa (Turner and Hartman, 1925) and wheat (Nelson and Hasselbring, 1931), and appearing during citrate fermentation in the mold *Aspergillus* (Challenger *et al.*, 1927) — and be metabolized by certain microorganisms, such as *Escherichia coli* (Grey, 1924).

Our present concepts of the inhibitory action of malonate, however, arose from the work on bacterial dehydrogenations by Quastel and Whetham (1925) at Cambridge. They tested the abilities of various dicarboxylic acids to reduce methylene blue in suspensions of *E. coli* and found that only succinate is active. Malonate inhibited this reduction by succinate. As stated in their own words, "Oxalic, glutaric, and adipic acids (when mixed with succinic acid) do not retard the reduction due to the succinic acid, but malonic acid has a definite retarding effect. It is difficult to explain the anomalous behaviour of malonic acid, but there is no doubt as to the reality of the effect." They found the methylene blue reduction time with succinate to be tripled in the presence of 77 m*M* malonate. Quastel and Wooldridge (1928) extended this work to show that the action on succinate oxidation is rather specific in that malonate does not appreciably inhibit the oxidation of several other substrates by *E. coli*. But in addition they demonstrated that increasing succinate concentrations would counteract the malonate inhibition, leading them to suggest that both substances are adsorbed to the enzyme reversibly, probably competing for the same active site. Finally, Quastel and Wheatley (1931), now at the Cardiff City Mental Hospital, reported that the malonate inhibition of succinate oxidation occurs in many bacteria, and in mammalian brain and muscle as well, the enzymes from the mammalian tissue being even more sensitive.

The concept of the competitive inhibition of an enzyme by a substance structurally related to the normal substrate was first clearly demonstrated

and expressed for this inhibition of succinate oxidation by malonate in the work of Quastel and Wooldridge, although competition specifically was not mentioned. Cook (1930), also at Cambridge, however, stated that a "competitive" mechanism had been established, presumably referring to the work of Quastel inasmuch as Cook performed no experiments indicating a competitive relationship. The competitive nature of the malonate inhibition has been substantiated many times and placed on a quantitative basis, so that malonate has come to be recognized as the classical example of inhibition by a purely competitive mechanism. The development and applications of this concept will be discussed in more detail in Chapter 2. For 20 years malonate was the only available specific inhibitor of succinate dehydrogenase, and later of the tricarboxylic acid cycle, and actually played an important role in the elucidation of the cycle sequence. Other cycle inhibitors have been described recently, but no other inhibitors of the succinate oxidation step as specific and useful as malonate have been found.

CHEMICAL PROPERTIES

Malonic acid and its salts when obtained commercially are often not sufficiently pure for accurate work and it has been the practice in our laboratory to recrystallize all material. Malonic acid may be recrystallized from ethyl acetate and benzene (Adell, 1940), or ether and benzene containing 5% light petroleum (Vogel, 1929), or simply from a hot concentrated benzene solution by cooling to 5°–10°. The sodium and potassium salts may be dissolved in small amounts of warm water and precipitated by the addition of ethanol, as is commonly done with other dicarboxylate salts (Potter and Schneider, 1942), or, with somewhat less yield, may be crystallized by cooling hot concentrated aqueous solutions. In all cases we have decolorized with activated charcoal in the solutions before recrystallization and have washed the products with ether preparatory to drying. It should be emphasized that the choice of the sodium or the potassium salt will depend on whether the preparation to be tested is cellular or subcellular.

Stability

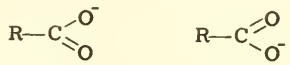
Malonic acid and its salts are quite stable and chemically unreactive. Decarboxylation to acetate proceeds very slowly under ordinary conditions. Aqueous solutions of sodium malonate heated to 125° for 48 hr show no perceptible decomposition (Fairclough, 1938), and the half-life of sodium hydrogen malonate in solutions 5–50 mM is at 80° around 40 days, calculated from the rate constant for decarboxylation (Hall, 1949). The free energy change for the reaction

$$\text{Malonate} \rightarrow \text{acetate} + CO_2$$

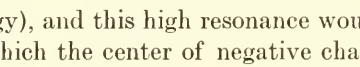
is approximately −7 kcal/mole but the activation energy is 27.9 kcal/mole (Gelles, 1956). Thus at physiological temperatures one may consider malonate as completely stable. Nevertheless, there are enzyme systems which catalyze the decarboxylation (see page 227) and one should be reasonably certain in the use of malonate that it is stable in the system investigated, since the formation of acetate might well confuse the results.

Molecular Structure

In crystals of malonic acid the molecules are arranged in zigzag chains with the carboxyl groups linked through two hydrogen bonds (Goedkoop and MacGillavry, 1957). The following bond parameters were observed (the two values refer to the two carboxyl groups, since the molecule in the crystal is apparently not symmetrical): C—C—C angle = 110°; C—C distance = 1.54, 1.52; C—O distance = 1.29, 1.31; C=O distance = 1.24, 1.22; O—C—O angle = 128°, 128°; and H-bond distance = 2.68–2.71. The malonate ion in solution would probably deviate somewhat from this configuration but not a great deal inasmuch as malonate is fixed in a rather rigid structure, because the C—C—C angle is determined by the electronic tetrahedral orbitals and can be distorted only with difficulty. The carboxylate groups can rotate around the C—C axis but they presumably sterically interfere with each other when both lie in the plane of the molecule, since the centers of the oxygen atoms would be 2.2 Å apart and the van der Waals' radius of the oxygen atom is 1.4 Å (Goedkoop and MacGillavry, 1957). In malonic acid crystals one carboxyl seems to be in the molecular plane and the other is at right angles; in the malonate ion it may well be that neither is in the C—C—C plane. However, another factor must be considered; it is possible that in solution there is intramolecular hydrogen bonding (Gelles, 1956), at least for the hydrogen malonate ion. When the carboxyl group ionizes, the equivalence of the structures:



allows a greater resonance than in the unionized state (equivalent to an extra 8 kcal/mole energy), and this high resonance would indicate an intermediate structure in which the center of negative charge lies midway between the two oxygen atoms. Although keto-enol tautomerism occurs (Höfling *et al.*, 1952) in the esters of malonic acid:



it is probably not significant in the malonate ion because it would reduce the electronic resonance.

The distance between the two centers of negative charge in malonate is of importance in the binding to succinate dehydrogenase. Calculation of this distance (using the following values: C—C—C angle = 111.7°; O—C—O angle = 125.8°; C—C distance = 1.544, and C—O distance = 1.273) leads to a value of 3.28 Å. Intercharge distances for various dicarboxylates and other compounds known to inhibit succinate dehydrogenase are given in Table 1-1, and these values will be of interest in comparisons of inhibitory activity. Dicarboxylic acids with more than one methylene group are flexible and the intercharge distances may vary between the limits of greatest bending and extension of the molecules. As the chain length increases there will be greater tendency for the intercharge distance in the ions to be less than that of the maximal extension, since the electrostatic repulsion will decrease. The mean statistical intercharge distance in the succinate ion will probably be closer to 4.75 Å than the contracted distance of 3.81 Å. Indeed, it may be calculated that it would require at least 2.3 kcal/mole to bring succinate from the extended to the contracted configuration, using the dielectric constant obtained from $D = 6d - 7$ (Eq. I-6-72)*; since the dielectric constant is probably less due to the hydrocarbon groups between the charges, this would be a minimal energy value. The mean intercharge distance in the succinate ion may be estimated as not less than 4.20 Å (Gane and Ingold, 1931; Eyring, 1932; Westheimer and Shookhoff, 1939) and possibly closer to 4.75 Å. The glutarate intercharge distance is probably around 5.2 Å. These considerations are of importance in comparing the interactions of these substances with the active center of succinate dehydrogenase. It must be remembered that the bound ions are undoubtedly held in a configuration different from the statistical mean in solution. The flexibility of the higher homologs allows them to adjust to a specified configuration, but at the expense of the energy and entropy changes necessary to bring them from their free configurations.

Acidic Ionization

The ionizations of malonic and succinic acids are important in the interactions with succinate dehydrogenase and with regard to the penetration of these substances into cells. The pK_a's for the simple dicarboxylic acids, including various derivatives of succinate and malonate, and related succinic dehydrogenase inhibitors, are given in Table 1-2. The dissociation constants change slightly with ionic strength; for malonic acid, $dpK_{a_1}/ds = -0.32$, and $dpK_{a_2}/ds = -0.98$, for ionic strengths around 0.15, where s is the

* In cross references of this type, Eq. I-6-72, the roman number indicates the volume of this treatise in which the equation, table, or figure (as the case may be) may be found, the first arabic number indicates the chapter number, and the second arabic number the equation, table, or figure number.

TABLE 1-1

INTERCHARGE DISTANCES IN DIANIONS OF INTEREST[a]

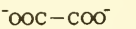
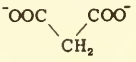
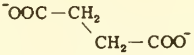
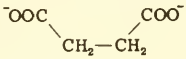
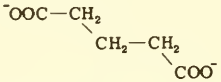
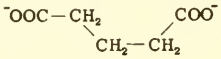
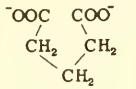
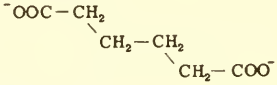
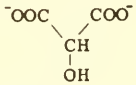
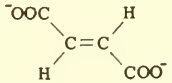
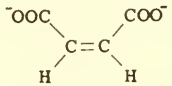
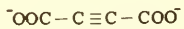
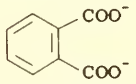
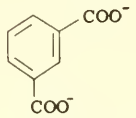
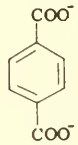
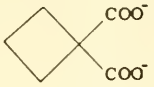
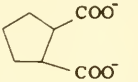
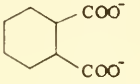
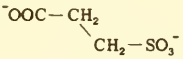
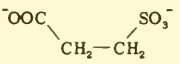
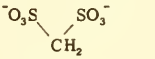
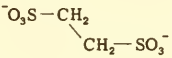
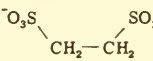
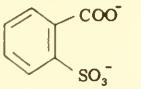
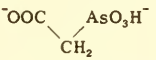
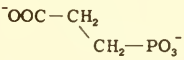
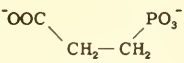
Compound		Structure	Distance (Å)
Oxalate			2.42
Malonate			3.28
Succinate	(a)		4.75
	(b)		3.01
Glutarate	(a)		5.83
	(b)		4.84
	(c)		1.70
Adipate			6.87
Hydroxymalonate (tartronate)			3.28
Fumarate			4.87
Maleate			3.66
Acetylene-dicarboxylate			5.16
o-Phthalate			3.55
Isophthalate			5.84
Terephthalate			7.33

TABLE 1-1 (*continued*)

Compound	Structure		Distance (Å)
Cyclobutane-dicarboxylate			3.39
Cyclopentane-dicarboxylate		*cis*-	3.52
		trans-	4.91
Cyclohexane-dicarboxylate		*cis*-	3.05
		trans-	4.51
β-Sulfopropionate (a)			5.05
β-Sulfopropionate (b)			3.13
Methanedisulfonate (methionate)			3.40
1,2-Ethanedisulfonate (a)			5.38
1,2-Ethanedisulfonate (b)			3.25
o-Sulfobenzoate			3.72
Arsonoacetate			4.05
β-Phosphonopropionate (a)			5.09
β-Phosphonopropionate (b)			3.14

[a] The distances were calculated on the basis of bond lengths and angles given in Tables I-6-12 and I-6-13 except where direct measurements were available. It was assumed that the center of negative charge lies midway between the resonating oxygen atoms. For *o*-phthalate and cyclohexane-dicarboxylate a 3° distortion of the bond angles due to electrostatic repulsion was assumed. For cyclopentane-dicarboxylate a further 5° widening was estimated from the bending of the ring angles. The value for cyclobutane-dicarboxylate is only approximate and is based on a 7° distortion of the bond angle in comparison to malonate. It should be pointed out that the values in this table are smaller than generally used; one reason is that intercarboxylate distances have usually been based on the distances between associating or dissociating protons, rather than between centers of negative charge.

ionic strength. Since ΔH for the dissociation of weak acids is quite small, the constants do not change much with temperature; $dpK_{a_1}/dT = 0.0031$, and $dpK_{a_2}/dT = 0.0038$, approximately. Three species will be present in any solution of malonate — HOOC—CH_2—COOH, HOOC—CH_2—COO^-, and $^-$OOC—CH_2—COO^- — the relative concentrations being determined

TABLE 1-2

IONIZATION CONSTANTS OF DICARBOXYLIC ACIDS [a]

Acid	pK_{a_1}	pK_{a_2}
Oxalic	1.09	3.79
Malonic	2.58	5.17
Succinic	3.95	5.16
Glutaric	4.07	4.93
Adipic	4.17	4.95
Pimelic	4.23	4.98
Methylmalonic	2.86	5.24
Ethylmalonic	2.79	5.34
Dimethylmalonic	2.97	5.59
Diethylmalonic	2.04	6.90
n-Propylmalonic	2.83	5.38
iso-Propylmalonic	2.78	5.46
Methylethylmalonic	2.71	6.07
Di-*n*-propylmalonic	1.92	7.13
Phenylmalonic	2.43	4.68
Hydroxymalonic (tartronic)	2.93	—
Maleic	1.67	5.75
Methylmaleic (citraconic)	2.23	5.89
Fumaric	2.85	4.00
Methylfumaric (mesaconic)	2.93	4.82
Malic	3.21	4.62
Tartaric	2.83	3.88
2,2′-Dimethylsuccinic	3.77	5.82
2,2′-Diethylsuccinic	3.34	6.22
Tetramethylsuccinic	3.33	6.90
Methylenesuccinic (itaconic)	3.67	5.19
Cyclopropane-1,1-dicarboxylic	1.77	7.37
Cyclobutane-1,1-dicarboxylic	3.08	5.82
Cyclopentane-1,1-dicarboxylic	3.18	6.02
Cyclohexane-1,1-dicarboxylic	3.40	6.05
trans-Cyclopropanedicarboxylic	3.49	4.75
cis-Cyclopropanedicarboxylic	3.16	6.09
trans-Cyclopentanedicarboxylic	—	4.22
trans-Cyclohexanedicarboxylic	—	4.45
Phthalic	2.95	5.23
Isophthalic	2.15	4.49

[a] These values have been obtained from a variety of sources and have been corrected to a temperature of 37° and an ionic strength of 0.15 so as to be applicable to physiological conditions. These corrections were obtained from studies on the variations of dicarboxylic ionization constants with temperature and ionic strength (e.g. Adell, 1940). They are not absolutely correct but probably allow a closer approximation than the values in the literature, which are usually for 25° and extrapolated to zero ionic strength.

by the pH (see Eqs. I-14-6 to I-14-8). The variations of these species with pH are shown in Fig. 1-1, and actual concentrations are given in Table 1-3 for malonate and succinate at a total concentration of 10 m*M*. In the usual range of physiological pH, over 95% of these acids are in the form of the completely dissociated doubly-charged anion; this is the active form for the inhibition of succinate dehydrogenase. However, it is the concentrations of the other forms which are important in the rates and degrees of penetration into cells, and these change appreciably with pH (e. g., from pH 7.4 to 6.8 there is a 4-fold increase in the singly dissociated form and a 16-fold increase in the undissociated form).

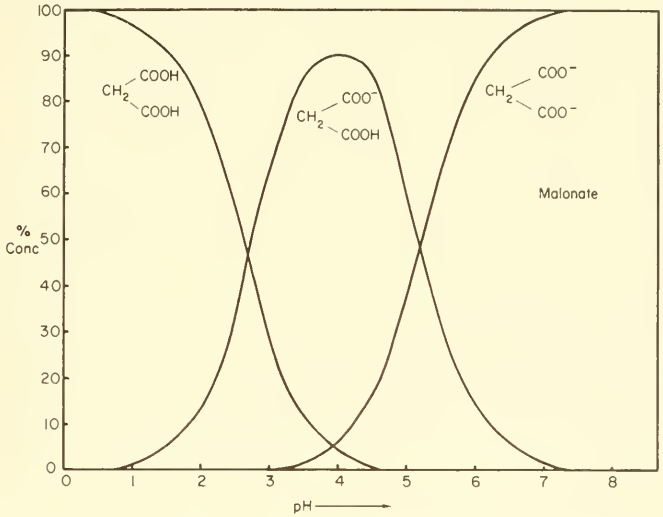


Fig. 1-1. Concentrations of the various ionic species of malonic acid at different values of the pH expressed as per cent as of the total concentration.

Das and Ives (1961) on the basis of thermodynamic evidence suggested that an internal symmetrical hydrogen bond occurs in H-malonate$^-$, and that this would affect to some extent the pK_a values and reduce hydration. However, Lloyd and Prince (1961) examined the infrared spectra of malonic acid and its ions in D_2O, compared the data with those obtained with fumaric acid (in which no hydrogen bonds could occur), and concluded that if hydrogen bonding exists in H-malonate$^-$, the bond is very weak and not symmetrical. Eberson and Wadsö (1963) determined the ionization enthalpies in water and ethanol, and also concluded that hydrogen bonding is not important in the dicarboxylates when ΔpK_a is less than 4. It would thus appear that intramolecular hydrogen bonding is not a significant factor in stabilizing the H-malonate$^-$ ion.

TABLE 1-3

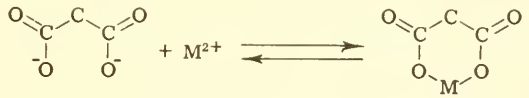
CONCENTRATIONS OF IONIZATION FORMS OF MALONIC AND SUCCINIC ACIDS AT DIFFERENT pH VALUES (WHEN THE TOTAL CONCENTRATION IS 10 m*M*)

pH	Malonic acid			Succinic acid		
	(HOOC–CH_2–COOH)	(HOOC–CH_2–COO^-)	($^-$OOC–CH_2–COO^-)	(HOOC–CH_2CH_2–COOH)	(HOOC–CH_2CH_2–COO^-)	($^-$OOC–CH_2CH_2–COO^-)
3.0	3.28	6.68	0.0384	8.98	1.01	0.0070
3.5	1.32	8.52	0.160	7.74	2.22	0.037
4.0	0.444	9.04	0.516	5.20	4.56	0.236
4.5	0.130	8.35	1.52	2.39	6.51	1.10
5.0	0.031	6.32	3.65	0.715	6.08	3.205
5.5	0.0055	3.54	6.46	0.132	3.71	6.16
6.0	0.00073	1.48	8.52	0.019	1.60	8.38
6.5	0.000081	0.520	9.48	0.0020	0.585	9.41
6.8	0.000021	0.268	9.73	0.00054	0.292	9.71
7.0	0.0000084	0.171	9.83	0.00022	0.187	9.81
7.4	0.0000013	0.069	9.93	0.000035	0.075	9.92
8.2	0.000000034	0.011	9.99	0.00000088	0.012	9.98

Metabolic studies of the substituted malonates and malonic esters will be taken up after the actions of malonate have been discussed (see page 235). It is interesting to note (Table 1-2) that the pK_{a_2}'s of the substituted malonic acids are generally higher than for malonic acid itself. This is mainly the result of the reduction of the dielectric constant of the region between the interacting carboxyl groups, and is particularly evident for the disubstituted ethyl and *n*-propyl derivatives. This increase in pK_{a_2} should facilitate penetration of these compounds into cells; in addition they are more lipid-soluble, which will also favor penetration. The esters are not active inhibitors of succinate dehydrogenase, at least by the same mechanism as malonate, but have been used because of their ability to enter cells and tissues readily, some hydrolysis to active malonate within the cells being assumed. The presence of two keto groups on either side of the methylene group makes this latter group more reactive and, indeed, imparts some acidic character to it, malonic diethyl ester having a pK_a of approximately 5×10^{-14} (Pearson and Mills, 1950). The rate of ionization is, however, quite slow ($k = 1.8 \times 10^{-3}$ min^{-1}).

Chelation with Metal Cations

Malonate is able to form fairly stable complexes with various cations normally present in media used in metabolic studies. The importance of this in malonate inhibition will be discussed later (see page 66), and in the present section we shall investigate the magnitudes of the effects expected. These complexes are chelates with a six-membered ring structure, accounting



for the relatively high stability compared to complexes with the monocarboxylates. The chelate dissociation constant is given by:

$$K = \frac{(M^{++})\,(A^{=})}{(MA)} \qquad (1\text{-}1)$$

where $A^{=}$ represents the anion of any dicarboxylic acid. The values for the pK's of some of the more important chelates are given in Table 1-4. These constants are dependent on temperature and ionic strength. The pK for Mg-malonate is related to the temperature at zero ionic strength in the following way: pK = 2.92 − 0.008 (35 − t°C) (Evans and Monk, 1952). Thus the pK's at 37° are approximately 0.1 unit higher than at 25°, the temperature at which the constants are most commonly determined. It was calculated from the data on the complexes of malonate with Mg^{++}, Ca^{++}, Ba^{++}, and Zn^{++} that the pK at an ionic strength of 0.15 is about

TABLE 1-4

DISSOCIATION CONSTANTS FOR SOME METAL COMPLEXES OF ORGANIC ACIDS [a]

Anion	pK								
	Ca^{++}	Mg^{++}	Mn^{++}	Zn^{++}	Cd^{++}	Cu^{++}	Co^{++}	Fe^{++}	Fe^{+++}
Oxalate	2.12	2.65	3.05	4.02	3.11	4.55	3.90	4.2	9.1
Malonate	1.51	2.01	2.64	2.88	2.45	4.68	3.07	3.22	4.59
Succinate	1.22	1.30	—	1.88	1.75	2.49	0.46	—	—
Glutarate	0.65	1.18	—	1.70	—	2.32	—	—	—
Adipate	1.35	—	—	—	—	2.51	—	—	—
Fumarate	0.58	—	—	—	—	1.67	—	—	—
Maleate	1.20	—	—	—	—	3.06	—	—	—
Malate	2.03	1.65	—	2.90	—	—	—	—	—
Tartrate	1.90	1.46	—	2.78	—	3.50	—	—	—
Citrate	3.28	3.20	—	—	—	13.3	—	3.6	12.2

[a] The dissociation constant is defined by $K = (M^{++})(A^{=})/(MA)$ and has been corrected as accurately as possible to a temperature of 37° and an ionic strength of 0.15 so as to be applicable to the usual physiological conditions. These constants are rather sensitive to ionic strength changes, and hence the values given must be considered as only approximations in most cases. However, they may serve as qualitative bases for considerations of the mutual effects of these ions on their concentrations in experiments on inhibition.

0.74 unit lower than at an ionic strength of zero. The free energy of formation of Mg-malonate is − 3.90 kcal/mole, while ΔH^o = 3.2 kcal/mole, and ΔS^o = 23.9 cal/degree (Evans and Monk, 1952; Chaberek and Martell, 1959, p. 139). The entropy term is large and probably results mainly from displacement of water from the charged groups.

The importance of this chelation in inhibition work lies in the reductions in the concentrations of the free ions it may bring about, both the metal ions and malonate. The decrease in metal ion concentration can easily alter enzyme activity or cellular function and such changes are apt to be attributed to a direct action of malonate. Conversely, the malonate concentration may be reduced appreciably. Examples of mutual concentration reduction are given in Table 1-5. The concentrations of Mg^{++} chosen approximate those in Tyrode solution (0.11 m*M*), Krebs-Ringer phosphate medium (1.18 m*M*), the usual media for mitochondria (5 m*M*), and sea water (53.6 m*M*), while the concentrations of Ca^{++} correspond to Krebs-Ringer phosphate medium (2.54 m*M*) and sea water (10.24 m*M*). It may be noted that very significant reductions in Mg^{++} and Ca^{++} can occur with concentrations of malonate commonly used; e.g., malonate at 10 m*M* will reduce the Mg^{++} 49% and the Ca^{++} 23% in Krebs-Ringer medium, and higher concentrations may almost deplete these ions from the solution. When the concentrations of these cations are high, as in sea water, the effective malonate concentration may be reduced markedly; e. g. malonate added to sea water at a total concentration of 10 m*M* will result in a 1.5 m*M* solution of free malonate ion. Such phenomena have usually been ignored or forgotten despite their possibly large magnitudes. One way of determining the importance of cation reduction in malonate studies is to calculate the reduction to be expected in the medium used and at the malonate concentration, and then to test the effects of lowering the cation concentration to this extent (Rice and Berman, 1961). Other metal cations may be reduced to a greater extent than Mg^{++} and Ca^{++}. Media initially 1 m*M* in Co^{++}, Mn^{++}, or Cu^{++} will in the presence of 5 m*M* malonate contain these ions at concentrations of 0.65 m*M*, 0.34 m*M*, and 0.0052 m*M*, respectively. If such metal ions are normally bound to enzymes, the degree of removal from the enzyme will, of course, depend on the relative affinities of the metal ion for the enzyme and malonate. There is also the possibility that malonate may chelate with metal ions combined with the enzyme, inactivating them for their catalytic role. It should also be remembered that similar phenomena may occur with succinate, and the inhibition kinetics may be distorted when malonate is used due to the differential reduction in the concentrations of these anions.

Detection and Determination of Malonate

Methods have been developed for the separation and identification of organic acids from animal and plant tissues. Earlier determinations involved

TABLE 1-5

MUTUAL REDUCTIONS OF THE CONCENTRATIONS OF MALONATE AND METAL CATIONS DUE TO CHELATION [a]

Added malonate (m*M*)	Final free ion concentration (m*M*)											
	Original concentration of Mg^{++}:								Original concentration of Ca^{++}:			
	0.1 m*M*		1 m*M*		5 m*M*		50 m*M*		2.54 m*M*		10 m*M*	
	(Mg^{++})	($malon^{=}$)	(Mg^{++})	($malon^{=}$)	(Mg^{++})	($malon^{=}$)	(Mg^{++})	($malon^{=}$)	(Ca^{++})	($malon^{=}$)	(Ca^{++})	($malon^{=}$)
0.1	0.0987	0.0987	0.991	0.091	4.967	0.067	49.92	0.0165	2.533	0 0927	9.975	0.075
1	0.090	0.990	0.914	0.914	4.677	0.677	49.16	0.165	2.416	0.876	9.760	0.760
5	0.065	4.970	0.677	4.677	3.642	3.642	45.88	0.877	2.207	4.667	8.883	3.883
10	0.044	9.944	0.507	9.507	2.783	7.783	41.89	1.890	1.947	9.407	7.953	7.953
20	0.030	19.93	0.336	19.34	1.836	16.84	34.42	4.422	1.572	19.03	6.516	16.52
50	0.015	49.91	0.165	49.16	0.877	45.88	23.06	23.06	0.989	48.45	4.119	44.12
100	0.007	99.91	0.090	99.09	0.465	95.46	7.26	57.26	0.600	98.06	2.500	92.50

[a] The concentrations chosen for Mg^{++} and Ca^{++} are similar to those commonly occurring in various media for both cellular and subcellular preparations.

oxidative titrations with hot acid permanganate or ceric sulfate (Willard and Young, 1930; Christensen and Ross, 1941) and such methods have been used for the analysis of malonate in the presence of proteins (Ross and Green, 1941). Colorimetric tests, for example with tetrahydroquinoline-*N*-propenal to form blue-violet compounds (sensitive to 0.01 mg malonic acid) (Dieterle and Wenzel, 1944), have been used, and several microcolorimetric and spot tests are available (Feigl, 1960). However, the most valuable methods are chromatographic. A large variety of solvents and spraying agents have been utilized, and different techniques have been applied: ion-exchange resin columns and partition chromatography (Stark *et al.*, 1951; Phares *et al.*, 1952; Owens *et al*, 1953; Shkol'nik, 1954; Reinbothe, 1957), strip paper chromatography, both one- and two-dimensional (Buch *et al.*, 1952; Cheftel *et al.*, 1952; Denison and Phares, 1952; Duperon, 1956; van Duuren, 1953; Jermstad and Jensen, 1950; Kalyankar *et al.*, 1952; Ladd and Nossal, 1954; Overell, 1952; Smith and Spriestersbach, 1954), and circular paper chromatography (Airan and Barnabas, 1953; Airan *et al.*, 1953; Barnabas, 1955). The use of chromatographic methods for the determination of succinate and malonate in animal tissues is well illustrated in the work of Busch and Potter (1952 a, b; Busch *et al.*, 1952). A moderately sensitive method for estimating 10–100 μg of malonate by applying iodine vapor to paper chromatograms was developed by Dittrich (1963).

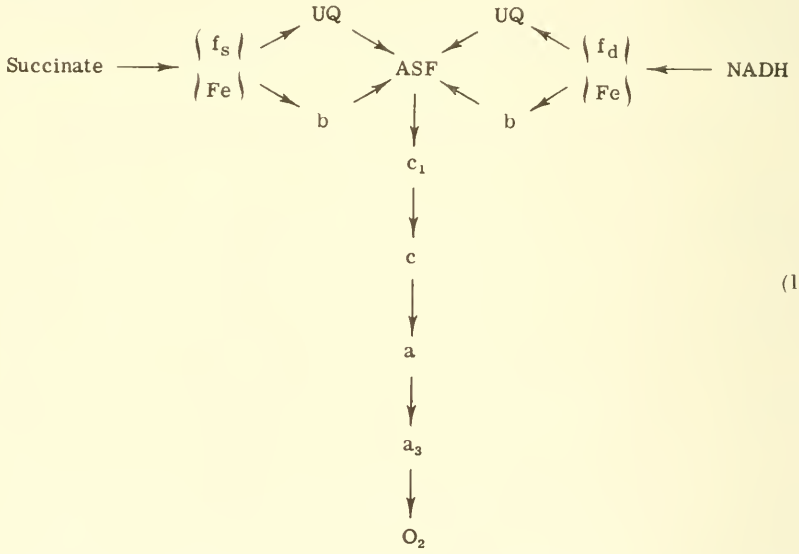
INHIBITION OF SUCCINATE DEHYDROGENASE

The oxidation of succinate in cells is catalyzed by a mitochondrial multienzyme system which is structurally organized into units that can be isolated as a particulate suspension capable of transferring electrons from succinate to oxygen: this complex is known as *succinate oxidase*. Evidence will be presented that the inhibition of this system by malonate is related to the binding of the malonate to the most proximal site in the sequence, namely, the active center for the attachment and dehydrogenation of succinate: this component is known as *succinate dehydrogenase*. It is impossible at the present time to define the succinate dehydrogenase accurately, because it is assayed with various electron-accepting dyes and the basic minimal unit has not been completely characterized. The inhibition of succinate dehydrogenase by malonate has generally been determined with various preparations of succinate oxidase and not with the isolated dehydrogenase, and thus it is necessary to discuss briefly the nature of the entire system.

Properties of Succinate Oxidase

Knowledge of the components of succinate oxidase and the pathways of electron flow has been advanced markedly in the past few years (Mahler,

1956; Singer, *et al.*, 1957; Green and Crane, 1958; Singer and Lara, 1957; Green and Fliescher 1960; Green, 1960; Redfearn, 1960). The particulate *electron transport particle* (ETP) of Green, obtained from heart mitochondria, is a succinate oxidase preparation and has been shown to contain the following components (per molecular weight of about 5×10^6): flavin dinucleotide 2, nonheme iron 64, heme (equal amounts of cytochrome a, cytochrome b, and cytochromes c + c_1) 6, copper 8, ubiquinone (coenzyme Q) 10, and lipid 34,5%. In addition, there are the several proteins with which these substances are bound. Such preparations also oxidize NADH, ascorbate, *p*-phenylenediamine, and hydroquinones, these substrates supplying electrons at various sites in the electron transport chain. The use of various electron donors and acceptors, the application of specific inhibitors, and the fragmentation of the succinate oxidase complex have led to several postulates of the pathways of electron flow. One difficulty is the probable difference in pathways between mitochondrial phosphorylating systems and submitochondrial nonphosphorylating preparations. A second difficulty is the possibility of alternate pathways of electron flow rather than a single linear sequence. Scheme 1-2, (Green, 1960; Redfearn, 1960) might be assumed provisionally:



(1-2)

Fe represents nonheme iron, f_s is the flavoprotein associated with succinate dehydrogenase, f_d is the flavoprotein associated with NADH dehydrogenase, UQ is ubiquinone (coenzyme Q), ASF is the antimycin-sensitive factor, and the other symbols indicate the usual cytochromes. It is possible that cytochrome b and ubiquinone are common to the two pathways from succinate and NADH, but Green believes the evidence points to fusion of the chains

only at the antimycin-sensitive factor. It is also possible that ubiquinone and cytochrome b are on a linear pathway rather than on alternate pathways. In nonphosphorylating systems, the role of cytochrome b is debatable. The sites of oxidation of *p*-phenylenediamine and ascorbate are distal to ASF. It should also be pointed out that succinate oxidase from other sources may have different ratios of components and somewhat different pathways. Thus the oxidation of succinate and NADH by the electron transport system of *Azotobacter* is not sensitive to antimycin (Bruemmer *et al.*, 1957). Two recent observations have extended our knowledge of succinate oxidase and related pathways. Azzone and Ernster (1961) have demonstrated an ATP requirement for mitochondrial phosphorylating succinate oxidation and propose reactions such as the following:

$$\text{Succinate} + \text{A} + \text{ATP} \rightarrow \text{fumarate} + \text{AH} \sim \text{P} + \text{ADP}$$
$$\text{AH} \sim \text{P} + \text{B} + \text{ADP} \rightarrow \text{A} + \text{BH}_2 + \text{ATP}$$

$$\text{Succinate} + \text{B} \rightarrow \text{BH}_2 + \text{fumarate}$$

A is possibly a flavoprotein, a quinone, or some other component of succinate dehydrogenase, and B may be cytochrome b. In nonphosphorylating systems the electrons may reach B more directly. An alternate pathway for AH $\sim$ P is the reduction of NAD:

$$\text{AH} \sim \text{P} + \text{NAD} \rightarrow \text{A} + \text{NADH} + \text{P}_i$$

Succinate has been shown to reduce NAD in mitochondria by Chance and Hollunger (1961 a, b, c) and this is dependent on ATP. Since endogenously formed succinate is more effective than added succinate, it is possible that this reaction may involve succinyl-CoA. The reduction of NAD passes through antimycin-sensitive and Amytal-sensitive links and seems to involve a third flavoprotein component. These findings are not only important with respect to the behavior of succinate oxidase, but also have possible bearing on the responses to malonate in mitochondrial and cellular systems.

The succinate oxidase particles have been fragmented in various ways to yield smaller particles or soluble preparations with different compositions and properties. One such preparation is the *succinate dehydrogenase complex* (SDC) from heart mitochondria or ETP, which perhaps represents that fraction of the complex up to cytochrome c, since it oxidizes both succinate and NADH and possesses an antimycin-sensitive step. Of more interest for malonate inhibition are the several forms of soluble succinate dehydrogenase that have been prepared. The purest contain no heme or lipid; four atoms of tightly bound nonheme iron and one flavin occur in a molecule (assuming a molecular weight of around 200,000). The flavin is apparently not riboflavin but occurs in a dinucleotide form covalently attached to peptide chains of the apoenzyme (Kearney, 1960). There is spectral evidence that both

flavin and nonheme iron are reduced by succinate, and the functional role of iron in the catalysis is further indicated by the inhibition produced by complexing the iron with 1,10-phenanthroline or β_1-globulin (Singer *et al.*, 1957). Electron spin resonance studies of succinate oxidase have demonstrated signals when succinate is added; these free radicals seem to be associated with the dehydrogenase and possibly reflect changes in the states of iron or flavin (Commoner and Hollocher, 1960; Hollocher and Commoner, 1960). The high sensitivity of succinate dehydrogenase to most sulfhydryl reagents indicates the presence of an SH group at or near the succinate-binding site. One may, therefore, characterize succinate dehydrogenase from our present knowledge as containing two cationic groups for the binding of succinate, an SH group nearby, some nonheme iron, and a unique flavin dinucleotide in a tight peptide complex.

Relationship of Malonate Inhibition to the Electron Acceptor Used

Measurement of malonate inhibition involves either the oxygen uptake of the complete succinate oxidase system, or the determination spectroscopically of the reduction of one of the normal components (such as cytochrome c), or the reduction of some artificial electron-acceptor dye. The complete system can reduce a variety of substances in the presence of succinate, and malonate has been shown to inhibit such reductions whatever the acceptor used: methylene blue (Quastel and Whetham, 1925; Hopkins *et al.*, 1938; Forssman, 1941; Franke 1944 a; Kaltenbach and Kalnitsky, 1951 a; Wadkins and Mills, 1955), ferricyanide (Stoppani, 1948; Thorn, 1953), tetrazolium dyes (Barker, 1953; Becker and Rauschke, 1951; Zöllner and Rothemund 1954; Waterhouse, 1955), manganese dioxide (Hochster and Quastel, 1952), 2,6-dichlorophenolindophenol (Repaske, 1954; Wadkins and Mills, 1955, Millerd, 1951), janus red (Agosin and von Brand, 1955), brilliant cresyl blue (Agosin and von Brand, 1955), and *N*-methylphenazine sulfate (Singer *et al.*, 1956 b). Inhibition of cytochrome c reduction has also been observed Seaman, 1954). There is thus substantial evidence that malonate blocks electron flow very early in the sequence, as would be expected if it prevents the binding of succinate to the dehydrogenase. The most proximal location of the site of malonate inhibition comes from the work of Ziegler (1961) on electron transport particles from heart mitochondria. Some of the nonheme iron is reduced by succinate and this is blocked by 20 m*M* malonate. It may also be noted that succinate reduces NAD and NADP in submitochondrial particles from heart through an ATP-dependent system and this is readily inhibited by malonate (Snoswell, 1962; Hommes, 1963; Lee *et al.*, 1964), which possibly indicates that succinate dehydrogenase is involved. The addition of malonate to succinate dehydrogenase brings about changes in the absorption spectrum in the flavin region, as do succinate, fumarate, and other competitive inhibitors (Dervartanian and Veeger, 1962). There is

a decrease in absorption between 400 and 470 mμ and an increase between 480 and 540 mμ, with a maximum at 510 mμ in the difference spectrum. It is not known what this implies relative to the site of malonate binding.

The dyes may accept electrons from various sites in the succinate oxidase system. The fragments obtained from the oxidase particles differ in their abilities to reduce these dyes, and as one approaches the purest succinate dehydrogenase the number of possible electron acceptors is reduced. Indeed, soluble succinate dehydrogenase reduces only the *N*-alkylphenazines and ferricyanide at appreciable rates. Some dyes accept electrons chiefly from the nonheme iron, some accept more efficiently from the flavin component, and some, such as the indophenol dyes, involve ubiquinone. It may well be that none of the commonly used dyes is completely selective. Ferricyanide, for example, may react at other sites down the chain in addition to the nonheme iron, since its reduction has a partially antimycin-sensitive component, and even the *N*-alkylphenazines, considered to be the most reliable acceptors at the dehydrogenase level, may be able to react at other sites.

The question of importance with respect to malonate inhibition is whether all of the methods of measuring succinate dehydrogenase activity are equivalent for the purpose of obtaining accurate kinetic results. Unfortunately, there have been very few reliable investigations wherein different methods or acceptors have been compared. The inhibitions of succinate oxidase (manometric) and succinate dehydrogenase (dye acceptors) at the same concentrations of succinate and malonate have been reported sporadically. It has generally been found that the oxidase is inhibited somewhat more strongly: *Arum* spadix (Simon, 1957), *Limulus* gill cartilage (Person and Fine, 1959), and potato tubers (Millerd, 1951). But in the enzymes from *Xanthomonas*, this is reversed (Madsen, 1960). The results of Millerd are particularly difficult to understand, inasmuch as she found a 40% inhibition of the oxidase at 0.1 m*M* malonate whereas the dehydrogenase (using 2,6-dichlorophenolindophenol as acceptor) was not inhibited at all. The author is not aware of any quantitative studies comparing malonate inhibition with different dye acceptors. It would appear that most workers have assumed there would be no difference.

Actually, from kinetic consideration, it is not at all necessary that the inhibition produced by a chosen concentration of malonate be the same when different acceptors are used. In fact, the inhibition may depend on the acceptor concentration. Thorn (1953) showed that the inhibition of pig heart succinate dehydrogenase by 0.536 m*M* malonate increases with the concentration of methylene blue: at 0.15 m*M* methylene blue the inhibition is 15.9% and at 3 m*M* methylene blue it is 28.8%. Succinate oxidase, is inhibited 52.6% at the same malonate concentration. The transfer of hydrogen atoms from succinate to a dye acceptor always involves the interaction

of these two substances with the enzyme surface, undoubtedly at different sites. At low concentrations of the acceptor, or with weak acceptors, the over-all rate may not be determined by the rate at which the hydrogen atoms are removed from the succinate, but may depend also on the rate of transfer to the acceptor. The malonate inhibition will thus vary with the degree of saturation of the enzyme with acceptor. On this basis it would seem reasonable to use those acceptors which are the most active and react with sites closest to the succinate site. A further consideration is the inhibition produced by the acceptors themselves. Both ferricyanide and *N*-methylphenazine begin to inhibit succinate dehydrogenase as the concentration is raised above certain levels. Such systems would then constitute examples of multiple inhibition and the kinetics of the inhibition due to malonate alone may be distorted. The choice of the acceptor and its concentration is thus of some significance.

Site of Inhibition by Malonate in the Succinate Oxidase Sequence

The results discussed in the preceding section point clearly to the site of inhibition as succinate dehydrogenase. Indeed, inhibition of soluble succinate dehydrogenase by malonate has been demonstrated. The competitive nature of the inhibition, to be treated in the following section, indicates the inhibition to be at the active site at which succinate is bound. There is thus no question but that the major site of inhibition is at the very beginning of the electron transport sequence in succinate oxidase. The question that now must be considered is whether malonate can inhibit at any other step of the electron transport chain.

There are two obvious ways to examine this. One is to test the action of malonate on the succinate oxidase system, using substrates that donate electrons at more distal sites than succinate. The other way is to determine the response of other oxidases that utilize most of the electron carriers in the succinate oxidase. Quastel and Wheatley (1931) observed that malonate at 67 m*M* does not inhibit the oxidation of *p*-phenylenediamine and hence concluded that the cytochrome region of the sequence is immune in their preparations. Actually, many oxidases, comprising varying segments of the electron transport chain, have been found to be insensitive to malonate at concentrations from 25 m*M* to 50 m*M*. All this evidence points to a rather specific action on the dehydrogenase. However, there are data in the literature which indicate that, at least in some species and at high enough malonate concentrations, inhibition at other sites may occur. Although 30 m*M* malonate does not inhibit NADH oxidation in beet mitochondria (Wiskich *et al.*, 1960), some inhibition has been observed in mosquito particles (10–15% at 1–10 m*M*) (Gonda *et al.*, 1957) and in *Tetrahymena* NADH oxidase (35% at 6.2 m*M* and 70% at 18 m*M*) (Eichel, 1959). Such inhibition could, of course, be on the NADH dehydrogenase rather than on

enzymes common to the succinate pathway. Malonate does not usually interfere with ascorbate oxidation, but in the silkworm it was found both spectroscopically and manometrically that malonate inhibits the oxidations of succinate and ascorbate equally (Sanborn and Williams, 1950). Observations such as these, coupled with those showing greater inhibition of succinate oxidase compared to the dehydrogenase, make it necessary to exert some caution in assuming a completely specific action in all cases.

Competitive Nature of the Inhibition

It has been often stated that competitive inhibition was first demonstrated by Quastel and Wooldridge (1928) for the inhibition of *E. coli* succinate dehydrogenase by malonate. However, inhibitions were not calculated and the data presented do not lend themselves to quantitative interpretation. Indeed, when their data are plotted on a $1/v_i - 1/(S)$ graph (Fig. 1-2) a straight line is not obtained. Since no comparable control studies were done in the absence of malonate, the fact alone that increasing the succinate concentration increases the rate in the presence of malonate does not prove competition. These points are brought out not to criticize pioneering work but to illustrate that conclusions about the type of inhibition cannot be made so readily as many imagine.

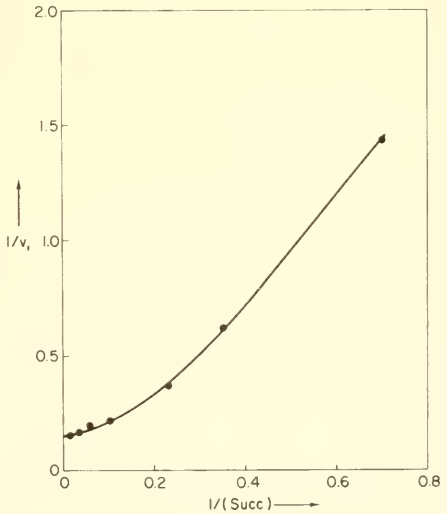


FIG. 1-2. A double reciprocal plot for the inhibition of *E. coli* succinate dehydrogenase by malonate at 1.43 m*M*. Succinate concentrations are in m*M*. (Data from Quastel and Wooldridge, 1928).

Competition between succinate and malonate has been claimed to occur in the following: rat liver homogenates (Potter and DuBois, 1943), oyster muscle homogenates (Humphrey, 1947), oyster egg homogenates (Cleland, 1949), carrot root (Hanly *et al.*, 1952), yeast (Krebs *et al.*, 1952), pig heart particulates (Thorn, 1953), cockroach muscle homogenates (Harvey and Beck, 1953), the trypanosome *Crithidia* (Hunter, 1960), and the soluble succinate dehydrogenase from heart (Keilin and King, 1960). In all cases, the inhibition produced by a certain malonate concentration is reduced by increasing the succinate concentration, but a quantitative analysis of the data has been seldom carried out. Most of these studies were made with the usual assay methods, but competition has recently been shown by measuring the reductions in the electron spin resonance signals produced by malonate at different succinate concentrations (Commoner and Hollocher, 1960).

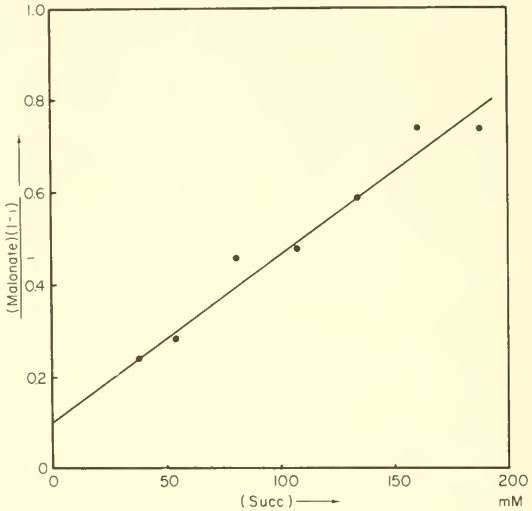


FIG. 1-3. A single-curve plot for the inhibition of cockroach muscle succinate oxidase by malonate at 0.33 mM. $K_i = 0.105$ mM and $K_m/K_i = 275$. (Data from Harvey and Beck, 1953).

Single-curve plots (type F, see Chapter I-5) were made for two of the inhibitions mentioned above (Figs. 1-3 and 1-4). In the case of *Crithidia*, Hunter showed competition with a $1/v$-1/(S) plot and the single-curve plot confirms this, K_i having the value of 0.22 mM and K_m/K_i calculated from the slope a value of 53. The results with cockroach muscle likewise fall roughly on a straight line, giving K_i as 0.11 mM and K_m/K_i as 275, values differing somewhat from those calculated by Harvey and Beck using a

different plotting procedure ($K_i = 0.13$ mM, and $K_m/K_i = 200$). In most studies insufficient data are available for plotting. It must be emphasized that a change in inhibition observed at two or three succinate concentrations is not adequate to prove a purely competitive inhibition. It seems to be rarely considered that an inhibition may not be either completely competitive or completely noncompetitive. The conditions for partially competitive inhibition were given (Eqs. I-3-14 and I-3-15) and the types of plot to be expected discussed (Chapter I-5). An interesting example of this is provided by the work of Honda and Muenster (1961) on the inhibition of succinate oxidation in lupine mitochondria. Here the osmolarity of the preparation and assay media was varied with sucrose, and it was found that the interaction constant, α, defined in Eqs. I-3-5 and I-3-6,

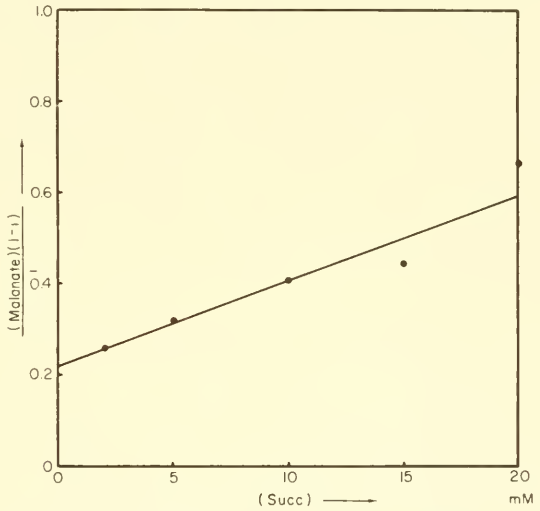


Fig. 1-4. A single-curve plot for the inhibition of *Crithidia* succinate dehydrogenase by malonate at 1 mM. $K_i = 0.22$ mM, and $K_m/K_i = 53$. (Data from Hunter, 1960).

varies quite markedly from values indicating nearly completely competitive inhibition to those showing noncompetitive inhibition. This work will be discussed in greater detail in a later section (see page 46), but it suffices to show that partial competitive inhibition by malonate is possible and that the type of inhibition may vary with the experimental conditions.

The most elegant treatment of malonate inhibition is by Thorn (1953) at the St. Thomas's Hospital Medical School in London, using succinate oxidase preparations from pig heart muscle. The activity was measured by the reduction of ferricyanide in the presence of cyanide to block the cyto-

chrome pathway. The usual $1/v - 1/(S)$ plots (Fig. 1-5) show apparently completely competitive inhibition, and values of the substrate and inhibitor constants, to be discussed in the next section, were calculated. Using average values of K_m and K_i obtained by Thorn, the inhibition curves in

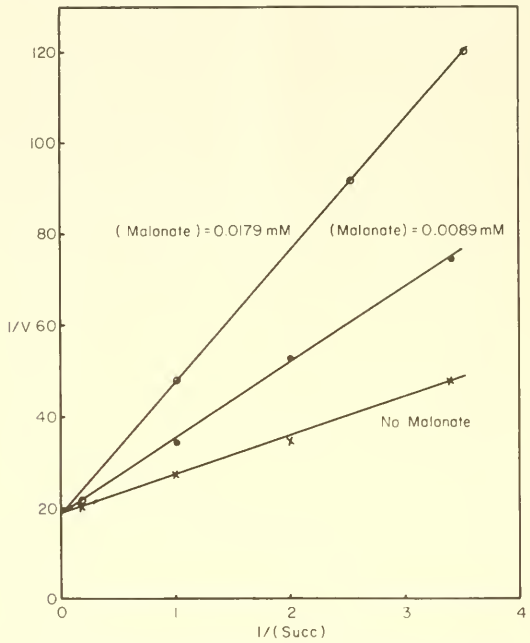


FIG. 1-5. Double reciprocal plots for the inhibition of pig heart succinate dehydrogenase by malonate, showing pure competitive inhibition. Succinate concentrations are in mM and v in spectrophotometric units. (Data from Thorn, 1953).

Fig. 1-6 were plotted. These curves show the expected reduction in inhibition as the succinate concentration is increased at constant values of malonate concentration. It may be noted that overcoming the inhibition is much more difficult at high inhibitor concentrations and this must be taken into account in experiments designed to show a competitive type of action. K_m was used rather than K_s because under the usual experimental conditions it is this constant that determines the behavior.

A word must now be said about malonate reversibility. One of the criteria of competitive inhibition is that the inhibitor should leave the active site readily when its concentration is reduced, or that it should be displaced rapidly when more substrate is added. These points have seldom been

tested with malonate. However, it was demonstrated quite early that the inhibition of rabbit muscle succinate dehydrogenase is reversible by washing; the preparation was incubated for 30 min. with 100 mM malonate anaerobically and then washed 3 times on a filter — the reduction times were 15 min for the control, 61 min with the malonate, and 17 min for the washed preparation (Hopkins *et al.*, 1938). A trypanosomal succinate dehydrogenase, however, showed no reversal of the inhibition, even when succinate was added to 50 times the malonate concentration, which is particularly surprising since in the living cells a good reversal was observed (Agosin and von Brand, 1955). The concentrations of malonate used here were not unduly high and so these data are unexplainable.

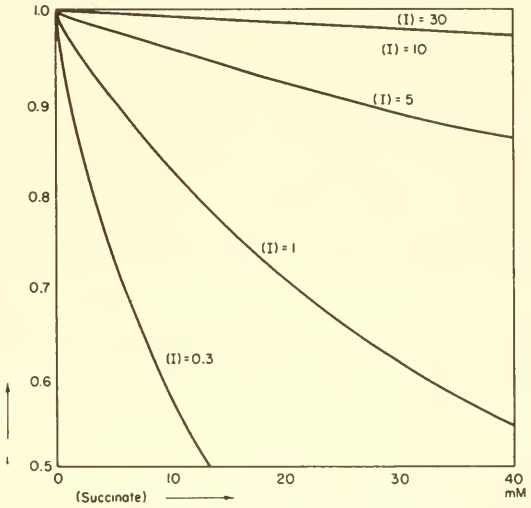


Fig. 1-6. Curves showing the calculated reductions of the malonate inhibition of succinate dehydrogenase by various concentrations of succinate, using $K_m = 0.366$ mM, and $K_i = 0.0076$ mM, as given by Thorn (1953).

Constants of the Inhibition

It is not surprising that the values of the inhibitor constant, K_i, for malonate inhibition are quite variable in the literature, because not only do the experimental conditions affect this constant markedly but the succinate dehydrogenase varies in its properties from species to species. From the values in the accompanying tabulation and from reasonable estimates based on assumed or determined substrate constants for the data in Table 1-6, it is seen that the K_i's for most preparations vary between 0.005 mM

TABLE 1-6

INHIBITION OF SUCCINATE DEHYDROGENASE BY MALONATE

Organism	Tissue	Preparation	Succinate (m*M*)	Malonate (m*M*)	(I)/(S)	% Inh.	Reference
Escherichia coli		Extract	59	5	0.085	20	Kaltenbach and Kalnitsky (1951a)
				10	0.17	55	
				20	0.34	72	
		Toluene-treated	7.14	14	1.96	95	Quastel and Wooldridge (1928)
				71.4	10	>95	
		Toluene-treated	4.3	6.6	1.53	97	Ajl and Werkman (1948)
		Extract	0.83	0.83	1	88	Okunuki and Sekuzu (1955)
		Sonic lysate	16.7	1.7	0.1	65	Asnis *et al.* (1956)
				17	1	100	
Azobacter vinelandii		Sonic lysate	9	3	0.33	77	Repaske (1954)
		Particulate	115	10	0.09	100	Bruemmer *et al.* (1957)
Micrococcus lysodeikticus		Extract	0.05	2	40	12	Saz and Krampitz (1955)
				6	120	26	
				12	240	72	
Rhodospirillum rubrum		Extract	0.33	3.3	10	90	Woody and Lindstrom (1955)
Mycobacterium tuberculosis		Extract	17	11	0.65	81	Yamamura *et al.* (1954)
		Particulate	33.3	16.7	0.5	40	Kusunose *et al.* (1956)
Xanthomonas phaseoli		Sonic lysate	20	10	0.5	92	Madsen (1960)
Penicillium chrysogenum		Extract	16	7	0.44	80	Godzeski and Stone (1955)

Aspergillus niger		Particulate	94	13	0.14	88	Martin (1954)
				30	0.32	100	
Aspergillus oryzae		Mitochondria	1	1	1	100	Imamoto *et al.* (1959)
Allomyces macrogynus		Suspension	20	1	0.05	54	Bonner and Machlis (1957)
				2	0.1	69	
				5	0.25	86	
				10	0.5	92	
Yeast		Frozen	6.25	2.5	0.4	96	Krebs *et al.* (1952)
			62.5	2.5	0.04	70	
		Frozen	2.1	3.3	1.57	95	Lynen (1943)
			4.2	3.3	0.79	86	
			8.3	3.3	0.40	74	
			16.7	3.3	0.20	48	
Ulothrix zonata		Homogenate	50 (?)	1	0.02	38	Hunter and Hunter (1957)
Cape barley (*Hordeum vulgare*)	Roots	Particulate	20	20	1	85	Honda (1955)
Pea (*Pisum sativum*)	Leaves	Particulate	10	1	0.1	57	Smillie (1956)
Pine (*Pinus lambertiana*)	Seedlings	Mitochondria	16.7	16.7	1	74	Stanley and Conn (1957)
Tomato	Stems	Mitochondria	25	25	1	89	Wu and Scheffer (1960)
Potato	Tubers	Particulate	6.7	0.1	0.015	0	Millerd (1951)
				1	0.15	29	
				10	1.5	73	
Beet	Roots	Mitochondria	50	30	0.6	69	Wiskich *et al.* (1960)
				50	1	100	

TABLE 1-6 (*continued*)

Organism	Tissue	Preparation	Succinate (m*M*)	Malonate (m*M*)	(I)/(S)	% Inh.	Reference
Tobacco	Leaves	Mitochondria	3.8	3.8	1	100	Pierpoint (1959)
			25	3.8	0.15	68	
			112	3.8	0.034	31	
Cuckoopint (*Arum maculatum*)	Spadix	Mitochondria	20	10	0.5	98	Simon (1957)
Euglena		Homogenate	50	15	0.3	30	Danforth (1953)
Trypanosoma cruzi		Mitochondria	16.7	0.1	0.006	35	Agosin and von Brand (1955)
				1	0.06	74	
				5	0.3	97	
Crithidia fasciculata		Particulate	8.3	3.33	0.4	93	Scaman (1956)
Paramecium caudatum		Homogenate	50	80	1.6	98	Humphrey and Humphrey (1948)
Stylonychia pusulata		Homogenate	37.5	0.25	0.007	57	Hunter (1958)
Physarum polycephalum (myxomycete)		Mitochondria	50	16.7	0.33	70	Johnson and Moos (1956)
				33.3	0.66	78	
				50	1	86	
Hymenolepis diminuta (cestode)		Homogenate	50	50	1	68	Read (1952)
				100	2	82	
Oyster (*Saxostrea commercialis*)	Muscle	Homogenate	10	1	0.1	15	Humphrey (1947)
				10	1	68	

				100	10	84	
			30	1	0.033	17	
				10	0.33	43	
				100	3.3	60	
	Eggs	Homogenate	10	1	0.1	10	Cleland (1949)
				10	1	35	
				100	10	64	
Horseshoe crab (*Limulus polyphemus*)	Gill cartilage	Homogenate	50	0.5	0.01	14	Person and Fine (1959)
				5	0.1	24	
				50	1	100	
Milkweed bug (*Oncopeltus fasciatus*)	Whole	Homogenate	50	7	0.14	91	Collias *et al.* (1952)
Woodroach (*Leucophaea maderae*)	Muscle	Homogenate		5		41	McShan *et al.* (1954)
				10		60	
Cockroach (*Periplaneta americana*)	Muscle	Homogenate	37.4	0.33	0.0088	58	Harvey and Beck (1953)
Mosquito (*Aedes aegypti*)	Whole	Particulate	30	1	0.033	38	Gonda *et al.* (1957)
				10	0.33	83	
Silkworm (*Samia cecropia*)	Midgut	Particulate	13	10	0.77	31	Sanborn and Williams (1950)
Chicken	Kidney	Homogenate	34	2.1	0.062	9	Potter and Elvehjem (1937)
				8.5	0.25	52	
				34	1	89	
Pigeon	Muscle	Extract	1.16	6.3	5.4	85	Straub (1937)
	Breast muscle	Extract	50	2.5	0.05	44	Barron and Singer (1945)
Mouse	Liver	Homogenate	15	29	1.9	98	Tietze and Klotz (1952)

TABLE 1-6 (*continued*)

Organism	Tissue	Preparation	Succinate (m*M*)	Malonate (m*M*)	(I)/(S)	% Inh.	Reference
	Liver	Homogenate	1	0.046	0.046	15	Fishgold (1957)
				0.1	0.1	26	
				0.21	0.21	42	
				0.46	0.46	59	
				1	1	80	
	Hepatoma 134	Homogenate	1	0.046	0.046	24	Fishgold (1957)
				0.1	0.1	48	
				0.21	0.21	76	
				0.46	0.46	88	
				1	1	100	
Rat	Liver	Homogenate	50	1	0.02	43	Ackermann and Potter (1949)
		Extract	0.63	1	1.6	77	Hochster and Quastel (1952)
		Homogenate	50	0.1	0.002	10	McShan and Meyer (1946)
				0.4	0.008	37	
				0.5	0.01	41	
				1	0.02	70	
		Homogenate	33	0.33	0.01	38	Potter and DuBois (1943)
				1	0.03	65	
				3.3	0.1	88	
			67	0.33	0.005	22	
				1	0.015	40	
				3.3	0.049	71	
		Homogenate	20	200	10	95	Zöllner and Rothemund (1954)

		Mitochondria	5	5	1	86	Montgomery and Webb (1956 b)
	Kidney	Extract	0.63	1	1.6	90	Hochster and Quastel (1952)
		Mitochondria		13		50	Chari-Bitron (1961)
		Mitochondria	5	5	1	85	Montogomery and Webb (1956 b)
	Pituitary	Homogenate	50	0.5	0.01	33	McShan and Meyer (1946)
	Muscle	Mitochondria	5	5	1	82	Montgomery and Webb (1956 b)
	Brain	Mitochondria	5	5	1	93	Montgomery and Webb (1956 b)
	Heart	Mitochondria	1	5	5	82	Montgomery and Webb (1956 b)
			5	0.01	0.002	3	
				0.5	0.1	13	
				1	0.2	31	
				5	1	80	
				10	2	97	
			10	5	0.5	67	
Guinea pig	Liver	Extract	0.63	1	1.6	100	Hochster and Quastel (1952)
	Brain	Homogenate	0.63	1	1.6	100	Hochster and Quastel (1952)
	Seminal vesicle epithelium	Particulate	50	0.33	0.0066	39	Levey and Szego (1955)
				0.67	0.013	50	
				1.67	0.033	66	
				3.33	0.067	75	
	Seminal vesicle muscle	Particulate	50	0.33	0.0066	30	Levey and Szego (1955)
				0.67	0.013	47	
				1.67	0.033	61	
				3.33	0.067	70	
Rabbit	Muscle	Mince	67	67	1	91	Quastel and Wheatley (1931)
	Brain	Mince	67	67	1	95	Quastel and Wheatley (1931)
	Heart	Mitochondria	5	50	10	100	Packer (1958)
Pig	Heart	Homogenate	7.4	7.4	1	100	Potter (1940)

TABLE 1-6 (*continued*)

Organism	Tissue	Preparation	Succinate (m*M*)	Malonate (m*M*)	(I)/(S)	% Inh.	Reference
		Mitochondria	40	1.2	0.03	57	Bonner (1954)
		Particulate	25	0.4	0.016	50	Thorn (1953)
Dog	Kidney	Homogenate		1.32		50	Shideman and Rene (1951 a)
	Heart	Particulate	10	10	1	99	Stoppani (1948)
Calf	Thymus	Nuclei	20	1	0.05	94	McEwen *et al.* (1963 a)
Ox	Heart	Solubile	10	10	1	90	Kearney and Singer (1956)
		Particulate			0.1	72	Rosen and Klotz (1957)
					1	94	
					5	100	
Horse	Muscle	Extract	67	3.6	0.054	50	Das (1937 b)
		Extract	2.9	7.1	2.45	93	Thunberg (1933)
			5.7	7.1	1.24	93	
			11.4	7.1	0.62	90	
	Heart	Extract	10	20	2	86	Franke (1944 a)
		Particulate	14.3	1.32	0.092	82	Holton and Colpa-Boonstra (1960)
Man	Brain	Mince	67	67	1	87	Quastel and Wheatley (1931)
	Epithelial carcinoma	Extract		1.5		61	Barban and Schulze (1956)
				3.1		81	
				6.2		86	

Preparation	K_i (mM)	Reference
Pig heart	0.0076	Thorn (1953)
Rat heart	0.01	Pylin and Matsumoto (1964)
Yeast	0.0105	Ryan and King (1962 a)
Rhizobium japonicum	0.017	Cheniae and Evans (1959)
Claviceps purpurea	0.03	McDonald *et al.* (1963)
Corynebacterium diphtheriae	0.037	Strauss and Jann (1956)
Beef heart	0.041	Kearney (1957)
Beef heart	0.045	Keilin and King (1960)
Mytilus edulis	0.06	Ryan and King (1962 a)
Beef heart	0.13	Lee *et al.* (1964)
Micrococcus lactilyticus	0.23	Warringa and Giuditta (1958)
Phaseolus vulgaris	0.24	Hiatt (1961)
Lupinus albus	0.91	Honda and Muenster (1961)

and 0.05 mM, and mammalian tissues generally yield quite low constants. The interest in the K_i lies in its relationship to the binding energy of malonate to the active center, so that variations in the K_i, unless due to experimental conditions, may be attributed to differences in the topography of the enzyme surface. The values of K_m for different preparations are not particularly significant, except for characterizing a certain preparation under specified conditions, because K_m does not usually equal K_s. Inasmuch as the ratio of K_s to K_i is of some significance in interpreting interactions with the active center, it will be worthwhile to discuss the only instance in which this has been determined.

The ratio, K_m/K_i, has been given by various investigators as ranging from 10 to 60. Thorn (1953) pointed out that K_m for succinate dehydrogenase is often quite different than K_s (Slater and Bonner, 1952). K_m thus equals $(k_1 + k_2)/k_1$, and k_2 is dependent on the experimental conditions. Thorn obtained values of K_m/K_i from 3 to 60 by varying the electron-acceptor dye and the reaction rate. The faster the rate, the greater the deviation of K_m from K_s. Thus extrapolation to zero rate from a series of experiments at different methylene blue concentrations enabled Thorn to determine the true ratio of succinate and malonate affinities; K_s/K_i turned out to be 3. Since K_i is approximately 0.0076 mM. $K_s = 0.023$ mM, a value which checks well with directly determined values of the rate constants ($k_1 = 3.35 \times 10^4$, and $k_{-1} = 0.99$). Similar low ratios would probably be found in most succinate dehydrogenase preparations. The difference in binding between succinate and malonate is thus not so great as previously believed.

It is of great practical importance to realize that the degree of inhibition of succinate oxidase by malonate varies with the rate of succinate oxidation and the electron acceptors present. This may explain some of the

differences in Table 1-6. If one is to compare the inhibitory potencies of malonate on succinate oxidases from different tissues or species, equivalent rates of succinate oxidation should be used and similar methods of determining the rate should be employed.

Inhibition of Succinate Dehydrogenase by Other Dicarboxylates

Before discussing the more intimate nature of the inhibition and the possible ways by which malonate is bound to the active center, it will be useful to consider the inhibitory potencies of other dicarboxylate ions. The configuration of an active center may often be approached by comparing the relative affinities of analogous compounds for the enzyme. At the end of this chapter a more complete discussion of inhibitors related to malonate will be given; for the present we shall be interested only in the inhibition of succinate dehydrogenase. Inhibitions and inhibitor constants are summarized in Tables 1-7 and 1-8.

(*a*) *Unsubstituted dicarboxylate ions.* It was stated by Quastel and Whetham (1925) that oxalate, glutarate, and adipate do not interfere with succinate oxidation, in contrast to malonate, and, although in later work they found some inhibition, this has generally been confirmed. Accurate comparisons must be made using inhibitor constants, and these are not available, but there is no doubt that in the series $^{-}OOC—(CH_2)_n—COO^{-}$ the inhibition reaches a sharp maximum at $n = 1$. One must assume that malonate best fits the intercationic distance on the active center of succinate dehydrogenase, and this includes succinate itself since K_s is generally larger than K_i for malonate.

(*b*) *Unsaturated dicarboxylate ions.* It is interesting to compare the two isomers, fumarate and maleate, since they differ in the intercarboxylate distance (Table 1-1). For mammalian succinate dehydrogenase it would appear that fumarate is bound much less tightly than either succinate or malonate (Table 1-8), but this does not necessarily hold for the bacterial enzymes, providing evidence that the active center configurations may be quite different in different dehydrogenases. One might expect a rather low affinity for fumarate because of the fairly long intercarboxylate distance, but it is surprising that maleate is such a poor inhibitor inasmuch as its intercarboxylate distance in only 0.38 Å greater than in malonate. A complicating factor is the ability of maleate to react with SH groups, and actually the inhibitions observed by Hopkins *et al.* (1938) and Morgan and Friedmann (1938 b) were obtained only after prolonged incubation. The possible significance of these observations will be discussed in the next section. On the other hand, acetylene-dicarboxylate, with a distance of 5.16 Å between carboxylate groups and a restriction to linearity, does inhibit and this is completely competitive (Thomson, 1959).

TABLE 1-7

INHIBITIONS OF SUCCINATE DEHYDROGENASE BY VARIOUS DICARBOXYLATE IONS

Compound	Organism	Tissue	Succinate (m*M*)	Inhibitor (m*M*)	% Inhibition	Reference
Oxalate	Chick	Kidney	34	34	51	Potter and Elvehjem (1937)
	E. coli		7.14	35.7	14	Quastel and Wooldridge (1928)
				71.4	29	
	Ox	Heart	24	24	38	Rosen and Klotz (1957)
Glutarate	*E. coli*		7.7	77	0	Quastel and Whetham (1925)
			7.14	71.4	43	Quastel and Wooldridge (1928)
	Chick	Kidney	34	34	25	Potter and Elvehjem (1937)
	Horse	Muscle	2.84	28.4	52	Thunberg (1933)
	Rat	Heart	5	20	4	Montgomery and Webb (1956 b)
				50	44	
				100	47	
Adipate	Chick	Kidney	34	34	35	Potter and Elvehjem (1937)
	E. coli		7.7	77	0	Quastel and Whetham (1925)
Fumarate	Chick	Kidney	34	34	69	Potter and Elvehjem (1937)
	Rat	Heart	50	2.8	58	Swingle *et al.* (1942)
	Pig	Heart	14.3	0.53	73	Holton and Colpa-Boonstra (1960)
Maleate	Rabbit	Muscle		100	56	Hopkins *et al.* (1938)
	Pig	Heart		80	48	Morgan and Friedmann (1938 b)

TABLE 1-7 (*continued*)

Compound	Organism	Tissue	Succinate (m*M*)	Inhibitor (m*M*)	% Inhibition	Reference
	Pigeon	Brain	67	16.5	0	Peters and Wakelin (1946)
				33	7	
	Rat	Heart	5	5	Stim	Montgomery and Webb (1956 b)
				20	37	
Acetylene-dicarboxylate	Pigeon	Muscle	20	5	30	Dietrich *et al.* (1952)
	Rat	Heart	5	5	4	Montgomery and Webb (1956 b)
				20	22	
				50	86	
Malate	Chick	Kidney	34	34	53	Potter and Elvehjem (1937)
	Rat	Heart	50	2.8	57	Swingle *et al.* (1942)
Oxalacetate	*Mycobacterium*		33.3	1.67	78	Kusunose *et al.* (1956)
				3.33	86	
	Penicillium		16.7	6	100	Godzeski and Stone (1955)
	Rat	Heart	50	0.056	28	Swingle *et al.* (1942)
				0.56	88	
		Brain	30	0.2	22	Dickens (1946 b)
				2	97	
		Kidney	50	0.02	50	Pardee and Potter (1948)
Hydroxymalonate (tartronate)	*E. coli*		7.14	71.4	0	Quastel and Wooldridge (1928)
Aspartate	Chick	Kidney	34	34	24	Potter and Elvehjem (1937)

Dimethylmalonate	Horse	Muscle	2.84	28.4	50	Thunberg (1933)
		Heart	10	20	0	Franke (1944 a)
Ethylmalonate	Horse	Muscle	2.84	28.4	73	Thunberg (1933)
Diethylmalonate	Horse	Muscle	2.84	28.4	62	Thunberg (1933)
Allylmalonate	Horse	Muscle	2.84	28.4	76	Thunberg (1933)
Butylmanolate	Horse	Heart	10	20	0	Franke (1944 a)
Dibutylmalonate	Horse	Heart	10	20	0	Franke (1944 a)
Nonylsuccinate	Horse	Heart	7.4	30	12	Franke (1944 a)
Decylsuccinate	Horse	Heart	7.4	30	30	Franke (1944 a)
Undecylsuccinate	Horse	Heart	7.4	30	80	Franke (1944 a)
Dodecylsuccinate	Horse	Heart	7.4	30	92	Franke (1944 a)
Cyclobutane-1,1-dicarboxylate	Mouse	Liver	10	29	47	Tietze and Klotz (1952)
cis-Cyclopentane-1,2-dicarboxylate	Mouse	Liver	10	30	53	Tietze and Klotz (1952)
trans-Cyclopentane-1,2-dicarboxylate	*Tetrahymena*		20	10	47	Seaman and Houlihan (1950)
				40	74	
	Mouse	Liver	10	29	47	Tietze and Klotz (1952)
Phthalate	Mouse	Liver	10	10	22	Tietze and Klotz (1952)
				50	59	
Terephthalate	Pigeon	Muscle	20	5	25	Dietrich *et al.* (1952)

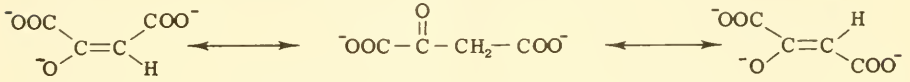
TABLE 1-8

INHIBITOR CONSTANTS FOR DICARBOXYLATE IONS ON SUCCINATE DEHYDROGENASE

Source of enzyme	Inhibitor	K_i (mM)	Reference
Corynebacterium diphtheriae	Malonate	0.037	Strauss and Jann (1956)
	Fumarate	1.8	
Micrococcus lactilyticus	Malonate	0.23	Warringa and Giuditta (1958)
	Fumarate	0.22	
Claviceps purpurea (ergot)	Malonate	0.03	McDonald *et al.* (1963)
	Fumarate	0.93	
Yeast	Malonate	0.0105	Ryan and King (1962 a)
	Fumarate	1.03	
Mytilus edulis (mussel)	Malonate	0.06	Ryan and King (1962 a)
	Fumarate	0.15	
Rat kidney	Oxalacetate	0.0015	Pardee and Potter (1948)
	Acetylene-dicarboxylate	0.81	Thomson (1959)
Pig heart	Malonate	0.05	Hellerman *et al.* (1960)
	Fumarate	3.5	
	Oxalacetate	0.0016	
	Ethyloxalacetate	0.04	
	Diethyloxalacetate	No inh.	
	Acetylene-dicarboxylate	1.4	
	cis-Cyclohexane-1,2-dicarboxylate	132	
Beef heart	Malonate	0.041	Kearney (1957)
	Fumarate	1.9	
	Itaconate	1.8	Dervartanian and Veeger (1962)

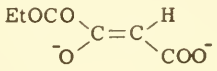
(*c*) *Oxalacetate.* The marked inhibition exerted by this substance was first noted by Das (1937 b) and this has been confirmed on the succinate dehydrogenases from a variety of organisms. It is at present recognized as the most potent succinate dehydrogenase inhibitor among the dicarboxylate ions. The binding of the oxalacetate to the enzyme would appear to be at the active center because the inhibition is competitive with succinate (Pardee and Potter, 1948; Kearney and Singer, 1956; Hellerman *et al.*, 1960), and the presence of oxalacetate on the enzyme protects the active center from various sulfhydryl reagents (Stoppani and Brignone, 1957). The reason for

this strong inhibition is still not clear but Hellerman and his group have advanced some interesting speculations. Oxalacetate in aqueous solution may exist around neutrality in three forms in equilibrium. It was suggested that the enolate form may be the potent inhibitor, especially the *trans*-



cis-Enolate tautomer | Keto tautomer | *trans*-Enolate tautomer

enolate tautomer where the enolate group and the carboxylate group are on the same side. Evidence for this was provided by showing that the oxalacetate monoethyl ester is inhibitory (about as potent as malonate)



trans-Enolate tautomer of monoethyl ester

(Table 1-8). The diethyl ester in inactive. Actually there is a difference in binding energy of about 2.1 kcal/mole between oxalacetate and either malonate or the monoethyl ester. This extra energy might be due to the doubly negative charge on one end of the molecule. It could well be that both the *cis*- and *trans*-enolate tautomers are bound, the charge distributions being almost equivalent if one takes the center of negative charge as lying between the carboxylate and enolate groups. It is unfortunate that the per cent of the oxalacetate in the enolate forms at pH 7.6 and 30° in aqueous solution is not known. Another possibility is that the keto tautomer combines with the enzyme, the extra binding energy arising from an interaction between the keto group and the enzyme; a hydrogen bond could account for the 2.8 kcal/mole difference between the binding energies of oxalacetate and succinate. One difficulty in assuming the enolate form as the active inhibitor is the fact that maleate inhibits very poorly and fumarate not a great deal better. The difference in binding energy between oxalacetate and fumarate for the pig heart succinate dehydrogenase is close to 4.8 kcal/mole, and it would be difficult to account for this large difference simply on the basis of additional ionic interactions. A final possibility must be entertained, although no evidence for it exists, namely, the complexing of the enolate group with one of the nonheme iron atoms near the cationic groups of the enzyme surface, since this could provide the extra energy for the binding of oxalacetate. It should be noted that although oxalacetate strongly inhibits succinate oxidation in rapidly respiring liver mitochondria, both coupled and uncoupled, it stimulates when the mitochondria are in a

state of respiratory control (Kunz, 1963). Malonate does not stimulate under these conditions and thus the effects of oxalacetate on intact mitochondrial succinate oxidation differ in some manner from those of malonate. This, however, is probably explained by other reactions of oxalacetate, *e.g.*, the oxidation of NADH to NAD, which would release the mitochondria from respiratory control.

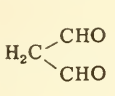
(*d*) *Substituted malonates and succinates.* Franke (1944 a) found that alkylmalonates are inactive on heart succinate dehydrogenase, confirming the weak inhibitions reported by Thunberg (1933). Alkylsuccinates likewise are inactive until the length of the alkyl chain is greater than eight carbon atoms. These higher alkylsuccinates may exhibit some degree of competitive inhibition but there is also inhibition of the oxidation of *p*-phenylenediamine and, hence, of the cytochrome system. Therefore, it is clear that additions of even small alkyl groups to malonate and succinate depress or abolish the normal inhibitory activity. Hydroxymalonate (tartronate) also is essentially inactive. These results indicate the importance of that part of the molecule between the two carboxylate groups and would seem to argue against a simple interaction of the molecules with cationic groups on a flat surface, the substituted groups protruding outward. Of course, it is necessary that the $—CH_2CH_2—$ grouping of succinate interact with the enzyme in order for dehydrogenation to take place. The failure of short-chain alkylmalonates to inhibit appreciably must be attributed to some manner of steric interference by the alkyl groups.

(*e*) *Cyclic dicarboxylate ions.* Cyclobutane and cyclopentane dicarboxylates are weak inhibitors of succinate dehydrogenase. Since the intercarboxylate distance in cyclobutane-1,1-dicarboxylate and *cis*-cyclopentane-1,2-dicarboxylate are not too far from that in malonate (Table 1-1), the poor inhibition may be due to the bulkiness of the rings interfering sterically, as do the alkyl groups discussed in the previous section. It is strange, however, that there is no inhibitory difference between the *cis* and *trans* isomers of cyclopentane-1,2-dicarboxylate, since the intercarboxylate distances differ by 1.39 Å. Thus the inhibition may not be by the same mechanism as for malonate; indeed, *cis*-cyclohexane-1,2-dicarboxylate inhibits succinate dehydrogenase noncompetitively (Hellerman *et al.*, 1960).

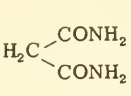
Nature of the Active Center and the Binding of Malonate

The evidence indicates the presence at the active center of two cationic groups and a nearby SH group. The cationic groups, perhaps 3–4 Å apart, are suggested by the very weak inhibitions exerted by monocarboxylates (Quastel and Wooldridge, 1928; Dietrich *et al.*, 1952) and the complete lack of a competitive inhibition by compounds in which the negative charges on the carboxylate groups are eliminated. Malondialdehyde (Holt-

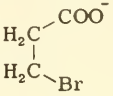
kamp and Hill, 1951), malondiamide (Fawaz and Fawaz, 1954), and various derivatives of succinate, in which one or both of the carboxylate groups have been replaced with nonionic groups (Dietrich *et al.*, 1952),



Malondialdehyde



Malondiamide



β-Bromopropionate

β-Hydroxypropiononitrile

are all lacking in inhibitory activity. An SH group close to the cationic attachment points is proved by the high sensitivity of the dehydrogenase to substances reacting with SH groups and the protection that malonate affords against such substances. The latter is actually better evidence for the proximity of the SH group because sulfhydryl reagents can alter protein structure and exert effects for some distance over the enzyme, whereas the blockade of these substances by a small molecule such as malonate would be almost certain proof. Hopkins *et al.* (1938) showed that malonate, can protect succinate dehydrogenase from oxidized glutathione (GSSG), which is a potent inhibitor. Incubation of the enzyme with GSSG increased the methylene blue reduction time from 10 min to 3 hr; malonate at concentrations from 0.2 to 100 m*M* gave almost complete protection. That the SH groups are protected by malonate was also shown by titration of these groups with iodine. Potter and DuBois (1943) reported protection against quinone inhibition and Barron and Singer (1945) against arsenicals. It is interesting that malonate also protects against oxygen poisoning of succinate oxidase, which was interpreted to mean that the SH groups of the enzyme are involved in this inactivation (Dickens, 1946 b). Oxalacetate has also been shown to be protective against mercurials and arsenicals (Stoppani and Brignone, 1957), providing evidence that it binds to the same site as succinate and malonate. The degree of protection depends on several factors, including the concentrations of the inhibitors and the rates at which they react with the enzyme; the less protection seen against the mercurials is attributed to their comparatively rapid action whereas the

Inhibitor	Inhibitor concentration (m*M*)	Oxalacetate concentration (m*M*)	% Protection
p-MB	0.5	1.3	21.8
p-Chloromercuriphenol	0.76	3.4	24.0
$HgCl_2$	0.38	3.4	53.8
Methylarsenoxide	2.0	3.4	67.2
Oxophenarsine	0.36	1.3	81.2

arsenicals require at least 30 min to reach their maximal inhibition. Such protection on a competitive basis was called *interference inhibition* by Ackermann and Potter (1949). Once the sulfhydryl reagents have reacted with the enzyme, malonate will not reverse the inhibition, but only slows down the rate at which the substance acts on the enzyme. (I find it difficult to understand how, in some cases, such low concentrations of malonate afford protection against irreversible inhibitors. For example, Potter and DuBois reported that 0.33 mM malonate protects quite well against *p*-quinone, and yet this concentration of malonate inhibits only around 20%, showing most of the enzyme uncombined with malonate).

Succinate dehydrogenase also contains nonheme iron and flavin dinucleotide but the locations of these components relative to the succinate-binding site are not known. Since the iron and the flavin both participate in the electron transfer, it is reasonable to assume that at least one of them is close, or even part of the active center. Most formulations have pictured the initial step as a transfer of hydrogen atoms from succinate to the flavin; if this is so, the topography of the active center must be rather complex.

We shall now turn to the energetics of the binding of malonate in order to determine if the ionic interactions generally assumed are reasonable. An immediate difficulty is the variability in the values of K_i reported, even for the same tissue; for example, 0.0076 mM (Thorn, 1953) and 0.05 mM (Hellerman *et al.*, 1960) for the enzyme from pig heart, and 0.041 mM for beef heart (Kearney, 1957). A K_i of 0.0076 mM would indicate an over all binding energy of 7.24 kcal/mole, or 3.62 kcal/mole for each carboxylate interaction assuming only ion-ion contribution. This is a reasonably high value for the interaction of COO^- and NH_3^+ groups and corresponds to an intercharge separation of around 4.30 Å (Fig. I-6-16), which is not far from contact of the groups. It is unlikely that other types of interaction are important for malonate. The corresponding K_s for succinate is 0.0028 mM, giving an interaction energy of 3.28 kcal/mole per carboxylate group and a separation of 4.45 Å. A difference of fit of 0.15 Å would thus account for the relative bindings of malonate and succinate. However, in the case of succinate it is more likely that other energy terms are involved. There is undoubtedly some interaction between the $—CH_2CH_2—$ region and the enzyme, and it is probable that distortion of the succinate molecule occurs upon binding. In any event, these rough estimates point to a fairly close fit of malonate to the enzyme cationic groups for the pig heart enzyme. The ability of malonate to bind to the active center of succinate dehydrogenases from bacteria is apparently much less (Table 1-8).

The next question is: what is the most probable distance between the two enzyme cationic binding sites? Information on this must be obtained from the relative bindings of substances having negatively charged groups

different distances apart. Since malonate is usually bound more tightly than succinate, and much more than oxalate, it is reasonable to assume an intercationic distance approximating the intercarboxylate distance in malonate. It is by no means necessary that the substrate in its free configuration exactly fits the enzyme site. Pauling (1946, 1948) has suggested, " an active region of the surface of the enzyme... is closely complementary in structure not to the substrate molecule itself, in its normal configuration, but rather to the substrate molecule in a strained configuration, corresponding to the activated complex for the reaction catalyzed by the enzyme." Now, the fact that malonate fits the active site well does not mean that the enzyme cationic groups are the same distance apart as the carboxylate groups (3.28 Å). The calculations above indicate a distance of 4.3 Å between carboxylate and cationic groups and thus, depending on the geometry of the binding, the cationic groups could be much farther apart than 3.28 Å. Extreme situations are shown in Fig. 1-7, where the intercat-

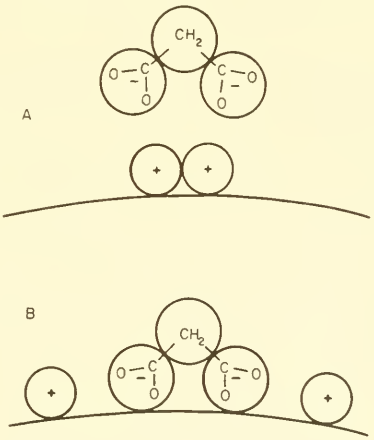


FIG. 1-7. Representations for the extreme situations in the interaction of malonate with the two cationic groups on the surface of succinate dehydrogenase. In both cases (A and B) the interaction distances and the energies between the ionic groups are the same.

ionic distance may vary from 3.28 to 13.1 Å, approximately the same energy of binding being expected in either case. Situation A is not very likely because it is improbable that protein cationic groups would occur so close, and, furthermore, in this case oxalate might be expected to bind quite well. Also, situation B would provide more opportunity for succinate to be dehydrogenated at the enzyme surface. Of course, the enzyme surface at the

active center may not be smoothly curved as shown, and we shall soon examine evidence that it is not.

The problem of the interactions of di-ionic substances with receptor groups has been recently treated by Schueler (1960, p. 448) and on the basis of statistical calculations he has concluded, "The most dramatic alteration in activity should occur upon approaching that agent in the series which possesses a length distribution just capable of overlapping the negative-charge spacing in the receptor, and this should be followed by a relatively slow rate of loss in activity with respect to increasing length" (he is assuming a positively charged drug). In view of what has just been said above, there is some doubt if predictions like this can be made with confidence. The large distances between the interacting charged groups make it very difficult to assign receptor configuration and much will depend on the over all configuration of the protein surface. Other factors, such as distortion of long-chain molecules, interactions of regions between the end charged groups the surface, and possible steric repulsions, must be considered.

Inasmuch as little accurate information on the intercationic distance can be obtained from K_i and K_s values alone, let us now turn to more profitable considerations of the topography of the active center. There is evidence from several lines that the active center is not a flat or slightly convex surface. In the first place, alkylsuccinates are bound to the enzyme very poorly; methylsuccinate is oxidized at 23% the rate for succinate, and ethylsuccinate at 18% the rate for succinate, while higher members are neither oxidized nor are they inhibitory (Franke, 1944 a). In the second place, as we have already seen, alkylmalonates are very poor inhibitors. Indeed, even the introduction of a hydroxyl group (tartronate) reduces the inhibition markedly. These observations indicate a rather close fit for malonate and succinate at the active center, additional groups giving rise to steric repulsion, as frequently reported for antigen-antibody reactions. In the third place, fumarate is bound fairly well while maleate is not, indicating again some steric repulsion since the intercarboxylate distances alone would certainly allow predictions that maleate would be bound more tightly. All molecules that bind appreciably to succinate dehydrogenase seem to be simple linear substances, or substances capable of assuming a linear configuration. All of this evidence points to a slit or tubular structure for the active center, such that compounds with added groups or rigid nonlinear molecules cannot enter. Such a situation is pictured in Fig. 1-8 in two dimensions. This is not to be construed as an attempt to represent the actual configuration but merely to show the steric barriers impeding attachment of larger or nonlinear molecules. Glutarate and adipate could not fit well, not because of unsatisfactory intercarboxylate distances, but because of the bulkiness of the longer hydrocarbon chains. Fumarate is able to bind because its configuration is much like that of succinate in the

extended form shown in the figure, whereas maleate might not fit because of its nonlinear structure. Acetylene-dicarboxylate would be expected to inhibit to some extent because of its linearity. Such a model would also explain why small alkyl groups added to succinate do not completely abolish the binding. It may also be mentioned that this type of configuration would allow the flavin and iron components of the dehydrogenase to be in positions close to the $—CH_2CH_2—$ group and thus able to participate in the removal of the hydrogen atoms.

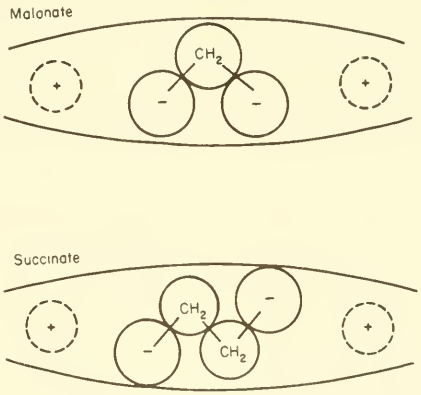


FIG. 1-8. Representations of the binding of malonate and succinate at the active site of succinate dehydrogenase, indicating the steric barriers possibly surrounding the region of the two cationic sites. The actual situation must be visualized in three dimensions.

Activation of Succinate Dehydrogenase by Malonate

Preparations of beef heart succinate oxidase obtained using borate buffer are not fully active but may be activated by the addition of phosphate. This interesting discovery by Kearney (1957, 1958) may have important bearings on the understanding of the active center of this enzyme, especially as she later found that succinate, fumarate, and malonate also activate, and indeed are much more potent than phosphate (see accompanying tabulation). Once the enzyme has been activated, the activator can be removed without loss of the activity; in fact, malonate must be dialyzed away if the full activity of the enzyme is to be measured. The activation constants are quite different from the Michaelis or inhibitor constants for these substances. It would appear that malonate binds more tightly to the less active form of the enzyme. Kearney favors the view that these activa-

tors convert a less active form of the enzyme to a more active form, and that this transformation may involve a localized change in the protein

Activator	K_{act} (mM)	K_m or K_i (mM)
Malonate	0.0072	0.025
Succinate	0.12	0.52
Fumarate	5.6	0.80
Phosphate	100	—

structure because of the high energy of activation. Whatever the explanation it is important to remember that malonate can exert an activating effect, as well as inhibiting, in media low in phosphate.

Effects of Various Factors on the Inhibition of Succinate Dehydrogenase

Very few illuminating studies on the modification of malonate inhibition are available. The *effects of temperature* were mentioned in Volume I, where the following thermodynamic parameters for the inhibition of beef heart succinate dehydrogenase at 38° were calculated: $\Delta F = -6.26$ kcal/mole, $\Delta H = -5.48$ kcal/mole, and $\Delta S = 2.6$ cal/mole/degree. The K_i for malonate was found to be 0.025 mM at 20°–23° and 0.041 mM at 38° (Kearney, 1957). The *effects of osmolarity* on the inhibition are surprisingly large (Honda and Muenster, 1961). Lupine mitochondria were prepared in media of different sucrose concentrations and assayed in media of two osmolarities (Table 1-9). These results were obtained on mitochondria and it is possible that the effects are not directly on succinate oxidase but on the permeability or structural properties of the mitochondria. In this connection it has been pointed out by Singer and Lusty (1960) that *N*-methylphenazine measures the full activity of succinate dehydrogenase in mitochondrial fragments, but in intact mitochondria it measures only a fraction of the activity. Various ways of damaging the mitochondria lead to increased succinate dehydrogenase activity (such as increase in Ca^{++} concentration). This was interpreted in terms of permeability barriers to *N*-methylphenazine (and also $FMNH_2$), limiting the rate in intact mitochondria. It could also be explained on the basis of structural changes in the enzyme complexes. In any case, these observations demonstrate the importance of the mitochondrial state in the functioning of succinate oxidase, and it would not be surprising if malonate inhibition were similarly sensitive. The inhibition of succinate oxidation by malonate in rat heart mitochondria in KCl medium is not altered by either halving or doubling the KCl concentration in the assay medium (Montgomery and Webb, 1956 b). However, the *effects*

TABLE 1-9

EFFECT OF OSMOLARITY IN THE PROPERTIES OF SUCCINATE OXIDASE IN LUPINE MITOCHONDRIA [a]

Preparation osmolarity (M)	Assay osmolarity (M)	K_m (mM)	K_i (mM)	K_m/K_i	a
0.15	0.22	5.10	0.91	5.6	2
	0.60	12.34	0.64	19.3	1
0.40	0.22	2.87	0.19	15.1	8
	0.60	5.47	0.16	34.2	191
0.60	0.22	1.20	0.05	24.4	36
	0.60	5.47	0.11	49.7	268

[a] K_m is the Michaelis constant for succinate, K_i the inhibitor constant for malonate, and a is the interaction constant defined in Eqs. I-3-5 and I-3-6, indicating the type of inhibition. The osmolarity is given in terms of sucrose concentration. (From Honda and Muenster, 1961.)

of Ca^{++} concentration on both succinate oxidation and malonate inhibition are complex (Fig. 1-9) and difficult to explain. The decrease in the rate of oxidation beyond Ca^{++} concentrations around 5 mM might be attributed to a complexing of the succinate, but for the same reason, namely, the complexing of malonate, which has a higher affinity for Ca^{++} than does succinate, the inhibition would be expected to decrease. Whether the

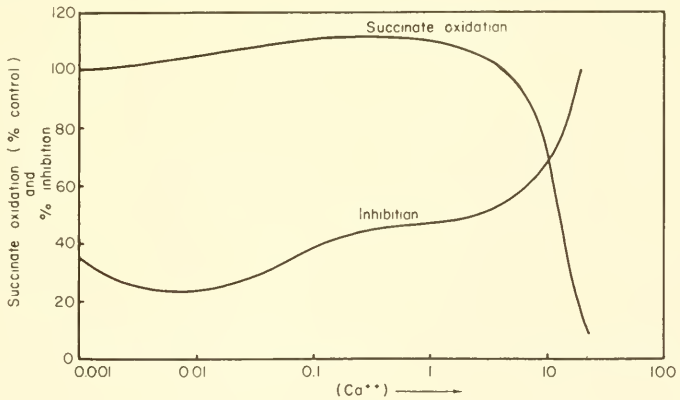


FIG. 1-9. Effects of Ca^{++} on the rate of succinate oxidation and the inhibition by malonate in rat heart mitochondria. Succinate is 5 mM and malonate is 1 mM. (From Montgomery and Webb, 1956 b).

modest stimulation of succinate oxidation by low concentrations of Ca^{++} is a permeability or structure-opening effect on the mitochondria, or due to a more direct effect on the enzyme, is not known; certainly the oxidations of other cycle substrates and pyruvate are strongly depressed by Ca^{++}. The *effects of* Mg^{++} *concentration* from 6.2 to 12.4 m*M* on trypanosomal succinate dehydrogenase inhibition by malonate have been reported as negligible (Agosin and von Brand, 1955). In connection with the relationship between mitochondrial structure and malonate inhibition, it is interesting to examine the *effect of ATP*, inasmuch as ATP protects or stabilizes mitochondria after isolation from cells. ATP at 1 m*M* has very little effect on the rate of succinate oxidation in rat heart mitochondria (in five experiments a mean depression of 3%), but in the presence of succinate (5 m*M*) and malonate (5 m*M*) it stimulated the rate 67%, thus antagonizing the inhibition by malonate (Montgomery and Webb, 1956 b). Similar results were seen with acetylene-dicarboxylate inhibition. If the effect of ATP is to reduce the permeability to these inhibitors, it is surprising that interference with succinate penetration does not also occur. Thus some other explanation may have to be sought.

Inhibition of Fumarate Reduction

If succinate, fumarate, and malonate bind at the same site on succinate dehydrogenase, the reverse reaction — the hydrogenation of fumarate to succinate — should be inhibited by malonate; that is, malonate should compete with fumarate as well as with succinate. The relative potencies of the inhibitions on the two reactions would depend on the Michaelis constants for succinate and fumarate, so that the inhibitions would not necessarily be identical. Malonate was, indeed, found to inhibit the reduction of fumarate by horse muscle succinate dehydrogenase, but less potently than the oxidation of succinate (18 m*M* malonate required to inhibit 50% in the former case and 3.6 m*M* in the latter) (Das, 1937 b). A more detailed analysis was made by Forssman (1941) in Lund, using pig heart succinate dehydrogenase and leucomethylene blue as a hydrogen donor, in this case the inhibition by malonate being essentially equivalent for both forward and backward reactions. More recent studies of beef heart succinate dehydrogenase, however, have given different values for K_i: 0.025 m*M* for succinate oxidation and 0.12 m*M* for fumarate reduction (Singer *et al.*, 1956 a). This difference is unexpected and the suggestion was made that the binding of malonate may be effected by the state of oxidation of the electron transport components adjacent to the binding site. The affinities of fumarate for the enzyme were also found to be different for each reaction. It may also be of significance that *N*-methylphenazine was the acceptor in the oxidation of succinate, and $FMNH_2$ or leucodiethylsafranin the donor in the reduction of fumarate.

The succinate dehydrogenase of *Micrococcus lactilyticus* behaves quite differently and is poorly inhibited by malonate, a ratio of (malonate)/(fumarate) = 20 being required for 29% inhibition (Peck *et al*, 1957). It might be thought that this enzyme is not succinate dehydrogenase, but another enzyme that could be called " fumarate reductase," especially as fumarate is reduced at a faster rate than succinate is oxidized, in contrast to the mammalian enzymes. However, it has been conclusively demonstrated it is not a separate enzyme and that the failure of malonate to inhibit is to be attributed to a very high affinity for fumarate coupled with a relatively low affinity for malonate (Warringa *et al.*, 1958). The configuration of the active center of the bacterial enzyme must differ from that of the mammalian enzymes. This may also explain the "fumarate reductases" obtained from yeast (Fischer and Eysenbach, 1937; Kováč, 1960) which are rather insensitive to malonate.

Variations of Malonate Inhibition of Succinate Dehydrogenases from Different Tissues and Species

The comparative biochemistry of enzyme inhibition is in its infancy and accurate comparison of results is usually impossible due to the different conditions under which the inhibitions were studied. Examination of Table 1-6 with a view to establishing phylogenetic relationships is made difficult by the different types of preparation and assay procedure used. A correlation graph, made by plotting inhibitions against (I)/(S) ratios, shows a very marked scatter of the points. For example, at an (I)/(S) ratio of 0.1. the inhibitions range from 10% to 100%. This variation cannot all be due to the differences in technique. All of those cases in which the malonate inhibition is significantly below the mean turn out to be in the bacteria, invertebrates, or plants. However, this is not a strict correlation because some of the potent inhibitions have been found in such organisms (*e.g.*, *Azotobacter*, *E. coli*, and *Trypanosoma*). The possibility of a relationship between succinate dehydrogenase type in the bacteria and the oxygen requirements for growth has been proposed. The enzyme from the obligate anaerobe *Micrococcus lactilyticus* has low affinities for succinate and malonate, as discussed in the previous section, whereas the enzyme from the facultative anaerobe, *Propionibacterium pentosaceum* is intermediate in properties between *Micrococcus* and the aerobic mammalian tissues (Singer and Lara, 1958). Certainly many invertebrate and plant tissues can withstand anaerobiosis better than mammalian tissues, but at the present state of our knowledge such a correlation is dangerous to make.

Comparisons of the malonate inhibitions of succinate dehydrogenases from different tissues have been reported in a few cases. The epithelium and muscle of guinea pig seminal vesicle were separated and the inhibitions by malonate at four different concentrations were similar (Levey and Szego,

1955). However, the inhibitions of epithelial dehydrogenase appear to be about 5% higher than for the muscle enzyme, although this may not be statistically significant. Malonate was found to inhibit Hepatoma 134 tumor succinate dehydrogenase more than the enzyme from normal mouse liver (Fishgold, 1957) over a range of five malonate concentrations; for example, 0.21 m*M* malonate inhibited the liver enzyme 42% and the hepatoma enzyme 76% at a succinate concentration of 1 m*M*. Killer and sensitive stocks of paramecia may have succinate dehydrogenases with different sensitivities to malonate, but it is difficult to draw conclusions from the data published (Simonsen and van Wagtendonk, 1956). The O_2 uptake of homogenates of the two strains was increased to different degrees by 50 m*M* succinate and malonate inhibited both quite well (see accompanying tabulation). The authors concluded that the enzyme from the killer strain, is inhibited more, based on absolute reduction, but actually

	Increase in O_2 uptake from succinate	
	Sensitive strain	Killer strain
Control	1.4	14.0
With malonate 80 m*M*	0.2	3.8
% Inhibition	86	73

the enzyme from the sensitive strain seems to be inhibited as well. Our work with succinate dehydrogenase from various rat tissues (Table 1-6) indicates no significant difference in susceptibility to malonate.

INHIBITION OF SUCCINATE OXIDATION IN CELLULAR PREPARATIONS

Attention will now be turned to the inhibition of the succinate oxidase system when it is located in the normal cellular structure and succinate is added exogenously to the preparations. When succinate is added to most cell suspensions, minces, or slices, there is an increase in the O_2 uptake, and this response is inhibited to varying degrees by malonate (Table 1-10). It is particularly important in cellular preparations to take account of the endogenous respiration and the effect of malonate on it (see Chapter I-9). In many studies this has not been done and this is one factor that makes it difficult to compare accurately the malonate inhibitions *in vitro* and *in vivo*. Since the endogenous respiration is generally inhibited less than succinate oxidation by malonate, failure to correct for endogenous respiration usually leads to low values for the inhibition. This is illustrated in

the four examples given in Table 1-11. The importance of the endogenous correction is seen to vary with the effect of malonate on the endogenous respiration. When malonate inhibits the endogenous O_2 uptake poorly, as in rat liver, or actually stimulates the O_2 uptake (due to its metabolism), as in *Euglena*, the true inhibition of succinate oxidation is much higher than would be calculated simply from the data on succinate and succinate + malonate.

Comparison of the inhibitions in Tables 1-6 and 1-10 for the same species or tissues, although this is qualitative only, shows that in several instances the inhibitory potency of malonate seems to be significantly less in cellular preparations. This is true for *E. coli*, *Rhodospirillum*, *Crithidia*, *Zygorrhynchus*, pigeon muscle, and rat liver. Moses (1955) points out that in *Zygorrhynchus* the inhibition of succinate oxidation in cell suspensions is very weak, even at the low pH of 3.4, but when the cells are treated with liquid nitrogen to destroy their structure, malonate inhibits normally. The oxidation of succinate by cell suspensions of *Bacterium succinicum* is not inhibited at all by 5 m*M* malonate whereas such oxidation in cell-free extracts is inhibited completely (Takahashi and Nomura, 1952). There are also several reports in which malonate was found to be ineffective but the extracted succinate oxidase system was not directly tested; however, in these cases one would certainly expect the enzyme to be sensitive to malonate. For example, malonate (2 m*M*) does not inhibit the oxidation of succinate by barley roots (Honda, 1957), or at 5–20 m*M* in dried cells of *Chlorella* (Millbank, 1957), while in beech roots malonate (28.6 m*M*) actually stimulates the rate of succinate oxidation (Harley and Ap Rees, 1959). However, in some cases comparable inhibitions have been observed *in vitro* and *in vivo*. Danforth (1953) showed in *Euglena* that malonate is quite effective in intact cells if the pH is low enough (around 4.5) and, although it is difficult to compare the results with those obtained from homogenates because of different concentrations of succinate and malonate, it would appear that malonate is equally inhibitory in the two preparations. Similar effects of malonate were also observed in our work (Montgomery and Webb, 1956 b) on rat heart slices and mitochondrial suspensions.

The failure of malonate to inhibit the oxidation of added succinate in cellular preparations well, or at all, has usually been attributed to permeability factors. However, it is difficult to understand how permeability could explain these results, inasmuch as the penetration of both succinate and malonate would be controlled by the same factors, presumably. That is, if there is some barrier to malonate reaching the succinate dehydrogenase, how can succinate pass this barrier? It is true that the pK_{a_1} for succinate is higher than for malonate but almost identical values of pK_{a_2} have been reported; thus the distribution of the ionic forms around neutrality would be approximately the same (Table 1-3). If only the uncharged forms of these acids —

TABLE 1-10

INHIBITIONS OF SUCCINATE OXIDATION IN CELLULAR PREPARATIONS BY MALONATE [a]

Organism	Tissue	Preparation	pH	Succinate (m*M*)	Malonate (m*M*)	% Inh.	Reference
Escherichia coli		Suspension	7.4	67	67	24	Quastel and Wheatley (1931)
		Suspension	—	10	200	86	Aubel *et al.* (1950)
Pseudomonas aeruginosa		Suspension	7.4	67	67	44	Quastel and Wheatley (1931)
		Suspension	6.9	10	10	80	Gray (1952)
Pseudomonas saccharophila		Suspension	5.9	25	1	0	Bernstein (1944)
					10	31	
					25	54	
					50	76	
Bacillus subtilis		Suspension	7.4	67	67	20	Quastel and Wheatley (1931)
Proteus vulgaris		Suspension	7.4	67	67	26	Quastel and Wheatley (1931)
Acetobacter pasteurianum		Suspension	6.0	17	70	50	King *et al.* (1956)
Alcaligenes fecalis		Suspension	7.4	67	67	54	Quastel and Wheatley (1931)
Serratia marscescens		Suspension	7.4	67	67	21	Quastel and Wheatley (1931)
Corynebacterium sp.		Suspension	6.0	16	24	50	Ladd (1956)

Rhodospirillum rubrum		Suspension	—	3.5	30 100 300	25 39 80	Clayton *et al.* (1957)
Rhizobium sp. (root nodule bacteria)		Suspension	6.5	13	40 200	26 78	Burris and Wilson (1939)
Neurospora (poky strain)		Suspension	—	4.6	10	100	Haskins *et al.* (1953)
Mycorrhiza (beech roots)		Suspension	5.5	28.6	28.6	38	Harley and Ap Rees (1959)
Streptomyces olivaceous		Suspension	7.2	3.3	3.3 6.7 10	40 65 85	Maitra and Roy (1961)
Schizophyllum commune	Mycelia	Suspension	—	—	1	20	Wessels (1959)
Zygorrhynchus moelleri		Suspension	3.4	10	20	26	Moses (1955)
Rhodotorula gracilis		Suspension	5.5	0.02	40	98	Litchfield and Ordal (1958)
Oat (*Avena sativum*)	Coleoptile	Mince	—	50	67	45	Berger and Avery (1943)
	Coleoptile	Whole	4.5	—	27.6	100	Bonner (1949)
Apple (*Malus malus*)	Fruit	Slice	4.5	20	20	93	Hatch *et al.* (1959)
Euglena		Suspension	4.5	10	10 30	65 100	Danforth (1953)
Plasmodium vivax		Suspension	7.4	—	20	100	Speck *et al.* (1946)

TABLE 1-10 (*continued*)

Organism	Tissue	Preparation	pH	Succinate (m*M*)	Malonate (m*M*)	% Inh.	Reference
Crithidia fasciculata		Suspension	7.4	2	1	79	Hunter (1960)
				5	1	76	
				10	1	71	
				15	1	69	
				20	1	60	
Ascaridia galli		Mince	7.3	10	10	74	Massey and Rogers (1950)
Nematodirus filicollis		Mince	7.3	10	10	82	Massey and Rogers (1950)
Moniezia benedeni (cestode)		Pulp	—	10	100	26	Van Grembergen (1945)
Snail (*Helix pomatia*)	Hepatopancreas	Slice	7.73	10	100	67	Baldwin (1938)
Oyster (*Crassostrea virginica*)	Mantle		8.0	5	25	38	Jodrey and Wilbur (1955)
Sea-hare (*Aplysia depilans*)	Gizzard	Slice	7.3	10	10	57	Ghiretti *et al.* (1959)
Locust (*Schistocerca gregaria*)	Fat body	Sheet	7.4	10	20	20	Clements (1959)
Chicken embryo	Cartilage		7.4	10	10	50	Boyd and Neuman (1954)
					100	75	

Pigeon	Muscle	Mince	7.4	24.2	0.354	12	Krebs and Johnson (1948)
					1.06	28	
					3.18	54	
					9.55	79	
					29.7	92	
					86	97	
				21	10	82	
				4.2	10	96	
				0.84	10	99	
	Muscle	Mince	7.4	1	1	0	Stare and Baumann (1939)
					10	58	
Rat	Brain	Mince	7.38	10	10	98	Cohen and Gerard (1937)
	Liver	Slice	7.4	10	20	69	Edson (1936)
	Heart	Slice	7.4	—	20	74	Fawaz and Fawaz (1954)
	Heart	Slice	7.4	20	5	55	Webb *et al.* (1949)
					20	94	
Rabbit	Brain	Mince	7.4	67	67	95	Quastel and Wheatley (1931)
	Muscle	Mince	7.4	67	67	91	Quastel and Wheatley (1931)
	Reticulocytes	Suspension	7.4	100	25	74	Rubinstein *et al.* (1956)
Man	Brain	Mince	7.4	67	67	87	Quastel and Wheatley (1931)
	Prostate	Slice	7.2	100	100	100	Andrews and Taylor (1955)
	Placenta	Slice	6.9	16.7	10	90	Hosoya and Kawada (1958)

[a] In some cases a correction for the malonate inhibition of endogenous oxygen uptake has been made and the inhibitions given may be somewhat different from those reported by the original authors.

Table 1-11

Effects of Endogenous Respiration on Calculations of Malonate Inhibition of Succinate Oxidation [a]

	Pigeon muscle	Rat kidney	Rat liver	*Euglena*
O_2 Uptake				
Endogenous	11.8	14.3	14.7	11.4
Malonate	3.0	4.5	12.4	13.9
Succinate	19.0	58.5	20.2	28.0
Succinate + malonate	6.0	15.8	14.1	19.7
% Inhibition				
Endogenous respiration	74.6	68.6	15.6	Stim 21.9
Total respiration in the presence of succinate	68.4	73.0	30.2	29.6
Succinate oxidation	58.3	74.4	69.1	65.1

[a] The true inhibition of succinate oxidation is given in the bottom row, and is to be compared to the figures in the row immediately above where the correction for the endogenous effect has not been made. The concentrations were: pigeon muscle — succinate 1 m*M* and malonate 10 m*M* (Stare and Baumann, 1939); rat kidney — succinate 20 m*M* and malonate 20 m*M* (Fawaz and Fawaz, 1954); rat liver — succinate 10 m*M* and malonate 20 m*M* (Edson 1936); *Euglena* — succinate 10 m*M* and malonate 10 m*M* (Danforth, 1953).

HOOC—R—COOH — penetrated, succinate would enter cells somewhat better than malonate, but, at least at pH's above 7, it seems unlikely that this is the situation. Also, the entrance of enough of the undissociated caid to be effective would presumably decrease the intracellular pH significantly (see Chaper I-14). Besides, some of these experiments in which malonate was inactive were done at low pH's (3.4 to 5.5). A question that must be considered is whether succinate oxidation is always entirely intracellular. It is possible that succinate oxidase occurs both in the mitochondria and in the plasma membrane. In many cases, malonate has little or no effect on tissue metabolism or function at concentrations capable of inhibiting the oxidation of added succinate completely. This is well seen in rat ventricle slices (Webb *et al.*, 1949) where the succinate may be oxidized at the cell surface, and it is interesting that in this tissue the potency of malonate *in vivo* and *in vitro* is the same. However, in most instances, the succinate oxidase seems to be protected in some manner in intact cells.

There are several possible factors that could modify the malonate inhi-

bition of succinate dehydrogenase when the enzyme is isolated from the cells. It will be well to mention some of these in order to emphasize that there are usually many ways, other than by permeability, by which unexpected phenomena may be explained.

(*a*) *The enzyme environment within the cell is different from the artificial media* used with isolated enzymes (see Chapter I-9). Many substances may be able to alter malonate inhibition; we have noted the effects of Ca^{++} and ATP. The concentrations of such substances may be different in cell and medium. The intracellular pH is also not that of most media used in enzyme study and may be easily changed by the addition of external substrate or inhibitor.

(*b*) *The addition of succinate to cells may influence the endogenous respiration*; that is, the change in O_2 uptake upon adding succinate may not represent accurately the rate of succinate oxidation. In other words, the correction for endogenous respiration may be in error. Also, the addition of succinate or malonate can secondarily alter the complex balance of the metabolic systems. For example, oxalacetate is a very potent inhibitor of succinate dehydrogenase and its concentration in the cell may be a controlling factor in the operation of the cycle. Since oxalacetate can be formed from succinate, and its formation inhibited by malonate, secondary changes in succinate dehydrogenase activity may occur that are easily interpreted as due to the direct effects of malonate.

(*c*) *The concentration of succinate in the cell may already be appreciable* and the addition of more may reduce the inhibition by malonate because of the competitive nature of the inhibition.

(*d*) *The rate of succinate oxidation may be limited by the rate at which it can enter into the cells or tissus*; that is, the succinate oxidase is so active that the succinate is oxidized as rapidly as it enters. In such a case, malonate would be quite ineffective until the enzyme is inhibited sufficiently to make it limiting.

(*e*) *The ratio* (*malonate*)/(*succinate*) *may be different in the cell than in the medium.* This could be due to differences in the permeabilities of the cell membrane towards these substances for, despite the fact that succinate and malonate have similar properties, many cases of differential permeabilities to more closely related ions are known. It could also be due to the somewhat different pK_a values, since the internal concentrations of the active ions are determined by the ionization constants (see Eq. I-14-146 for buffered cells).

Whether these factors, or others, are responsible for the anomalous results mentioned above is not known. It would be very useful to have data on the

rates of penetration of succinate and malonate into cells, obtained preferably with radioactive material and under the same conditions. Quantitative studies on the malonate inhibition of intracellular dye reduction resulting from succinate oxidation might also be informative in certain instances. It has been shown that malonate inhibits the succinate-induced reduction of neotetrazolium in adipose tissue cells (Fried and Antopol, 1957), but nothing otherwise is known about the succinate dehydrogenase from this tissue.

Competitive Nature of the Inhibition in Cellular Systems

It has been shown in several tissues that the addition of succinate will reverse the inhibition of respiration produced by malonate. Thus in *Avena* coleoptile the inhibition by 50 m*M* malonate is reduced from 57.4% to 25.8% upon adding succinate (Bonner, 1948), and in spinach leaves from 75.4% to 20.5% (Bonner and Wildman, 1946). In chick embryonic cartilage, the depression of respiration by 10 m*M* malonate is reversed by 100 m*M* succinate but not by 10 m*M* (Boyd and Neuman, 1954). Such results have occasionally been stated to prove the competitive nature of the inhibition but this reasoning is not completely valid. The mere increase in O_2 uptake seen on addition of succinate to malonate-inhibited tissues is alone not evidence for competition. The effects of succinate on uninhibited tissue must also be tested and it must be shown that the actual inhibition is decreased. A decrease in the inhibition brought about by increasing succinate concentrations has indeed been reported in two tissues, pigeon breast muscle (Krebs and Johnson, 1948) and the trypanosome *Crithidia* (Hunter, 1960) and in the latter a true competitive inhibition was demonstrated by $1/v$—$1/(S)$ plots. The data are given in Table 1-10. It is probable that the inhibition of succinate oxidation in cellular systems by malonate would frequently not obey strictly competitive kinetics, due to the various complexities that arise, as discussed in the previous section, even though the primary inhibition on the succinate dehydrogenase were competitive. Some of the problems involved in the determination of the type of inhibition in cells have been discussed in Chapter I-9.

INHIBITIONS OF ENZYMES OTHER THAN SUCCINATE DEHYDROGENASE

It is very important to establish the degree of specificity that may be achieved in the use of malonate under various conditions. To this end we shall first discuss the direct evidence for the inhibition of enzymes other than succinate dehydrogenase, and then proceed to the effects on the operation of the tricarboxylic acid cycle, the accumulation of succinate and other intermediates, and finally the antagonism of the malonate inhibition

by fumarate. We shall then be in a position to evaluate the specificity of malonate. There are other reasons, of course, for taking up these subjects; for example, malonate is frequently used to block the cycle in living tissue and in this connection it is essential to understand how malonate can alter cycle activity under various conditions.

Some effects of malonate on miscellaneous enzymes are presented in Table 1-12. There are three major difficulties in the establishment of the over all spectrum of action of malonate. First, there are many quite important enzymes whose response to malonate has never been investigated directly. Second, inspection of the table will show that in few cases has more than one concentration been used, and often the single concentration reported is either too high or too low to be of much value. Third, the same enzyme from different species often shows widely varying susceptibility to malonate (e.g. NADH oxidase, β-glucuronidase, lactate dehydrogenase, oxalacetate decarboxylase, and others in the table), making it clear that there are many different spectra of malonate inhibition and that statements on specificity must be qualifield by naming the source of the enzymes in question.

Ideally, the results on the inhibition of an enzyme by malonate should be given for several concentrations, preferably convering the range from that concentrations just sufficient to produce some inhibitions, through that causing approximately 50% inhibition to higher inhibitions (unless these latter concentrations are unreasonably high). Another way of looking at the problem is to consider that range of malonate concentrations most likely to give useful information when tested on enzymes. This will depend on the source of the enzymes. For example, in mammalian tissues it usually requires malonate concentrations between 2 and 5 mM to inhibit succinate dehydrogenase around 90% in the presence of 5–10 mM succinate. In the interests of establishing the degree of specificity, it would thus be most important to test the effects of malonate at concentrations around 5 mM on enzymes from such sources. When the organism studied possesses a succinate dehydrogenase less sensitive to malonate, correspondingly higher concentrations must be applied to the other enzymes.

Instances of Competitive Inhibition

Until it is time to discuss the matter of specificity, there is little to say about these inhibitions since the results in the table speak for themselves. It is evident that several enzymes other than succinate dehydrogenase are readily inhibited. One would not be surprised if enzymes attacking the dicarboxylate anions, where the carboxylate groups are separated by two carbon atoms, were inhibitable by malonate to some extent, since it is likely that these enzymes also possess cationic groups appropriately spaced. Actually, fumarase, malate dehydrogenase, the malic enzyme, oxalacetate

TABLE 1-12

INHIBITIONS OF ENZYMES OTHER THAN SUCCINATE DEHYDROGENASE BY MALONATE

Enzyme	Source	Malonate (m*M*)	% Inhibition	Reference
Acetylenecarboxylate hydrase	*Pseudomonas* sp.	10	0	Yamada and Jakoby (1959)
Aconitase	Pigeon muscle	67	0	Breusch and Keskin (1944)
Adenosinetriphosphatase	Liver	10	0	Myers and Slater (1957 a, b)
Alanine dehydrogenase	*Mycobacterium tuberculosis*	1	0	Goldman (1959 b)
D-Amino acid oxidase	Lamb kidney	3	10	Frisell *et al.* (1956)
L-Amino acid oxidase	Rat kidney	10	0	Blanchard *et al.* (1944)
Carboxypeptidase	Pancreas	500	0	Smith *et al.* (1951)
Choline oxidase	Rat liver	30	5	Rothschild *et al.* (1954)
Creatine kinase	Rabbit muscle	120	100	Kuby *et al.* (1954)
Cysteine desulfurase	Dog liver	10	0	Fromageot and Grand (1944)
		100	11	
Cytochrome oxidase	*Arum maculatum* spadix	10	11	Simon (1957)
	Lamb liver	50	Stim 10	Eichel and Wainio (1948)
Diamine oxidase	Pig kidney	10	17	Zeller (1938)
Fructose-1,6-diphosphatase	*E. coli*	200	69	Aubel and Szulmajster (1950)
Fumarase	*Avena* coleoptile	67	21	Berger and Avery (1943 a)
	Pig heart	40	50	Massey (1953 b)

Glucose dehydrogenase	Lamb liver	50	8	Eichel and Wainio (1948)
	Beef liver	100	0	Nakamura (1954)
β-Glucuronidase	Mouse liver	15	0	Karunairatnam and Levvy (1949)
	Beef spleen	10	40	Mills *et al.* (1953)
Glutamate dehydrogenase	Beef liver	8	7	Caughey *et al.* (1957)
γ-Glutamyl transferase	Pumpkin seedlings	5	0	Stumpf *et al.* (1951)
Glycero-P dehydrogenase	Fly muscle	2	0	Klingenberg and Bücher (1961)
Glycolate oxidase	Tobacco	1	0	Clagett *et al.* (1949)
		10	30	
Glyoxylate reductase	Tobacco	10	0	Zelitch (1955)
Hydrogenase	*Proteus vulgaris*	80	0	Hoberman and Rittenberg (1943)
D-α-Hydroxy acid dehydrogenase	Rabbit kidney	$K_i = 1.2$		Tubbs (1962)
β-Hydroxybutyrate dehydrogenase	Pig heart	60	29	Green *et al.* (1937 a)
Isocitrate dehydrogenase	Pig heart	—	0	Adler *et al.* (1939)
Isocitrate lyase	*Pseudomonas indigofera*	8.5	70	McFadden and Howes (1963)
	Castor beans	1 × (S) [a]	10	Carpenter and Beevers (1959)
		5 × (S)	94	
α-Ketoglutarate oxidase [b]	Pea seedlings	1	15	Price (1953)
		10	40	
		30	60	
	Mosquito	1	28	Gonda *et al.* (1957)
		10	43	
	Pigeon muscle	3.3	0	Stumpf *et al.* (1947)
	Rat heart	10	0	Slater and Holton (1954)

TABLE 1-12 (*continued*)

Enzyme	Source	Malonate (m*M*)	% Inhibition	Reference
	Rabbit kidney	33	>12	Grafflin *et al.* (1952)
		40	>28	
	Cat heart	50	0	Ochoa (1944)
D-Lactate: cytochrome c reductase	Yeast	5	0	Nygaard (1961 b)
Lactate dehydrogenase	*E. coli*	71	15	Quastel and Wooldridge (1928)
	Pigeon muscle	2.2	50	Das (1937 b)
Lactate oxidase	*Mycobacterium tuberculosis*	40	0	Yamamura *et al.* (1952)
Lipase	Pig liver	24	20	Gajdos (1939)
		48	40	
		72	80	
		96	90	
Malate decarboxylase	*Lactobacillus arabinosus*	10	0	Nossal (1952)
Malate dehydrogenase	*Mycobacterium tuberculosis*	74	16	Yamamura *et al.* (1954)
	Moraxella lwoffi	10	10	Lwoff *et al.* (1947)
		20	20	
		50	23	
	Pigeon liver	20	30	Scholefield (1955)
	Pigeon muscle	106	50	Das (1937 b)
	Pig heart	30	11	Green (1936)
		300	47	

Malate dehydrogenase (decarboxylating)	Beef lens	8.3 33	55 100	Van Heyningen and Pirie (1953)
Malcate dehydrogenase	Pigeon muscle	10	Inh	Laki (1935)
NADH oxidase	*Tetrahymena pyriformis*	6.2 18	35 70	Eichel (1959)
	Beet root	30	0	Wiskich *et al.* (1960)
Oxalacetate decarboxylase	*Mycobacterium tuberculosis*	25	0	Yamamura *et al.* (1954)
	Azotobacter vinelandii	10	0	Plant and Lardy (1949)
	Moraxella lwoffi	10	Inh	Lwoff *et al.* (1947)
	Pigeon liver	10	100	Evans *et al.* (1943)
	Pigeon liver	10	85	Salles and Ochoa (1950)
	Pigeon liver	25	90	Liebecq and Peters (1949)
	Rat liver	1	0	Corwin (1959)
Oxalacetate transacetase (condensing enzyme)	Rat liver	20	55	Pardee and Potter (1949)
Oxalate decarboxylase	*Coriolus hirsutus*	1	0	Shimazono (1955)
Oxalosuccinate decarboxylase	Pigeon liver	25	0	Liebecq and Peters (1949)
Phosphatase (acid)	Mouse liver	50	0	Macdonald (1961)
	Rat liver	100	0	Goldberg *et al.* (1962)
	Human prostate	10	0	Anagnostopoulos (1953 a)
	Human prostate (S = phenyl-P)	10	0	Nigam *et al.* (1959)
	(S = *p*-nitrophenyl-P)	10	11	
	(S = β-glycero-P)	10	66	
3-Phosphoglyceraldehyde dehydrogenase	Rabbit muscle	7.4	0	Potter (1940)

TABLE 1-12 (*continued*)

Enzyme	Source	Malonate (m*M*)	% Inhibition	Reference
Pyrophosphatase	Human erythrocytes	2	10	Naganna and Menon (1948)
		10	30	
		20	70	
Pyruvate kinase	Rabbit muscle	30	67	Fawaz and Fawaz (1962)
		60	86	
Pyruvate oxidase	Pigeon muscle	3.3	0	Stumpf *et al.* (1947)
Succinate: methylmalonate isomerase	Pig heart	5	100	Flavin *et al.* (1955)
Transaminases				
Alanine: α-ketoglutarate	Wheat germ	10	7	Cruickshank and Isherwood (1958)
γ-Aminobutyrate: α-ketoglutarate	Beef brain	1	0	Baxter and Roberts (1958)
Aspartate: α-ketoglutarate	Wheat germ	10	0	Cruickshank and Isherwood (1958)
Kynurenine: α-ketoglutarate	Pig heart	40	0	Jenkins *et al.* (1959)
	Rat kidney	6	0	Mason (1959)
Urease	Jack bean	52	47	Kistiakowsky *et al.* (1952)
Urocanate oxidase	*Pseudomonas aeruginosa*	1	0	Ota *et al.* (1956)

[a] Concentrations of malonate given in terms of succinate concentration.

[b] Most of the studies with α-ketoglutarate oxidase include the oxidation of the succinate formed (see text).

decarboxylase, and the condensing enzyme are inhibited by malonate, although usually not as potently as is succinate dehydrogenase. Enzymes catalyzing reactions of the dicarboxylates in which the charges are farther apart (e.g. α-ketoglutarate) would be expected to be less susceptible. It is likely that these inhibitions are mostly competitive but sufficient data to establish this are generally lacking.

The inhibition of *fumarase* by malonate has been shown to be competitive in the thorough study of Massey (1953 b) but the affinity of the enzyme for malonate is not very high (K_i = 40 mM). This implies either a very different intercationic distance in this enzyme from that in succinate dehydrogenase or a different configuration of the enzyme surface surrounding these cationic groups, probably the latter. Both directions of the reaction catalyzed by the *malic enzyme* are inhibited by malonate, which competes with either malate (Stickland, 1959 b) or pyruvate (Stickland, 1959 a) (see accompanying tabulation). The inhibition of this enzyme might well alter

Malate (mM)	% Inhibition by malonate at:			Pyruvate (mM)	% Inhibition by malonate at:	
	2 mM	5 mM	20 mM		1 mM	10 mM
0.1	71	88	100	1	43	62
0.3	48	74	91	10	37	70
1	19	48	84	50	10	46

the operation of the tricarboxylic acid cycle under certain conditions. The situation is different for *lactate dehydrogenase*, since malonate is competitive with respect to lactate but noncompetitive with respect to pyruvate (Ottolenghi and Denstedt, 1958), leading to the suggestion that these two substrates react with different sites on the enzyme. The K_i's are 6.4 mM for the oxidation of lactate and 27 mM for the reduction of pyruvate. Oxalate and tartronate are much better inhibitors of this enzyme. The *D-α-hydroxy acid dehydrogenase* of yeast, which oxidizes lactate, malate, α-hydroxybutyrate, and glycerate, is competitively inhibited by malonate with a K_i of 0.9 mM (Boeri *et al.*, 1960). In this case, oxalate is a very potent inhibitor (K_i = 0.0025 mM) while tartronate is of similar potency to malonate. Finally, in reducing systems in which succinate can serve as an electron donor, malonate may inhibit competitively. This is the case for the particulate *nitrate reductase* of soybean root nodules, the K_i for malonate being 0.017 mM (Cheniae and Evans, 1959). The nitrate reductase is not inhibited by malonate directly but the results on the over all system might make it appear to be the case. In all cases where malonate inhibits competitively, the susceptibility of the reaction in complex systems will depend on the concentration of the substrate, and thus may be quite high in living systems where the concentrations of intermediates are frequently low.

Inhibition Due to Chelation with Metal Ion Cofactors

Many enzymes are dependent on dissociable metal ions for their activity and the operation of most of the important metabolic systems thus requires the presence of these cofactors. The list of enzymes requiring Mg^{++} is a long one and includes the oxidases and decarboxylases for the keto acids, most of the enzymes involved in phosphate metabolism (e.g., the kinases, the transphosphorylases, the phosphatases, and the acyl—CoA synthetases), some dehydrogenases e.g., phosphoglucose dehydrogenase, phosphogluconate dehydrogenase, and isocitrate dehydrogenase), some peptidases, phosphoglucomutase, and enolase. Several enzymes require Zn^{++}, such as lactate dehydrogenase, glutamate dehydrogenase, alcohol dehydrogenases, carboxypeptidase, and carbonic anhydrase. Since malonate is able to chelate effectively with these metal ions, inhibition may result from the reduction of metal ion concentration in the medium or the removal of the metal ions from the enzyme. The ability of malonate to inhibit by this mechanism will depend on the affinity of the enzyme for the metal ion. The binding of Zn^{++} to enzymes is usually rather strong and it is difficult for malonate to deplete the enzyme of this metal, but Mg^{++} is more loosely bound in most cases and the activity of enzymes dependent on it is generally related to the Mg^{++} concentration in the medium. The effect of malonate on Mg^{++}-dependent enzymes will thus depend on the concentrations of Mg^{++} and malonate, and on the relationship between enzyme activity and Mg^{++} concentration. In the use of malonate, especially at higher concentrations, it is imperative to consider the possibility of such effects. It is likely that some of the inhibitions in Table 1-12 are due to metal ion depletion.

Inhibition by reaction with an activator was discussed briefly in Chapter I-3 and it was seen that in the general case no simple expression for the inhibition is possible. Nevertheless, it should be reasonably easy to determine if the inhibition is purely the result of activator depletion, since the concentration of free activator can be calculated from the dissociation constant of the activator-inhibitor complex by an equation similar to Eq. I-3-72:

$$(\mathrm{Mg}) = \frac{1}{2}\sqrt{[(\mathrm{I}_t) - (\mathrm{Mg}_t) + K]^2 + 4(\mathrm{Mg})K} - \frac{1}{2}[(\mathrm{I}_t) - (\mathrm{Mg}_t) + K] \quad (1\text{-}3)$$

If the effect on the enzyme is only to reduce the Mg^{++} concentration, the addition of malonate should bring the activity to that value corresponding to the reduced free Mg^{++}. Another possibility is that the Mg-malonate complex is the active inhibitor, in which case the kinetics should be investigated with the calculated concentrations of this complex at different concentrations of Mg^{++} and malonate.

The most thorougly studied instance of the possible relationship of Mg^{++} to inhibition by malonate is the work on the utilization of oxalacetate

in rat tissue homogenates by Pardee and Potter (1949). The formation of citrate here probably involves decarboxylation of some of the oxalacetate to pyruvate, with subsequent condensation to enter the cycle. Although a single enzyme was not studied, it is possible that the utilization of oxalacetate was limited by the decarboxylase. This reaction is activated by low concentrations of Mg^{++} and inhibited by higher concentrations (upper curve in Fig. 1-10). It is clear that the inhibition by malonate de-

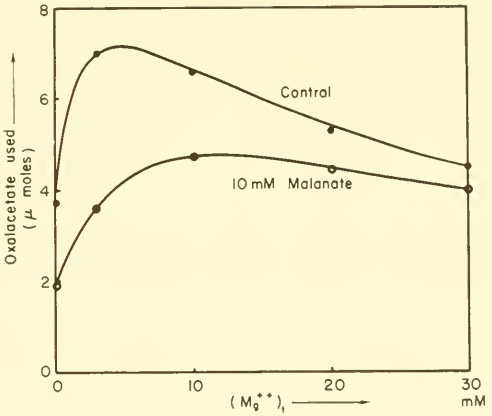


FIG. 1-10. Utilization of oxalacetate by a rat kidney homogenate at different concentrations of Mg^{++}, with and without malonate. (From Pardee and Potter, 1949).

creases as the Mg^{++} concentration is raised (lower curve). This was interpreted to mean that the inhibition is mainly due to depletion of Mg^{++}. Some arguments may be brought against this interpretation. Table 1-13 gives my calculations of the concentrations of free Mg^{++}, free malonate, and the complex at the different levels of total Mg^{++} used in the experiment shown in Fig. 1-10. It is seen that the malonate at 10 m*M* does indeed reduce the free Mg^{++}, but in the higher range of Mg^{++} concentrations this should increase the activity rather than decrease it, because in this range Mg^{++} is somewhat inhibitory. Pardee and Potter did not consider the reduction in free malonate concentration, which is very marked as shown in Table 1-13. This could well acount for the decrease in the inhibition from 47% at zero Mg^{++} concentration to 12% at 30 m*M* Mg^{++}. One might speculate that the reduction in rate at high Mg^{++} concentrations could be due to the complexing of oxalacetate so that it is unable to react with the enzyme. The question of the mechanism of the malonate inhibition is thus not settled. It was claimed that the inhibition is not typically competitive because increase in the oxalacetate concentration actually increases the inhibition somewhat,

Table 1-13

Effects of Total Mg^{++} Concentration on the Concentrations of Free Mg^{++}, Free Malonate, and Mg-Malonate Complex [a]

(Mg^{++}_t) (mM)	(Mg^{++}) (mM)	(Mg-malonate) (mM)	(Malonate) (mM)
0	0	0	10
3	1.59	1.41	8.59
10	6.13	3.87	6.13
20	14.09	5.91	4.09
30	22.98	7.02	2.98

[a] Conditions as in Fig. 1-10 from the work of Pardee and Potter (1949). The total malonate concentration is 10 mM in all cases. The values were calculated from Eq. 1-3 using 9.77×10^{-3} M for the dissociation constant of the Mg-malonate complex.

although no figures were given so the magnitude of the effect is unknown. An increase of oxalacetate might reduce the amount of Mg^{++} bound to malonate and thereby increase the free malonate concentration. It would appear unlikely that the Mg-malonate complex is inhibitory since its concentration increases with Mg^{++} concentration (Table 1-13), whereas the inhibition decreases. However, it is evident that malonate inhibits this enzyme system in some manner directly, not only from the above considerations but also because of the marked inhibition observed in the absence of Mg^{++}.

Dialkylfluorophosphatase is activated by Mn^{++} and its inhibition by malonate was attributed by Mounter and Chanutin (1953) to the chelation of the activator. Since no concentrations of either Mn^{++} or malonate were given, it is impossible to evaluate the results. However, their data show that malonate inhibits just as well, if not better, in the absence of Mn^{++} (21% with Mn^{++} added and 28% without Mn^{++} at 15 min). These results might better be interpreted as due to removal of free malonate by the Mn^{++}. If Mn-malonate is incubated with the Mn-free enzyme, activity slowly appears, indicating that the Mn^{++} is transferred from the malonate to the enzyme, which is not surprising since the affinity of the enzyme for the Mn^{++} is much greater, as shown by the dissociation constants (pK for enzyme-Mn^{++} complex is 7.7). It would be very surprising under these circumstances if malonate were able to reduce the Mn^{++} sufficiently to inhibit the enzyme.

There are several other claims for this mechanism of malonate inhibition but in all cases there is either inadequate evidence or no evidence at

all. The examples discussed above indicate the impossibility of establishing such a mechanism without considering the changes in malonate concentration or treating the data quantitatively. Despite this lack of positive evidence, there is certainly no doubt but that this type of inhibition can occur and may be sometimes very important. In the oxidation of pyruvate by rat heart mitochondria, the Mg^{++} concentration must fall below 1 m*M* before there is any significant decrease in the rate (Montgomery and Webb, 1956 b). We usually used 5 m*M* Mg^{++} in the medium so that it would have required at least 40 m*M* malonate to produce a detectable inhibition by this mechanism. Malonate at 50 m*M* did indeed inhibit around 50% but this must certainly be due to other actions to a large extent. These experiments were done with the α-ketoglutarate oxidase blocked by parapyruvate so that any inhibition of succinate oxidation would not be involved.

EFFECTS OF MALONATE ON THE OPERATION OF THE TRICARBOXYLIC ACID CYCLE

Malonate is usually assumed to produce its major effects on cellular metabolism and function by disturbing the operation of the cycle* and reducing the rate of formation of ATP. Malonate has often been used to establish if the cycle is operative in a tissue or if a particular functional activity is dependent on the cycle. It is thus important to examine critically the nature of the cycle block and the effects it may have on the over-all oxidative metabolism. There are two aspects that are especially relevant to this question. There is the problem of the specificity of action of malonate on succinate dehydrogenase and this will be considered later. In the present section we shall assume that the only inhibition is on the oxidation of succinate and discuss the problems relative to the interpretation of such a block. Before treating the actual results obtained with malonate, the nature of the cycle and its responses to inhibition will be outlined.

Some General Principles of Cycle Block

The primary function of the cycle is to incorporate and oxidize acetyl-CoA, whether this arises from pyruvate, acetate, fatty acids, or elsewhere, and thus it is particularly important to discuss the effects of malonate block on this. The situation is relatively clear in suspensions of isolated mitochondria, in which the concentrations of the cycle intermediates are low and the endogenous respiration is generally negligible. Pyruvate, or other substances giving rise to acetyl-CoA, may be oxidized through the cycle only if some

* In this chapter the term "cycle" will always refer to the tricarboxylic acid cycle for convenience and other cycles will be designated by their special names.

cycle intermediate (sparker) is provided to furnish oxalacetate to condense with the acetyl-CoA. A very small amount of such a sparker may suffice to initiate the entry of the acetyl-CoA into the cycle, and the cycle will then perpetuate itself through the continuous formation of oxalacetate, in which case pyruvate will be completely oxidized to CO_2 and water. Such a system should be quite sensitive to malonate, because an inhibition of the oxidation of succinate will reduce the amount of oxalacetate formed and consequently the amount of acetyl-CoA entering the cycle. On the other hand, if an approximately molar equivalent of fumarate, malate, or oxalacetate is initially present with the pyruvate, there will be an adequate concentration of oxalacetate to incorporate acetyl-CoA at a rapid rate, and the process will not depend on a regeneration of oxalacetate. This system will not be very sensitive to malonate, because a block of succinate oxidase will not appreciably reduce the amount of oxalacetate present. The first important principle is, therefore, that the degree of cycle inhibition by malonate will depend on the source of oxalacetate.

When the cycle is operating in a steady state, the concentrations of intermediates are low, and oxalacetate is formed just as rapidly as pyruvate is incorporated, the cycle rate being limited by the entry of acetyl-CoA. This may be the normal state of the cycle (Krebs and Lowenstain, 1960) but probably in cells, and certainly in isolated preparations, there are times when the cycle is not in a steady state. There is an initial rise in citrate concentration during the oxidation of pyruvate by heart mitochondria in the presence of malate (Montgomery and Webb, 1956 a), indicating that the tricarboxylates cannot be handled as rapidly as pyruvate can enter the cycle when the supply of oxalacetate is not limiting. The rate of oxygen uptake is initially very high, falls to a new level during the first 40 min, maintains this level for 2–3 hr, and then suddenly fails when the pyruvate is completely utilized. The first phase occurs when oxalacetate is readily available, and corresponds to the accumulation of citrate; the rate of oxygen uptake during this period is not an accurate measure of the rate of operation of the entire cycle — block of succinate oxidation may have very little effect on the oxygen uptake because relatively little of the respiration arises from this region of the cycle. The second steady-state phase should be more sensitive to malonate because the oxidations of succinate and malate now contribute 2 of the total of 5 oxygen atoms taken up per molecule of pyruvate. The second principle is thus that malonate inhibition will sometimes depend on the time interval during which the oxygen uptake is measured, particularly whether it is the initial rate or the total oxygen consumed.

Succinate usually accumulates during malonate inhibition (see page 90) and this will progressively reduce the degree of inhibition due to the competitive nature of the inhibition. Eventually a new steady state may be reached during which the succinate concentration remains constant. This

will always tend to lessen the effect of malonate and under certain circumstances it might effectively overcome the inhibition. The third principle is that the degree of malonate inhibition will depend on the level of succinate accumulation in the system studied.

Oxalacetate can often be formed by reactions outside the cycle. Pyruvate and phosphoenolpyruvate can be carboxylated to oxalacetate in the presence of oxalacetate decarboxylases or oxalacetokinase (Bandurski, 1955), and transamination between α-ketoglutarate and aspartate may also give rise to oxalacetate. In such cases the inhibition of pyruvate oxidation in the cycle by malonate will be reduced because the incorporation of pyruvate will not be dependent only on the regeneration of oxalacetate (Holland and Humphrey, 1953). Such reactions may occur in isolated mitochondria, as well as in cells, since in heart mitochondria, where pyruvate alone is not oxidized at all, the presence of bicarbonate or CO_2 allows a substantial rate of pyruvate oxidation (Montgomery and Webb, 1956 a), presumably through the carboxylation of some of the pyruvate to oxalacetate. The fourth principle is that the degree of malonate inhibition will depend on noncycle sources of oxalacetate.

Alternate metabolic pathways involving cycle substrates or intermediates may occur in some tissues. There are many opportunities for the metabolism of pyruvate, in addition to its oxidation through the cycle, and in the presence of malonate these pathways may become important. This is particularly true in microorganisms but the ability to decarboxylate pyruvate to acetate is common to most species and tissues. Thus, in the presence of high concentrations (50 mM) of malonate, pyruvate is quantitatively transformed into acetate by rabbit heart mitochondria (Fuld and Paul, 1952). An alternate pathway for succinate that would circumvent a malonate block is the cleavage of succinate (in the presence of NADH, CoA, and ATP) to 2 acetyl-CoA molecules. This succinate-cleaving enzyme was discovered in *Tetrahymena* (Seaman and Naschke, 1955) but it is also active in several rat tissues and in certain bacteria. This reaction will not, of course, restore cycle activity but it can lead to the formation of acetate or other products from acetyl-CoA, as well as reduce the concentration of succinate. Finally, the recently delineated glyoxylate cycle (Kornberg and Krebs, 1957) could bypass that region of the cycle containing succinate oxidase, malate being formed from isocitrate through the condensation of glyoxylate and acetyl-CoA, the over-all process being the formation of succinate from

$$2 \text{ Pyruvate} + 3/2\ O_2 \rightarrow \text{succinate} + 2\ CO_2 + H_2O$$

pyruvate. This shunt would allow a greater utilization of pyruvate and a greater oxygen uptake in the presence of malonate than would be the case with the tricarboxylic acid cycle alone. The glyoxylate cycle has been found in many microorganisms and there is some evidence for its occurrence

in certain plants, but its role in animal tissues is as yet unknown (Krebs and Lowenstein, 1960). As a result of these considerations, the fifth principle of cycle block is that the degree of inhibition by malonate will depend on the activity of various alternate pathways and shunts; in addition, it will depend on what is measured, e.g., oxygen uptake, CO_2 production, or pyruvate disappearance.

There is no doubt, therefore, tha the operation of the cycle and any ancillary pathways will vary with the experimental or physiological conditions, and that one must expect marked differences in the behavior of the cycle in different species or tissues. In addition to the factors discussed above, there are several other reasons for variability in response to malonate; the different susceptibilities of succinate dehydrogenase to inhibition (see page 49), the failure of malonate to penetrate readily into cells, and the possibility that malonate can inhibit other enzymes. The reliability of malonate as an indicator of cycle activity in a tissue must be evaluated in the light of these considerations. Certainly the lack of an expected response to malonate cannot be immediately interpreted as indicating the absence of the cycle, and the production of a significant effect by malonate should be substantiated by other more direct evidence before the operation of the cycle is established.

Inhibition of Cycle Substrate Oxidation by Malonate

A summary of some of the effects of malonate on cycle oxidations is given in Table 1-14. In many cases the concentration of malonate is too high to act specifically on succinate dehydrogenase, and the results are to some extent meaningless. It is very difficult to make any generalizations but malonate concentrations above 10 mM in subcellular preparations must be looked upon as probably not completely specific, whereas in cellular systems it is impossible to evaluate the specificity because the intracellular concentration is not known. In attempting to interpret the inhibitions observed, it is often necessary to know the pathway of metabolism of the substrate and how much oxygen is normally taken up per molecule utilized. For example, when α-ketoglutarate is added to a mitochondrial suspension, it may be oxidized to fumarate (or malate) taking up 2 atoms of oxygen, or to oxalacetate taking up 3 atoms of oxygen, or completely taking up 8 atoms of oxygen. If succinate oxidation is completely blocked by malonate, only 1 atom of oxygen will be taken up. Thus the maximal inhibitions in these three cases are 50%, 67%, and 87.5%, respectively. Similar reasoning applies to each substrate and in many cases the exact fate of the substrate is not known so that it is difficult to estimate what effect might be expected from malonate. It is also important in this connection, as pointed out in the previous section, to distinguish between inhibition over an initial short period of oxidation and inhibition of the total oxygen uptake.

(*a*) *Inhibition of pyruvate oxidation.* The oxidation or disappearance of pyruvate in cellular preparations is usually not depressed very much by malonate at concentrations less than 10 m*M*, whereas in mitochondrial preparations the expected degree of inhibition is usually observed. This may be partly explained by poor penetration into the cells and partly by the alternate pathways that may reduce the importance of the cycle. One type of correction that can be applied for a more accurate determination of cycle inhibition by malonate is that used by Speck *et al.*, (1946). In malarial parasitized erythrocytes, pyruvate is oxidized without the appearance of acetate, but in the presence of malonate, some acetate in formed. Correction was made for that pyruvate that went to acetate, since this fraction of the pyruvate utilization is not dependent on the cycle. The inhibition of over all pyruvate utilization was 12% but corrected for the acetate it was 31%. In the free parasites, the over all inhibition was 33% and the corrected inhibition 76%. Of course, pyruvate here or in other cells may be metabolized in other ways, so that the correction for acetate alone may not give the true cycle inhibition, but at least is provides a better value.

Malonate should inhibit the oxidation of pyruvate more strongly when there is a low concentration initially of oxalacetate or a substance forming oxalacetate (see page 70). This was shown in homogenates of rat tissues by Pardee and Potter (1949). In each case the inhibition by 4 m*M* malonate

Tissue	Substrates	% Inhibition
Heart	Pyruvate	91
	Pyruvate + oxalacetate	56
Kidney	Pyruvate	93
	Pyruvate + oxalacetate	55
Brain	Pyruvate	74
	Pyruvate + oxalacetate	47
Liver	Pyruvate	28
	Pyruvate + oxalacetate	15

is less when oxalacetate, is present. Since the oxygen uptake was determined from 10 to 30 min after the start of the experiments and inasmuch as the concentrations of pyruvate and oxalacetate were 3.5 m*M*, it is unexpected that so much inhibition would be exerted when the mixture is present. This might be due to the decarboxylation of sufficient oxalacetate so that it was less effective than anticipated, or even at this low concentration malonate may have been inhibiting some reaction other than the oxidation of succinate. In rat heart mitochondria we found that 5 m*M* malonate inhibits the oxidation of 5 m*M* pyruvate only about 10% in the presence of

TABLE 1-14

INHIBITIONS OF THE OXIDATIONS OF TRICARBOXYLATE CYCLE SUBSTRATES BY MALONATE

Substrate	Organism and preparation	Method [a]	Malonate (m*M*)	% Inhibition	Reference
Pyruvate	*Staphylococcus aureus*	O	33	11	Stedman and Kravitz (1955)
	Bacterium succinicum	O	5	Inh	Takahashi and Nomura (1952)
	Escherichia coli	O	200	51	Aubel *et al.* (1950)
	Pseudomonas saccharophila	O	10	0	Bernstein (1944)
			25	14	
			50	29	
	Pullularia pullulans	O	14	2	Clark and Wallace (1958)
			62	15	
			100	23	
	Pasteurella multocida	O	10	16	de Issaly and Stoppani (1964)
	Schizophyllum commune	O	1	15	J. G. H. Wessels (1959)
	Rhodotorula gracilis	O	40	66	Litchfield and Ordal (1958)
	Yeast (acid medium)	O	12.5	81	Krebs *et al.* (1952)
	Yeast	S	44	53	Stoppani *et al.* (1958 b)
	Tomato stem slices	O	50	87	Link *et al.* (1952)
	Avocado mitochondria	O	10	74	Avron and Biale (1957)
	Carrot mitochondria	O	20	64	Dalgarno and Birt (1962)
	Sugar pine seedling mitochondria	O	10	45	Stanley and Conn (1957)
	Paramecium caudatum	O	20	1	Holland and Humphrey (1953)
	Colpidium campylum	O	20	42	Seaman (1949)
	Plasmodium vivax	O	20	84	Speck *et al.* (1946)
		S	20	76	

Carp liver mitochondria	O	20	37	Gumbmann and Tappel (1962 b)
Pigeon muscle mince	S	5	51	Stare *et al.* (1941)
	S	1	77	Krebs and Eggleston (1940)
		25	95	
Pigeon brain brei	O	24	21	Banga *et al.* (1939)
Pigeon brain homogenate	O	24	71	Banga *et al.* (1939)
Duckling heart slices	S	10	46	Miller and Olson (1954)
Guinea pig brain dispersion	O	4.3	3	Dawson (1953)
		43	26	
Guinea pig brain slices	O	10	8	Weil-Malherbe (1937)
		40	44	
Rat liver homogenate	O	4	28	Pardee and Potter (1949)
		20	44	
Rat liver mitochondria	O	5	85	Montgomery and Webb (1956 b)
	O	20	59	Dalgarno and Birt (1962)
Rat kidney homogenate	O	4	93	Pardee and Potter (1949)
		20	95	
Rat kidney mitochondria	O	5	3	Montgomery and Webb (1956 b)
Rat kidney slices	O	40	41	Cremer (1962)
	C	40	84	
	S	40	73	
	O	20	Stim 46	Elliot and Greig (1937)
	S	20	42	
Rat mammary gland slices	O	5	71	Terner (1951)
	S	2.5	30	
		5	33	
		10	66	
Rat muscle mitochondria	O	5	7	Montgomery and Webb (1956 b)
Rat heart strips	C	5.6	Stim 11	Rice and Berman (1961)

TABLE 1-14 (*continued*)

Substrate	Organism and preparation	Method [a]	Malonate (m*M*)	% Inhibition	Reference
	Rat heart slices	O	20	70	Webb *et al.* (1949)
	Rat heart homogenate	O	4	91	Pardee and Potter (1949)
			20	93	
	Rat heart mitochondria	S	10	0	Reiss and Hellerman (1958)
		O	2	10	Montgomery and Webb (1956 b)
			5	33	
	Rat heart mitochondria (with α-ketoglutarate oxidation blocked)	O	10	8	Montgomery and Webb (1956 b)
			20	15	
			30	21	
			40	32	
			50	59	
	Rat brain slices	C	10	68	Kini and Quastel (1959)
		O	40	48	Cremer (1962)
		C	40	83	
		S	40	67	
	Rat brain homogenate	O	4	74	Pardee and Potter (1949)
			20	74	
	Rat brain mitochondria	O	5	27	Montgomery and Webb (1956 b)
		O	0.04 [b]	60	Lovtrup and Svennerholm (1963)
	Rabbit leucocytes	C	4	12	Coxon and Robinson (1956)
			20	30	
	Sheep thyroid slices	O	100	38	Dumont (1962)
			200	69	
		C	100	39	

			200	74	
	Sheep heart mince	S	1	21	Smyth (1940)
			10	73	
			25	82	
	Bull spermatozoa	O	10	Stim 43	Lardy and Phillips (1945)
	Human placenta	O	20	21	Hatch *et al.* (1959)
	Human heart slices	O	100	84	Burdette (1952)
Acetate	*Staphylococcus aureus*	O	33	32	Stedman and Kravitz (1955)
	Bacterium succinicum	O	5	Inh	Takahashi and Nomura (1952)
	Rhodospirillum rubrum	S	30	91	Clayton *et al.* (1957)
			100	91	
			300	97	
	Pullularia pullulans	O	14	3	Clark and Wallace (1958)
			62	5	
			100	11	
	Ashbya gossypii mycelia	O	40	100	Mickelson and Schuler (1953)
	Penicillium chrysogenum	O	10	0	Hockenhull *et al.* (1954 b)
		O	100	0	Beevers *et al.* (1952)
	Rhodotorula gracilis	O	40	72	Litchfield and Ordal (1958)
	Yeast	O	67	0	Lynen (1943)
		S	44	64	Stoppani *et al.* (1958 b)
			60	69	
	Yeast (cold-treated)	O	25	0	Krebs *et al.* (1952)
	Yeast (acid medium)	O	12.5	86	Krebs *et al.* (1952)
	Euglena	O	20	81	Danforth (1953)
	Schistocerca gregaria (desert locust) fat body	C	10	Stim 31	Clements (1959)
	Sheep spermatozoa	O	10	17	Scott *et al.* (1962)
		C	10	23	

TABLE 1-14 (*continued*)

Substrate	Organism and preparation	Method [a]	Malonate (m*M*)	% Inhibition	Reference
	Rat liver slices	O	45	96	Jowett and Quastel (1935 c)
	Rat kidney slices	C	5	48	Nakada *et al.* (1957)
			10	71	
			25	91	
	Rabbit kidney slices	O	10	50	Mudge (1951)
	Human placenta	O	20	1	Hosoya *et al.* (1960)
Lactate	*Escherichia coli* (toluene-treated)	O	13	35	Cook (1930)
	Rhodospirillum rubrum	S	30	0	Clayton *et al.* (1957)
			100	0	
			300	61	
	Penicillium chrysogenum	O	10	0	Hockenhull *et al.* (1954 b)
	Plasmodium vivax	O	20	67	Speck *et al.* (1946)
Citrate	*Micrococcus sodonensis*	S	75	5	Perry and Evans (1960)
	Aerobacter indologenes	C	30	Stim	Brewer and Werkman (1939)
	Proteus vulgaris (no NADP)	O	200	83	Kasamaki *et al.* (1963)
	Proteus vulgaris (with NADP)	O	200	48	Kasamaki *et al.* (1963)
	Schizophyllum commune	O	1	20	J. G. H. Wessels (1959)
	Cauliflower bud homogenate				
	1 m*M* Citrate	O	10	52	Laties (1953)
	5 m*M* Citrate	O	10	10	
	10 m*M* Citrate	O	10	0	
	Avocado mitochondria	O	10	41	Avron and Biale (1957)
	Phaseolus aureus mitochondria	O	10	21	Bonner and Millerd (1953)

	Apple fruit mitochondria	O	20	100	Hatch *et al.* (1959)
	Tobacco leaf mitochondria	O	21	62	Pierpoint (1959)
	Paramecium caudatum	O	20	57	Holland and Humphrey (1953)
	Nematodirus filicollis mince	S	20	37	Massey and Rogers (1950)
	Helix pomatia hepatopancreas	O	33	62	Ap Rees (1962)
	Pigeon muscle mince	O	1	51	Stare and Baumann (1939)
			10	57	
		S	5	Stim 24	Stare *et al.* (1941)
	Human heart slices	O	100	61	Burdette (1952)
cis-Aconitate	Avocado mitochondria	O	10	24	Avron and Biale (1957)
Isocitrate	*Proteus vulgaris* (no NADP)	O	200	100	Kasamaki *et al.* (1963)
	Proteus vulgaris (with NADP)	O	200	50	Kasamaki *et al.* (1963)
	Pasteurella multocida	O	10	9	de Issaly and Stoppani (1964)
	Carrot mitochondria	O	20	Stim 37	Dalgarno and Birt (1962)
	Rat liver mitochondria	O	20	Stim 35	Dalgarno and Birt (1962)
		O	20	20	Hawtrey (1962)
α-Ketoglutarate	*Escherichia coli*				
	Washed	O	6.7	Stim 4	Ajl and Werkman (1948)
	Acetone-treated	O	6.7	47	
	Extract	O	6.7	40	
	Neisseria gonorrhoeae	O	10	57	Tonhazy and Pelczar (1953)
	Streptomyces coelicolor	O	10	41	Cochrane and Peck (1953)
		C	10	13	
		S	10	Stim 9	
	Schizophyllum commune	O	1	27	J. G. H. Wessels (1959)
	Rhodotorula gracilis	O	40	82	Litchfield and Ordal (1958)
	Cauliflower bud homogenate	O	10	63	Laties (1953)
	Avocado mitochondria	O	10	48	Avron and Biale (1957)

TABLE 1-14 (*continued*)

Substrate	Organism and preparation	Method [a]	Malonate (m*M*)	% Inhibition	Reference
	Phaseolus aureus mitochondria	O	10	43	Bonner and Millerd (1953)
	Lupine mitochondria	O	10	63	Conn and Young (1957)
	Apple fruit mitochondria	O	20	74	Hatch *et al.* (1959)
	Tobacco leaf mitochondria	O	17	69	Pierpoint (1959)
	Sugar pine seedling mitochondria	O	10	73	Stanley and Conn (1957)
	Paramecium caudatum	O	20	85	Holland and Humphrey (1953)
	Nematodirus filicollis mince	O	10	33	Massey and Rogers (1950)
	Ascaridia galli mince	O	10	43	Massey and Rogers (1950)
	Helix pomatia hepatopancreas	O	33	100	Rees (1953)
	Aedes aegypti mitochondria	O	1	28	Gonda *et al.* (1957)
			10	43	
	Calliphora erythrocephala	O	10	10	Lewis and Slater (1954)
	(blowfly) mitochondria	S	10	Stim 25	
	Carp liver mitochondria	O	15	25	Gumbmann and Tappel (1962 a)
	Pigeon liver mince	S	2	37	Stern (1948)
			20	67	
	Guinea pig brain mince	O	25	25	Weil-Malherbe (1937)
			50	43	
			200	60	
	Guinea pig brain slices	O	5	6	Weil-Malherbe (1937)
			10	14	
			40	51	
	Rat heart mitochondria	O	10	33	Slater and Holton (1954)
		S	10	Stim 8	

		O	1	9	Montgomery and Webb (1956 b)
			5	34	
			20	62	
	Rat brain homogenate	O	3.3	59	Christie *et al.* (1953)
		S	3.3	29	
	Rat brain mitochondria	O	0.04 [b]	73	Lovtrup and Svennerholm (1963)
	Rabbit kidney mitochondria	O	20	45	Keller and Lotspeich (1959 a)
	Beef retina homogenate	O	—	54	Burgess *et al.* (1960)
Fumarate	*Pseudomonas saccharophila*	O	1	0	Bernstein (1944)
			10	17	
			25	52	
			50	72	
	Pasteurella multocida	O	10	5	de Issaly and Stoppani (1964)
	Rhodospirillum rubrum	S	30	6	Clayton *et al.* (1957)
			100	14	
			300	83	
	Yeast	S	44	54	Stoppani *et al.* (1958 b)
	Avena coleoptile extract	D	67	20	Berger and Avery (1943 a)
	Avocado mitochondria	O	10	33	Avron and Biale (1957)
	Paramecium caudatum	O	20	72	Holland and Humphrey (1953)
	Helix pomatia hepatopanereas	O	33	84	Rees (1953)
	Pigeon muscle mince	O	1	3	Stare and Baumann (1939)
			10	51	
	Rat heart slices	O	20	0	Webb *et al.* (1949)
Malate	*Escherichia coli*	O	200	0	Aubel *et al.* (1950)
	Moraxella lwoffi	S	10	10	Lwoff and Audureau (1947)
			20	20	
	Schizophyllum commune	O	1	5	J. G. H. Wessels (1959)

TABLE 1-14 (*continued*)

Substrate	Organism and preparation	Method [a]	Malonate (m*M*)	% Inhibition	Reference
	Avocado mitochondria	O	10	21	Avron and Biale (1957)
	Apple fruit mitochondria	O	20	83	Hatch *et al.* (1959)
	Tobacco leaf mitochondria	O	21	0	Pierpoint (1959)
	Sugar pine seedling mitochondria	O	10	70	Stanley and Conn (1957)
	Carrot mitochondria	O	20	51	Dalgarno and Birt (1962)
	Paramecium caudatum	O	20	64	Holland and Humphrey (1953)
	Rat liver mitochondria	O	20	15	Dalgarno and Birt (1962)
	Rat heart mitochondria	O	20	34	Montgomery and Webb (1956 b)
	Human heart slices	O	100	100	Burdette (1952)
Oxalacetate	*Moraxella lwoffi*	S	50	100	Lwoff and Audureau (1947)
	Rat liver homogenate	O	4	18	Pardee and Potter (1949)
			20	53	
	Rat kidney homogenate	O	4	32	Pardee and Pother (1949)
			20	70	
	Rat heart homogenate	O	4	30	Pardee and Potter (1949)
			20	42	
	Rat brain homogenate	O	4	50	Pardee and Potter (1949)
			20	68	

[a] The methods used to determine the utilization of the substrates are designated as follows: O — oxygen uptake; C — CO_2 production, either volumetric or radioactive; D — dye reduction; and S — direct determination of the disappearance of the substrate.

[b] Although the malonate concentration is stated to be 40 μM, it is likely from the results that this actually signifies 40 millimoles in 2.3 ml and a concentration of 17.3 m*M*.

5 mM malate. However, when the malate concentration is between 0.1 and 1 mM, the inhibition is close to 50% (Montgomery and Webb, 1956 b).

(*b*) *Inhibition of α-ketoglutarate oxidation.* The possible inhibition of α-ketoglutarate oxidase by malonate is important not only because of the bearing it has on the effects of malonate on the operation of the cycle, but also because malonate has been frequently used to block succinate oxidation in order to study in particulate systems the oxidation of α-ketoglutarate uncomplicated by further oxidations. This technique was first proposed by Ochoa (1944), who showed that high concentrations (25–50 mM) of malonate would allow α-ketoglutarate to be oxidized to succinate in enzyme preparations from cat heart. In four experiments with 50 mM malonate, 0.86 mole of succinate was formed for every mole of α-ketoglutarate utilized, indicating that even here an appreciable fraction of the succinate formed is further oxidized. No data were given as to whether these concentrations of malonate inhibit the utilization of α-ketoglutarate. Slater and Holton (1954) used 10 mM malonate to study the oxidation of α-ketoglutarate in heart mitochondria, and it was shown that this concentration does not reduce the utilization of α-ketoglutarate, although 20–40 mM does inhibit it. Malonate was also used to investigate the formation of α-ketoglutarate from citrate in *Micrococcus sodonensis* (Perry and Evans, 1960), but the rationale for this is obscure since malonate by inhibition of succinate oxidation would not depress the disappearance of α-ketoglutarate. However, at the concentration of malonate used (75 mM), it is quite possible that the α-ketoglutarate oxidase was inhibited.

The oxygen uptake with α-ketoglutarate as the substrate in particulate preparations from several sources has been shown to be reduced by malonate as expected if the succinate formed is partially protected from oxidation (see Table 1-14). Malonate concentrations around 10 mM inhibit 40–60% in most cases. No definite information on the possible inhibition of the α-ketoglutarate oxidase can be obtained from such studies.

If the oxidation of α-ketoglutarate stops at fumarate, the system is a two-step linear chain (neglecting the other reactions involved in the formation of succinate). An atom of oxygen is taken up in each step:

$$\alpha\text{-Ketoglutarate} \xrightarrow[(1)]{1/2\ O_2} \text{succinate} \xrightarrow[(2)]{1/2\ O_2} \text{fumarate}$$

so that a complete and specific inhibition of reaction (2) would result in a maximal inhibition of 50% with respect to the oxygen uptake. However, in case the first reaction is much faster than the second, malonate would not inhibit the initial rate of the reaction, even though it inhibited the final total oxygen uptake. Therefore, the inhibition may theoretically vary from 0 to

50%, depending on the relative rates of the reactions and the period during which the oxygen uptake is measured. If the fumarate is further oxidized, more oxygen will be consumed and the inhibition by malonate may be greater than 50%. Furthermore, it is essentially impossible to inhibit the succinate dehydrogenase completely, especially as succinate will accumulate and progressively overcome the inhibition.

The use of malonate to study α-ketoglutarate oxidation in mitochondria involves the assumption that malonate does not significantly affect the α-ketoglutarate oxidase directly. Unfortunately, no investigations of the inhibition of α-ketoglutarate dehydrogenase by malonate have been reported, and thus it is difficult to compare the sensitivities of the two dehydrogenases. Several studies have determined the effects of malonate on the disappearance of α-ketoglutarate (α-KG) during periods when the oxygen uptake is depressed, and it is rather strange that some effect, either positive or negative, has always been reported. The pertinent data have been summarized in the accompanying tabulation, and it is seen that an inhibition

Preparation	Malonate (m*M*)	% Inhibition of	
		O_2 uptake	*α-KG* utilization
Streptomyces coelicolor	10	41	Stim 9
Pea seedling mitochondria	10	66	21
Blowfly sarcosomes	10	10	Stim 25
Heart mitochondria	10	33	Stim 8
Rat brain homogenate	3.3	59	29

of α-ketoglutarate disappearance is observed in some cases and a stimulation in others. The oxidation of α-ketoglutarate depends on Mg^{++} and it is possible that the differences are related to the degree of Mg^{++} requirement and the concentrations of Mg^{++} used in the assay media. Price (1953) found that even 1 m*M* malonate would inhibit α-ketoglutarate utilization in pea seedling mitochondria and that 30 m*M* had a very marked effect (60% inhibition). This is not a competitive type of inhibition because increasing the α-ketoglutarate concentration does not lower the inhibition, and even increases it somewhat. The possibility of Mg^{++} reduction was explored and it was found that the calculated drop in the Mg^{++} concentration could not have been responsible for the inhibition. Furthermore, it was not possible to reverse the inhibition by increasing the Mg^{++} concentration from 1 m*M* to 4 m*M*. An inhibition by a Mg-malonate complex was considered to be the most likely explanation, but yet no inhibition was seen when the concentration of Mg^{++} in the medium was reduced to zero, although the enzyme was still partially complexed with Mg^{++}. What-

ever the explanation, it is obvious that in such mitochondria malonate could not be used to isolate α-ketoglutarate oxidation.

Another quantitative study on the effects of malonate on α-ketoglutarate oxidation was made by Grafflin *et al.* (1952), who were attempting to find a good assay system for α-ketoglutarate oxidase in rabbit kidney homogenates. They concluded that the use of malonate is unsatisfactory and abandoned this procedure. The difficulty lies particularly in the inability, except at high malonate concentrations (around 30 m*M*), to inhibit completely the oxidation of succinate, as determined from the total oxygen uptake compared with the theoretical value for a one-step oxidation of α-ketoglutarate. Although no evidence on the effect of malonate on the α-ketoglutarate oxidase was presented, it would be surprising if concentrations of malonate above 20 m*M* had no effect. Lewis and Slater (1954) also remarked that even in the presence of 10 m*M* malonate, the oxygen uptake from the oxidation of α-ketoglutarate greatly exceeds the disappearance of α-ketoglutarate, .O/.α-KG ratios generally being above 2, in blowfly sarcosomes. Also, the oxygen uptake over 35–45 min is depressed only 10% at this concentration of malonate.

It is difficult to understand why the oxidation of succinate is so resistant to malonate under these circumstances. Taking the values of K_i that have been found in mammalian tissue studies, malonate at 10 m*M* should inhibit well over 95% even at succinate concentrations that might occur experimentally. For example, in beef heart preparations, where K_i is about 0.04 m*M*, 10 m*M* malonate would inhibit over 99% at a succinate concentration of 2 m*M*. It may be that in the intact system the oxidation of endogenously formed succinate from α-ketoglutarate via succinyl-CoA is kinetically different than the oxidation of exogenous succinate, or that local concentrations of succinate can reach much higher levels than predicted on the basis of over all analyses. One must conclude at least at the present time that the specific inhibition of succinate oxidation, even in these relatively simple systems, is generally impossible.

This problem has been approached recently by Jones and Gutfreund (1964), who experimentally determined the steady-state concentrations of succinate in guinea pig liver mitochondria during the oxidation of α-ketoglutarate. The O_2 uptake of uninhibited mitochondria is 40–45% due to α-ketoglutarate oxidation, 40–45% due to succinate oxidation, and 10–20% due to other oxidations. The effects of malonate on the oxidation of exogenous succinate were compared with the effects on the oxidation of succinate-C^{14} formed from α-ketoglutarate-C^{14}. The rate of utilization of α-ketoglutarate is not altered up to 8 m*M* malonate. The steady-state succinate concentration by total analysis is 0.04 m*M*, and this is not affected by malonate until its concentration is higher than 0.04 m*M*; half the succinate formed from α-ketoglutarate accumulates with 0.7 m*M* malonate, and

80% accumulates at 8 m*M* malonate. The O_2 uptake due to the oxidation of succinate formed from α-ketoglutarate is reduced 50% by 0.2 m*M* malonate. The data indicate that endogenously formed succinate is at a much higher concentration than that determined by total analysis. The inhibition studies indicate the steady-state succinate concentration in the mitochondria to be 4.6–13 m*M* and the isotopic studies suggest concentrations exceeding 4 m*M*. Such high succinate concentrations would protect the succinate dehydrogenase and result in less malonate inhibition than expected. Since there appear to be no permeability barriers in the mitochondria to succinate, the authors suggested that there is a spatial relation between the succinate dehydrogenase and the enzyme forming the succinate. If this is true, it raises the interesting possibility that certain enzymes are specific not only for their substrates but also for other enzymes with which they interact to form functional metabolic complexes.

(*c*) *Effects on tricarboxylate utilization.* Malonate has variable affects on the oxidation of citrate and isocitrate, often inhibiting rather well, but sometimes having little effect or actually stimulating (Table 1-14). The relationship between malonate inhibition and isocitrate oxidation is probably complex in most instances. There seems to be minimal direct inhibition of isocitrate dehydrogenase, although data are lacking. Hülsmann (1961) showed that malonate at 11 m*M* reduces quite potently the utilization of isocitrate by rabbit heart mitochondria when acetate or β-hydroxybutyrate is the additional substrate (these accelerating the utilization of isocitrate). The stimulation of isocitrate utilization by these substrates was claimed to be due to the formation of acetoacetyl-CoA, an intermediate in fatty acid synthesis, and this alters the oxidation-reduction states of NAD-NADH and NADP-NADPH through the transhydrogenase reaction, isocitrate oxidation rate being dependent on the concentration of NADP. Kasamaki *et al.* (1963) showed that malonate inhibits citrate and isocitrate oxidations in *Proteus vulgaris* much less when NADP is added. Chappell (1964 a) suggested that the effects of malonate on tricarboxylate utilization in rat liver mitochondria are due to the block in the formation of malate from succinate, malate being necessary for the oxidation of isocitrate, since it provides a means for reoxidation of NADPH mediated through the transhydrogenase and malate dehydrogenase. We see here a possibly important control in the operation of the cycle and how malonate can exert effects indirectly on steps distant from the succinate oxidation reaction.

(*d*) *Effect of cycle substrate concentration on the inhibition.* Some claims have been made that increases in the citrate concentration will tend to overcome inhibition of the oxygen uptake by malonate, and sometimes this has been attributed to a competitive effect, it being assumed that the higher concentration of substrate will give rise to a higher succinate level. Thus

Laties (1953) found that increasing the citrate from 1 mM to 10 mM would reduce the inhibition by 10 mM malonate from around 52% to zero in cauliflower homogenates, and Pierpoint (1959) reported that a 10-fold rise in citrate concentration brings about a decrease from 62 to 45% in the inhibition by 21 mM malonate in tobacco leaf mitochondria. Laties pointed out that this could not be due to a competitive effect because of an increased production of succinate, since at the malonate concentration used the oxidation of 10 mM succinate is completely inhibited. However, if what was suggested in the previous section regarding the difficulty in the inhibition of the oxidation of endogenously produced succinate is valid, data on the inhibition of exogenous succinate may not be applicable. Laties felt that the explanation might lie in the interference between electron-transport systems, so that when citrate concentration is high, the contribution made by succinate oxidation to the total respiration is less. Clear-cut effects of concentration on inhibition have not been observed with α-ketoglutarate (Grafflin *et al.*, 1952; Pierpoint, 1959), pyruvate (Smyth, 1940), acetate (Jowett and Quastel, 1935 c), or malate (Pierpoint, 1959).

In experiments of this type, it is well to remember that the pattern of oxygen uptake may change with the concentration of the substrate. The rela-

$$\begin{array}{ccccc} & x\mathrm{O} & & y\mathrm{O} & \\ & \downarrow & & \downarrow & \\ \mathrm{S} & \rightarrow & \text{succinate} & \rightarrow & \mathrm{P} \end{array}$$

tive amounts of oxygen taken up before and after the succinate step may be altered by substrate concentration due to factors previously discussed in this chapter. Thus the ratio x/y in the above equation will vary and hence the effect of an inhibitor of succinate oxidation. If the inhibition on succinate oxidation is i and the ratio $x/y = r$, the over all inhibition on the total oxygen uptake will be $i/(1 + r)$, so that anything that changes r will change the inhibition. If high concentrations of the substrate produce an accumulation of some intermediate (as citrate rises when pyruvate enters the cycle rapidly), r will increase, at least over the initial period, and the inhibition will be less at lower substrate concentrations.

(*e*) *Stimulation of cycle substrate utilization by malonate.* In a number of cases there is clearly a stimulation of the utilization of pyruvate, acetate, or citrate by malonate. Sometimes this is recognized in an increased oxygen uptake but occasionally the disappearance of the substrate is accelerated while the oxygen uptake is inhibited. Often this effect is very marked. In the mycelia of *Ashbya gossypii*, oxygen uptake due to addition of acetate is stimulated 48% by 4 mM malonate, whereas 40 mM malonate inhibits 53% (Mickelson and Schuler, 1953). The increase in the respiration from pyruvate in bull sperm by 10 mM malonate is almost as great (Lardy and Phillips, 1945). Table 1-14 cites a number of other instances.

The mechanisms for such stimulations must vary with the particular substrate used, but it may be useful to suggest some possible ways in which malonate could produce this apparently anomalous effect. (1) If the preparation has an active oxalacetate decarboxylase, this may reduce the concentration of oxalacetate for condensation with acetyl-CoA. Malonate is able to inhibit this enzyme in some cases (Table 1-12) (Pardee and Potter, 1949), in which case oxalacetate may be protected so that cycle entry of acetyl-CoA is facilitated. (2) If ADP concentration is low and ATP concentration high normally in the preparation, malonate by inhibiting certain phases of the cycle might increase ADP concentration and thus stimulate electron transport in other oxidative processes by providing more phosphate acceptor. (3) If there is competition between the different oxidative reactions in the cycle for some common coenzyme or cofactor (e.g. NAD, NADP, or CoA), inhibition of some oxidations by malonate might allow these factors to be used more readily by other systems. Such competition between the pyruvate and α-ketoglutarate systems has been suggested in heart mitochondria (Montgomery and Webb, 1956 a). (4) If malonate is metabolized by the preparation, it is possible that a product would accelerate the utilization of some cycle intermediate. In some organisms malonate can form acetyl-CoA and acetate, as well as other products. (5) In intact cells, malonate might increase the permeability of the cell membrane so that certain substrates, such as pyruvate, citrate, or α-ketoglutarate, could enter more readily. (6) By chelation with inhibitory metal ions that may occur in the preparation, malonate might accelerate the rates of certain reactions. All of these mechanisms are purely hypothetical, since in no case has the actual mechanism been established.

Intracellular Concentrations of Cycle Intermediates

Interpretation of intracellular inhibition by malonate and other inhibitors acting on the cycle should ideally involve in many cases a knowledge of the concentrations of certains intermediates. Data collected for different types of cells are shown in Table 1-15. These figures were calculated on the basis of intracellular water contents. However, it is likely that these substances are not distributed homogeneously throughout the cell water. There is evidence that some intermediates may occur within the mitochondria at different concentrations than in the surrounding medium. Thus the mitochondria/medium ratios for sheep kidney mitochondria under certain conditions were found to be: pyruvate 0.84, fumarate 7.42, α-ketoglutarate 1.0, citrate 0.83, and oxalacetate 0.13 (Bartley and Davies, 1954). Furthermore, the values given in the table are all abnormal since truly normal tissues were not used. The rats were fasted for 24 hr while the suspensions of *E. coli* and yeast were metabolizing acetate rather than a more normal substrate. In normal rat tissues the concentrations may well

Table 1-15

Concentrations of Cycle Intermediates in Cells [a]

Intermediate	Concentration (mM) in:					
	Brain	Liver	Kidney	Muscle	*E. coli*	Yeast
Pyruvate	4.3	0.63	0.56	0.38	—	—
Lactate	29.3	2.58	2.40	1.42	—	—
Citrate	0.60	0.23	0.25	0.008	0.081	0.85
cis-Aconitate	< 0.013	< 0.011	< 0.011	< 0.009	—	—
Isocitrate	0.057	0.070	0.073	0.008	—	—
α-Ketoglutarate	2.2	0.28	0.26	0.016	0.065	0.012
Succinate	0.74	0.29	0.29	0.013	0.28	4.9
Fumarate	2.6	1.41	1.22	0.080	0.027	1.3
Malate	0.52	0.22	0.16	0.012	0.023	5.1
Oxalacetate	1.4	0.12	0.11	0.012	—	—

[a] Data for rat tissues from Frohman *et al.* (1951). Concentrations in intracellular water calculated from water contents given by Lowry (1943). The animals were fasted for 24 hr before removal of tissues. The values for brain are approximate because of unavoidable damage to the tissue during removal. The values for skeletal muscle are so low that they are not as accurate as the other tissues.

Data for *E. coli* from Swim and Krampitz (1954). Concentrations in intracellular water estimated on the basis of 65% water content (water content of packed *E. coli* is 75%, and 10% is intercellular). The bacteria were metabolizing acetate.

Data for baker's yeast from DeMoss and Swim (1957). Concentrations in intracellular water estimated from a water content of 71% for yeast at 30°. The yeast were metabolizing acetate.

The concentrations are somewhat different than those given by Krebs and Lowenstein (1960), because they did not calculate the concentrations in intracellular water.

be higher, and in yeast metabolizing sugar the values may be lower. One value for oxalacetate in normal rat liver is available (0.036 m*M*) and in this case fasting for 24 hr did not alter this appreciably (Kalnitsky and Tapley, 1958).

These values do not represent a thermodynamic equilibrium based on differences in free energy, but rather a dynamic or kinetic equilibrium, depending mainly on the relative rates of the cycle reactions and competing processes. The higher concentration of fumarate compared to malate in rat tissues illustrates this because in a thermodynamic equilibrium there would be about one-fourth the concentration of malate. It is interesting that the values differ so widely from tissue to tissue and certainly this must be one factor in determining the different responses to malonate or other competitive inhibitors. The levels of succinate are generally low, except in yeast, but as pointed out above the concentrations within the mitochondria or at the region of the active center of succinate dehydrogenase may well be higher. The effects of malonate on the concentrations of these intermediates in cells will be taken up in the following section.

Analyses of plant tissues have not been presented here because there is some doubt as to the significance of the figures. Most plants contain large amounts of organic acids, including the cycle intermediates. Beevers (1952) postulated that the cycle in plants is less readily blocked than in animal tissues because of these high concentrations of succinate and other cycle intermediates. This could well be an important factor, but actually the concentrations of these acids in the plant cytoplasm are not known in most cases, total analyses including the vacuolar fluid, which is often of greater volume than the cytoplasm and contains most of the organic acids. There is another way by which these plant acids could protect against malonate. The presence of large amounts of fumarate or malate, or of any substance capable of forming oxalacetate, would allow pyruvate to be incorporated into the cycle even in the state of complete block of succinate oxidation. Examples of the overcoming of malonate inhibition by fumarate and malate will be presented shortly.

ACCUMULATION OF SUCCINATE DURING MALONATE INHIBITION

An effective inhibition of succinate oxidation should lead to a rise in the concentration of succinate under conditions in which succinate can still be formed. Such accumulation of succinate has been frequently observed and some of the more quantitative results are summarized in Table 1-16. In addition to the examples in the table, accumulation of succinate has been reported in the following species and tissues: *Shigella* (Yee *et al.*, 1958), *Nocardia* (Cartwright and Cain, 1959), *Aspergillus* (Shimi and Nour

El Dein, 1962), tobacco leaves (Vickery, 1959; Vickery and Palmer, 1957), potato slices (Romberger and Norton, 1961), avocado mitochondria (Avron and Biale, 1957), pea leaf particulates (Smillie, 1956), barley roots (Laties, 1949 b), *Colpidium* (Seaman, 1949), *Trypanosoma* (Bowman *et al.*, 1963), carp liver mitochondria (Gumbmann and Tappel, 1962 b), rat heart homogenates (Lehninger, 1946 b), rat liver slices (Elliott and Greig, 1937), human heart slices (Burdette, 1952), ascites carcinoma cells (Dajani *et al.*, 1961), and in many tissues of rats and rabbits (Busch and Potter, 1952 a; Forssman, 1941). In the experiments leading to the results in Table 1-16, the preparations were incubated for one to several hours with malonate and the succinate analyzed at the and of the incubation, so that the rates of succinate formation at any time are difficult to evaluate, and may well have been greater initially. The over all succinate concentrations may be estimated from the volumes in which the expriments were run and in most cases the final succinate concentrations range between 0.5 and 2.5 m*M*.

Several points are brought out by the results in Table 1-16. It is seen that succinate can be formed from essentially all the cycle substrates and intermediates in the presence of malonate. Rapid rates are found when oxalacetate or some substance forming oxalacetate is added with pyruvate, as would be expected, because in the absence of a source of oxalacetate, the malonate would reduce the incorporation of pyruvate into the cycle and hence the rate of formation of succinate. It may be noted in some cases that, in the absence of added substrates or malonate, some succinate accumulates (yeast, *Avena* coleoptile, spinach leaves, and dog heart), which implies that under the experimental conditions succinate is formed more rapidly than it can be oxidized. This is somewhat surprising because it is usually assumed that the activity of succinate oxidase is quite high in most tissues. The possibility of the accumulation of sufficient oxalacetate to inhibit succinate dehydrogenase when little acetyl-CoA is available cannot be ignored. This phenomenon is also evident in the analyses for succinate given in Table 1-15. The interesting effects of malonate concentration are seen in two investigations. In spinach leaves, the maximal succinate accumulation occurs at malonate concentrations around or below 50 m*M*; at higher concentrations, the malonate is apparently acting on other enzymes in the cycle and reducing the rate of formation of succinate. Likewise, in brain minces, the high concentration of 200 m*M* malonate is seen to depress succinate accumulation.

Quantitative conversion of cycle substrates to succinate in the presence of malonate is generally not observed. In fact, in most cases in which the disappearance of substrate was determined simultaneously with the formation of succinate, only a small fraction appeared as succinate. For example, Speck *et al.* (1946) found in malarial parasitized erythrocytes that only 22% of the pyruvate utilized in the presence of 20 m*M* malonate was

TABLE 1-16

ACCUMULATION OF SUCCINATE DURING MALONATE INHIBITION

Preparation	Substrates (mM)	Malonate (mM)	Succinate formed [a]	Reference
Corynebacterium creatinovorans	—	—	0	Barron *et al.* (1950)
	Acetate (8.2)	—	0	
	Acetate (8.2)	16.5	14.2	
Yeast	—	—	0.053	Lynen (1943)
	Fumarate (33)	67	0.156	
	Fumarate (33) + acetate (25)	67	0.230	
	Acetate (20)	—	0.152	Barron and Ghiretti (1953)
	Acetate (20)	17	2.50	
	—	—	2.85	Stoppani *et al.* (1958 b)
	—	44	3.42	
	Pyruvate (22)	—	5.32	
	Pyruvate (22)	44	9.95	
	—	—	1.90	Stoppani *et al.* (1958 b)
	—	44	2.85	
	Fumarate (12)	—	3.25	
	Fumarate (12)	44	4.75	
	Malate (12)	—	1.90	
	Malate (12)	44	3.95	
	—	—	4.37	Stoppani *et al.* (1958 b)

	—	44	4.30	
	Acetate (20)	—	4.80	
	Acetate (20)	44	4.67	
Plasmodium gallinaceum	—	—	0	Speck *et al.* (1946)
	—	20	0.2	
	Pyruvate (10)	—	0	
	Pyruvate (10)	20	1.5	
	Malate (10)	—	2.2	
	Malate (10)	20	4.9	
	Pyruvate (10) + malate (10)	—	10.5	
	Pyruvate (10) + malate (10)	20	11.5	
Malarial parasitized erythrocytes	—	—	0	Speck *et al.* (1946)
	—	20	3.7	
	Pyruvate (10)	—	1.1	
	Pyruvate (10)	20	4.6	
	Fumarate (10)	—	0	
	Fumarate (10)	20	3.0	
	Pyruvate (10) + fumarate (10)	—	1.0	
	Pyruvate (10) + fumarate (10)	20	3.9	
Avena coleoptile	—	—	0.93	Bonner (1948)
	—	50	1.63	
	Fumarate (15.4)	50	6.40	
	—	—	1.05	
	—	50	1.30	
	α-Ketoglutarate (27.8)	50	4.96	

TABLE 1-16 (*continued*)

Preparation	Substrates (m*M*)	Malonate (m*M*)	Succinate formed [a]	Reference
Spinach leaves	—	—	1.10	Laties (1949 b)
	—	48	2.64	
	—	96	2.20	
	—	144	1.75	
	—	192	0.81	
Nematodirus filicollis	Pyruvate (10) + fumarate (10)	—	0.40	Massey and Rogers (1950)
	Pyruvate (10) + fumarate (10)	10	1.25	
Ascaridia galli	Pyruvate (10) + fumarate (10)	—	0.51	Massey and Rogers (1950)
	Pyruvate (10) + fumarate (10)	10	1.03	
Pigeon muscle brei	Pyruvate (10) + fumarate (10)	—	0.28	Massey and Rogers (1950)
	Pyruvate (10) + fumarate (10)	10	1.33	
	—	12.5	0.80	Krebs and Eggleston (1940)
	Pyruvate (9.4)	12.5	1.29	
	Fumarate (1.25)	12.5	4.60	
	Pyruvate (9.4) + fumarate (1.25)	12.5	6.25	
	—	10	1.65	Krebs and Eggleston (1940)
	Pyruvate (10) + oxalacetate (2.5)	10	7.67	
	Citrate (2.5)	10	9.10	
Sheep heart mince	—	10	1.74	Smyth (1940)
	Fumarate (2.5)	10	5.68	

	Pyruvate (12.5)	10	2.18	Smyth (1940)
	Pyruvate (12.5) + fumarate (2.5)	10	6.37	
	Pyruvate (12.5) + fumarate (5.0)	10	7.73	
	Pyruvate (10)	25	4.65	Smyth (1940)
	Pyruvate (10) + fumarate (1.25)	25	8.04	
	Pyruvate (10) + fumarate (2.5)	25	11.3	
Dog heart (heart-lung)	—	—	0.51	Fawaz *et el.* (1958)
	—	30	2.07	
	—	60	2.49	
Ox brain mince	—	25	5.80	Weil-Malherbe (1937)
	Pyruvate (200)	25	8.05	
	—	50	4.74	
	Pyruvate (200)	50	9.56	
	—	200	7.62	
	Pyruvate (200)	200	7.45	
Horse brain mince	—	25	2.36	Weil-Malherbe (1937)
	α-Ketoglutarate (125)	25	14.0	
	—	50	1.88	
	α-Ketoglutarate (125)	50	12.3	
	—	200	4.60	
	α-Ketoglutarate (125)	200	5.54	

[a] No standard units are given for the succinate formed because the changes in the rates of formation only are significant. The values are almost always given as μmoles/hr, but no comparisons can be made between the different experiments since variable amounts of tissue were present.

recoverable as succinate. The highest conversion efficiency was observed by Krebs and Eggleston (1940) in pigeon muscle brei, where 75–85% of the pyruvate utilized in the presence of fumarate and 12.5 m*M* malonate went to succinate. Complete conversion to succinate would not, of course, be expected unless one could inhibit succinate oxidation completely and specifically, which in most instances cannot be done.

Factors Determining Succinate Accumulation

The effects of inhibition on the concentrations of intermediates in multienzyme systems have been treated in Chapter I-7. Some of the most important factors involved in malonate inhibition will be summarized.

(I) *Degree of inhibition of succinate oxidase*: hence, the concentration of malonate, the affinity of the enzyme for malonate, and the ability of malonate to penetrate if the preparations are cellular.

(II) *Rate of formation of succinate*: this will depend primarily on the availability of cycle substrates and their concentrations.

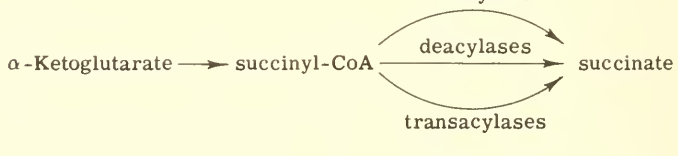
(III) *Action of malonate on enzymes other than succinate dehydrogenase*: such actions may slow down the formation of succinate, as discussed above.

(IV) *Other pathways of succinate metabolism*: several reactions of succinyl-CoA or succinate are known and these would tend to prevent accumulation.

(V) *Diffusion of succinate from cells*: when only cells or tissues are analyzed for succinate, intracellular accumulation will be reduced by the loss of succinate into the medium or the blood.

(VI) *Time after addition of malonate*: although this has never been studied, it is probable that the succinate concentration will follow characteristic time courses in each case, in some cases perhaps decreasing after a peak level has been reached.

Another factor, about which nothing is known, is the possible effect of rising succinate concentration on the reactions forming succinate. The oxidation of α-ketoglutarate forms succinyl-CoA, and succinate can arise in at least three different ways from succinyl-CoA.



What effects succinate concentration might have on these reactions are unknown, but some inhibition during succinate accumulation is possible, although a simple backing-up of the α-ketoglutarate → succinate reaction would be thermodynamically unlikely.

The relative potential rates of α-ketoglutarate oxidase and succinate oxidase under normal intramitochondrial conditions are not known, but the oxidation of succinate is certainly one of the most rapid reactions seen in mitochondrial suspensions. It may be that there is no accumulation of succinate in the cycle under physiological conditions, and that the small amounts of succinate found in tissues do not truly indicate the situation in the regions of succinate oxidase. Succinate oxidase is, perhaps, the one enzyme that has never been considered as normally limiting the cycle rate. If the maximal rate of succinate oxidation is much higher than the rate at which succinate can be formed, it would require a fairly high inhibition of the oxidase before succinate accumulates markedly. For example, if the rate of succinate formation is one-tenth the rate at which it can be oxidized, 90% inhibition of the succinate oxidase would make the rates equivalent, and the succinate concentration would not rise very much (probably not more than 0.02–0.05 mM). Under any likely conditions, calculations from Eqs. I-7-8 and I-7-9 make it clear that succinate oxidation must be inhibited fairly strongly to produce a significant succinate accumulation. The common assumption that succinate must accumulate rather quantitatively when sufficient malonate has been added to inhibit succinate oxidase 75–90% is thus unjustified. If alternate pathways for succinyl-CoA or succinate exist, the accumulation of succinate would be even less evident.

Several instances of failure of succinate to accumulate during malonate inhibition have been reported. Hanly *et al.* (1952) found that in only two of six experiments with carrot root slices did succinate accumulate, and in these the rise was insignificant. One might suspect a lack of penetration, but 15–50 mM malonate was used at pH 4; under these conditions, malonate depressed respiration strongly. Weil-Malherbe (1937) found no succinate accumulation in guinea pig brain slices with malonate 4–40 mM, respiration being markedly reduced at the higher concentrations. A depression of succinate level in *Streptomyces* due to 10 mM malonate was noted by Cochrane (1952), with either malate or citrate as substrate. Since the incubation was 16 hr and the pH 5.1–5.4, it is possible that a nonspecific acid damage from malonic acid penetration was responsible for the cycle depression, or it might be that in this organism malonate is not specific at 10 mM, or, as Cochrane suggested, succinate may not be formed via the cycle. The failure of succinate to accumulate, even when the respiration is suppressed by malonate, is difficult to explain except on the basis of actions other than on succinate dehydrogenase.

Succinate Accumulation in the Whole Animal

Some of the most interesting and suggestive experiments on succinate accumulation resulting from malonate inhibition have been performed with whole animals and, although such work is often difficult to interpret in a quantitative fashion, the results have demonstrated that malonate can partially block the succinate oxidase in various tissues of the living animal. Such inhibition has obvious implications for developments in the study of drug actions and chemotherapy. The first work of this type was done by Krebs *et al.* (1938), who determined the urinary excretion of succinate, citrate, and α-ketoglutarate following injections of cycle intermediates and malonate into rats and rabbits. Some of their results are shown in Table 1-17. The effects on citrate and α-ketoglutarate will be discussed later (see page 104). Although malonate increases the succinate excretion some 5-fold, only 2.9% of the injected fumarate is recovered as succinate compared to 0.6% in the controls. The injection of 10 millimoles of a substance into a rabbit will lead to a maximal extracellular concentration of approximately 30 m*M*, so that reasonably high concentrations of malonate were probably achieved. The effect of the malonate had mainly disappeared after 24 hr due to the excretion and destruction of the malonate. An almost 10-fold increase in the urinary succinate was seen in the more recent experiments of Thomas and Stalder (1958), in which 3.7 millimoles/kg of sodium malonate were fed to rats, the succinate over a 40-hr period rising from a control value of 2.35 mg to 28.0 mg.

The blood concentration of succinate is increased in rabbits following the injection of malonate (Forssman, 1941). Intravenous injection of 2.8 millimoles/kg of malonate leads to a slow rise in the blood succinate to around 0.20 m*M* at 3 hr, while injections of 3.5–5.1 millimoles/kg give levels as high as 0.77 m*M*. A lethal dose of 8.25 millimoles/kg produces death in 35 min and at the time of death the succinate concentration is 1.1 m*M*. The lower doses produce no obvious effects on the animals. The normal values for blood succinate are about 0.025 m*M*.

The succinate found in the urine and blood in these studies originated mainly in the tissues of the animals. Are malonate inhibition and succinate accumulation especially related to a particular tissue, or do all the tissues contribute to the metabolic disturbance? Can differences in the metabolic patterns of the various tissues be demonstrated by their responses to the administration of malonate? How do tumors compare with normal tissues in their susceptibility to malonate? It was to answer such questions as these that Busch and Potter (1952 a, b) at the McArdle Memorial Laboratory at Wisconsin undertook their excellent series of studies on the accumulation of succinate in various tissues of rats following injections of malonate. Analyses for malonate and succinate were made by anion exchange chromatography (Busch *et al.*, 1952) at various times after the subcutaneous

TABLE 1-17

URINARY EXCRETION OF CYCLE INTERMEDIATES IN RATS AND RABBITS RECEIVING INJECTIONS OF FUMARATE AND MALONATE [a]

Animal	Route	Day	Substrates (millimoles)	Malonate (millimoles)	Excretion (μmoles/day)		
					Citrate	α-Ketoglutarate	Succinate
Rabbit	IV	1	Fumarate (19.2)	—	300	394	116
		2	—	—	26	18	32
		3	Fumarate (20)	10	355	487	554
		4	—	—	25	31	40
		5	—	—	17	18	25
		6	—	20	49	161	210
Rat	SC	1	—	—	—	1.3	5.5
		2	—	2	—	51.4	324
		3	—	—	—	2.2	8.8
		4	—	—	9.7	5.3	12.4

[a] From Krebs *et al.* (1938). The rabbits were intravenously infused with 200 m*M* solutions at a rate of 1–2 ml/min. The rats were injected subcutaneously and four animals were used for each experiment.

injection of 12 millimóles/kg of malonate (Fig. 1-11). Maximal concentrations of malonate are reached in most tissues 1–2 hr after the injection and the time course of the succinate levels is well correlated with that of malonate. The tissue succinate concentration is linearly related to the malonate concentration, except in the Flexner-Jobling tumor (Busch and Potter,

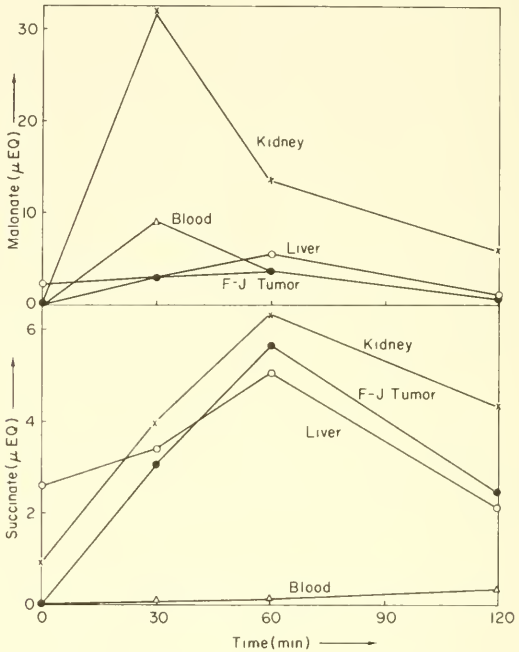


Fig. 1-11. Tissue levels of malonate and succinate in rats injected with sodium malonate (12 millimoles/kg). (From Potter *et al.*, 1952).

1952 a) (Fig. 1-12). The slopes of the lines give some measure of the degree of malonate effect in the particular tissue, but several factors are involved so that they are not quantitative indications of succinate oxidase inhibition. The urinary excretion of malonate, succinate, and citrate is shown in Fig. 1-13. The malonate and succinate levels in several normal tissues and tumors following 24 millimoles/kg malonate subcutaneously are given in Table 1-18. If the figures in the table are multiplied by approximately 0.7, one will obtain the millimolar concentrations in the cell water, except for kidney and blood, the former tissue having extracellular fluid high in succinate and malonate.

The ratios of (succinate)/(malonate) in the tissues give essentially the slopes of the lines in plots such as Fig. 1-12, except for tumors where a

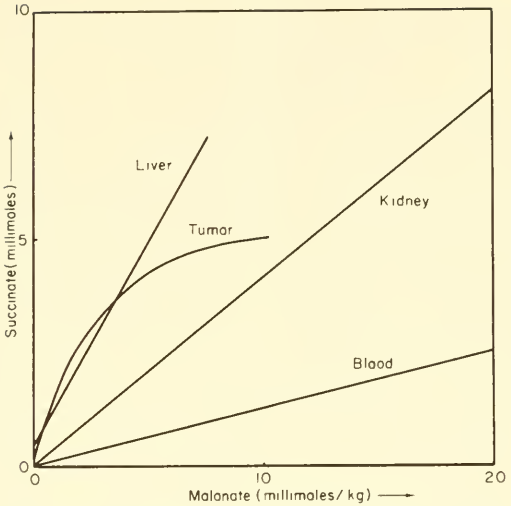


FIG. 1-12. Relationships between malonate and succinate concentrations in the tissues of rats injected with malonate. (From Busch and Potter, 1952 a).

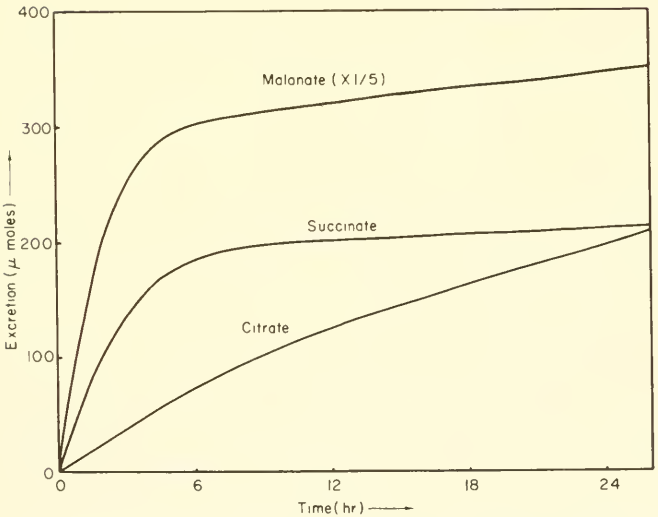


FIG. 1-13. Urinary excretions of malonate, succinate, and citrate following the injection of 12 millimoles/kg sodium malonate into rats. (From Busch and Potter, 1952 a).

TABLE 1-18

TISSUE SUCCINATE AND MALONATE CONCENTRATIONS FOLLOWING INJECTIONS OF MALONATE IN RATS [a]

Tissue	Succinate		Malonate		Ratio succinate / malonate
	Control	After malonate	Control	After malonate	
Spleen	0	12.8	0	12.8	1.00
Liver	2.2	12.0	2.4	14.2	0.85
Brain	0	2.0	0	2.8	0.71
Thymus	0	9.0	0	21.0	0.43
Kidney	0.6	16.4	0.3	44.0	0.37
Lung	0	5.6	0	16.0	0.35
Muscle	1.0	3.0	2.0	8.8	0.34
Heart	0	5.0	0	18.4	0.27
Blood	0	2.6	0	24.6	0.11
Tumors					
Flexner-Jobling carcinoma	0	9.2	0	17.6	0.52
Walker 256 carcinoma	0	8.0	0	5.2	1.54
Jensen sarcoma	0	8.8	0	8.0	1.10
Hepatoma	0	6.0	0	12.0	0.50
Papilloma	0	4.4	0	4.4	1.00
Average	0	7.3	0	9.4	0.78

[a] The figures are in μequivalents/g wet weight of tissue. Malonate was injected subcutaneously at a dosage of 12 millimoles/kg, and after 1 hr a similar amount was again injected; 1 hr following the last injection the animals were sacrificed. (From Busch and Potter, 1952 b.)

linear relationship may not be followed. These ratios indicate the amount of succinate formed per unit concentration of malonate and do not relate directly to the degree of inhibition of succinate oxidase but more to the ability of the tissue to form succinate in the presence of the inhibition. As discussed previously, for the same degree of block, succinate will be formed at greatly different rates in different tissues, depending on the succinate-forming substrates available and the activity of the pathways leading to succinate. The concentrations of malonate in the tissues vary greatly and this must be a reflection of the differing permeabilities of the tissues to malonate. It may be noted that the concentration in the brain is quite low,

a phenomenon seen with most ionic substances, and this must account for the poor accumulation of succinate in this organ and the relative lack of effect of malonate on central nervous system function.

The low concentration of succinate in the blood is interesting since it implies that succinate does not leave the tissues readily. The conclusion that must be reached is that the rate at which succinate diffuses out of the tissues into the blood is slower than the rate of renal excretion of the succinate. Substances that are not resorbed by the renal tubules are excreted rapidly and their concentrations in the blood can be maintained at a low level despite a continuous influx. Nevertheless, it shows that succinate leaves the tissue cells rather slowly under physiological conditions. The slow penetration of malonate into the tissues is suggested by the fact that the peak levels in the blood occur around 30 min after administration whereas the peak levels in liver and tumor occur 30 min later (Fig. 1-11), the kidney concentration paralleling the blood levels because of the excretory function of this organ.

The degree of succinate accumulation does not necessarily reflect the cycle activity in a tissue. For one reason, succinate can often be formed from other pathways. In certain tissues the amino acid content rises markedly (e.g. + 215% in thymus and + 160% in spleen) during malonate inhibition, while in others, especially the Flexner-Jobling carcinoma, the amino acids decrease as the succinate increases. It is likely in the latter tissues that some of the succinate is derived from amino acids, probably mainly glutamate. Thus the cycle activity in this tumor may be quite low and the normal accumulation of succinate due to other sources for the succinate. The other tumors do not show such marked decreases in amino acid content and this was attributed to their greater necrosis. It may be recalled that the incorporation of acetate by Flexner-Jobling tumor is slower than in most tissues and very little labeled succinate is formed from labeled acetate (Busch and Potter, 1953), indicating a low degree of cycle activity. We have seen that this tumor also differs from normal tissues in the nonlinearity of the plot of tissue succinate against tissue malonate (Fig. 1-12). At low levels of malonate the ratio (succinate)/(malonate) is near 3 but at high concentrations diminishes to 0.5. This means that as the malonate concentration rises, the ability of the tumor to accumulate succinate decreases. This is the type of curve expected if malonate at higher concentrations is inhibiting the reactions forming succinate. If the supply of cycle substrates in this tumor is low, a relatively small block of the cycle might reduce the formation of succinate through the cycle to zero. The slope approaches that of the blood, and it is possible that above the inflection point the slow rise in succinate may be due only to the rise in the blood.

This type of investigation could well be applied to other inhibitors and certain chemotherapeutic agents. First, one is able to correlate tissue

concentrations of inhibitor with the metabolic disturbance produced. Second, the inhibition occurs under physiological conditions, rather than in slices or minces or other preparations in which the cell metabolism may be very abnormal. Last, one is able to compare the different tissues with respect to their metabolic patterns and perhaps determine some of the reasons for the selective actions of inhibitors or drugs. These methods of investigation, called "*in vivo* metabolic blocking techniques" by Busch and Potter, if applied properly, would help to provide a more rational basis for development in chemotherapy and the selective depression of tumor growth.

ACCUMULATION OF CYCLE SUBSTRATES OTHER THAN SUCCINATE

Specific inhibition of succinate oxidase would be expected to lead to the accumulation of succinate but of no other cycle intermediates, because the free energy differences between them are of such magnitude that no backing-up from succinate would be anticipated. When other members of the cycle are found to accumulate in the presence of malonate, it is generally considered to be evidence that either the action of malonate is not specific or that secondary reactions are proceeding. Malonate has been shown many times to cause an accumulation of certain cycle intermediates, especially citrate and α-ketoglutarate, in cell suspensions, slices, and whole animals. The nature of these effects will first be summarized and then some possible mechanisms will be considered.

Accumulation of Citrate

The administration of malonate to dogs (Orten and Smith, 1937), rabbits (Krebs *et al.*, 1938), and rats (Busch and Potter, 1952 a) leads to an increased urinary excretion of citrate (Table 1-17 and Fig. 1-13). There is also a rise in plasma citrate following injections of malonate in rabbits (Forssman, 1941) and dogs (Stoppani, 1946). Tissue citrate also rises in mice injected with 10 millimoles/kg malonate: kidney (16 to 20), heart (40 to 70), liver (5 to 10), and diaphragm (70 to 225) (values in milligrams per kilogram wet weight) (Chari-Bitron, 1961). Brain, however, shows no increase in citrate, perhaps due to the poor penetration of malonate. Some accumulation of citrate has also been observed in suspensions of *Ashbya gossypii* mycelia metabolizing acetate and oxalacetate (Mickelson and Schuler, 1953), *Schizophyllum commune* mycelia metabolizing pyruvate and malate (J. G. H. Wessels, 1959), and Ehrlich ascites tumor cells metabolizing fumarate (Kvamme, 1958 c) in the presence of malonate. Thus this phenomenon is widespread, occurring in different types of organism and under a variety of conditions.

On the other hand, 4–10.5 millimoles/kg of malonate injected intravenously into rabbits does not increase plasma citrate appreciably (Forssman and Lindsten, 1946), and a number of reports have indicated a depression of citrate formation by malonate. Rat brain and liver homogenates oxidizing oxalacetate, or pyruvate and oxalacetate, form less citrate in the presence of 10 m*M* and 30 m*M* malonate, respectively, this being attributed to an inhibition of oxalacetate decarboxylase (Pardee and Potter, 1949). The formation of citrate from acetate in yeast is inhibited 73% by 17 m*M* malonate, while simultaneously succinate accumulates markedly (Barron and Ghiretti, 1953). The incorporation of C^{14} from glucose into citrate in potato tuber slices is also depressed 71% by 50 m*M* malonate (Table 1-19) (Romberger and Norton, 1961). Although there is an increase in citrate in excised tobacco leaves during culture with malonate, this increase is generally less than in the controls, so that this probably represents an inhibition of citrate formation (Table 1-20) (Vickery, 1959; Vickery and Palmer, 1957). Finally, malonate inhibits the formation of citrate from a variety of substrates in kidney and testis breis, the effects being surprisingly large for the reasonable concentrations of malonate used (Table 1-21) (Hallman, 1940). It is, therefore, evident that citrate levels may be affected by malonate in a variety of ways, depending on the malonate concentration, the type of preparation, and the conditions of the experiment. It is not difficult to explain the falls in citrate level brought about by malonate, since this could arise either by a depression of succinate oxidation (reducing the rate of entry of acetyl-CoA into the cycle) or inhibitions of other reactions (such as the condensation of oxalacetate and acetyl-CoA), especially at the high malonate concentrations often used. It is, on the other hand, difficult to interpret the accumulation of citrate and to this end we must direct our efforts, although only suggestions can be offered because of the lack of sufficient data.

Citrate is being formed and metabolized continuously and thus, generally speaking, a rise in the citrate level implies an inhibition of citrate utilization or an acceleration of its formation, or both. Although there is little evidence for a direct affect of malonate on isocitrate utilization, we have noted that an inhibition of succinate oxidation can interfere with isocitrate oxidation by depletion of NADP mediated by a fall in malate concentration (Jones and Gutfreund, 1964). This could certainly contribute to the accumulation of the tricarboxylates in some instances.

The inhibitions of citrate oxidation in Table 1-14 can be mostly explained on the basis of a block at the succinate oxidase step. On the other hand, there is certainly no evidence that the formation of citrate via the cycle can be stimulated by malonate, most data pointing instead to a depression if there is any effect. It would appear that effects of malonate on the cycle alone are not sufficient to explain an accumulation of citrate. Other path-

TABLE 1-19

DISTRIBUTION OF RADIOACTIVITY FOLLOWING 3-HR INCUBATION OF POTATO TUBER SLICES WITH GLUCOSE-u-C^{14} [a]

Component	Radioactivity (cpm)		
	Control	Malonate	% Change
Sucrose	344,000	306,000	− 11
Glucose	31,900	26,800	− 16
Fructose	5,950	5,830	− 2
Oligosaccharides	8,050	6,000	− 25
Weak acids			
Citrate	4,350	1,280	− 71
Isocitrate	235	757	+222
Succinate	925	6,300	+582
Fumarate	190	125	− 34
Malate	8,900	1,740	− 80
Glycolate	2,750	1,830	− 33
Total	67,300	41,500	− 38
Acidic amino acids	56,100	16,700	− 71
Neutral amino acids	32,100	56,200	+ 75
Basic amino acids	950	750	− 21
Phosphorylated compounds	28,000	10,000	− 64
Lipids	111	367	+230
Respiratory CO_2	22,600	17,300	− 23

[a] Fresh slices of potato tubers incubated at 28° and pH 5 for 3 hrs with uniformly labeled glucose, in the absence of and presence of 50 m*M* malonate. (From Romberger and Norton, 1961.)

ways for the formation of citrate are known, e.g., the citrase reaction from acetate and oxalacetate, or through isocitrate by the isocitrase reaction from succinate and glyoxylate. However, the free energy changes for these reactions are such that citrate would not accumulate in significant amounts even though the substrates for its formation accumulated. Also the results in Table 1-22 show that the effect is not specific for malonate, but is seen with succinate, malate, fumarate, and other organic anions. The marked effect of glutarate, which is a poor inhibitor of succinate oxidase, suggests that the citrate accumulation may not be related to the block of this enzyme. It may be noted that part of the augmented citrate excretion may be attri-

TABLE 1-20

EFFECTS OF MALONATE ON THE COMPONENTS OF EXCISED TOBACCO LEAVES [a]

Component	Control before incub.	Change during culture in:						Control before incub.	Change during culture in:			
		Water	K-succ (200 m*M*) (pH 5)	K-malonate (100 m*M*)					Water	K-succ (200 m*M*) (pH 6)	K-malonate (200 m*M*)	
				(pH 4)	(pH 5)	(pH 6)	(pH 7)				(pH 5)	(pH 6)
Change in fresh weight (%)	0	+ 9	+ 14	− 10	− 22	− 2	0	0	+ 8	+ 5	− 10	− 8
Ash (g/kg)	20	− 0.1	+ 15.2	+ 4.9	+ 8.6	+ 9.5	+ 9.4	17.6	+ 0.2	+ 14.3	+ 8.4	+ 11.8
Uptake of acid (meq/kg)	—	—	410	149	221	164	136	—	—	221	203	210
Total organic acids (meq/kg)	276	− 15	+223	+ 97	+171	+140	+134	201	− 5	+189	+151	+190
Malate (meq/kg)	158	− 73	+ 59	−112	−114	−101	− 98	166	− 31	+100	− 90	−100
Citrate (meq/kg	33.4	+ 50.2	+ 76.3	+ 19.2	+ 6.8	+ 30.2	+ 41.1	15.1	+ 23.9	+ 43.0	+ 7.9	+ 24.5
Oxalate (meq/kg)	44	+ 4	+ 3	− 9	− 11	− 11	− 10	12	+ 3	0	0	− 2
Succinate (meq/kg)	Trace	Trace	+ 25	+ 37	+ 52	+ 55	+ 49	Trace	Trace	+ 32	+ 45	+ 78
Malonate (meq/kg)	—	—	—	+120	+184	+139	+106	0.9	+ 0.5	(+ 11)	+185	+183
Protein-N (g/kg)	3.55	− 0.37	− 0.60	− 0.61	− 0.38	− 0.53	− 0.60	—	—	—	—	—
Starch (g/kg)	1.39	− 1.19	− 1.33	− 1.27	− 1.30	− 1.30	− 1.34	2.6	− 2.5	− 2.5	− 2.2	− 2.6
Malonate metabolized (% of uptake)	—	—	—	0	0	17	8	—	—	—	8.7	13
Malonate (final concentration m*M*)	—	—	—	80	140	80	60	—	—	—	120	110

[a] All values are for 48-hr incubation. The data for 100 m*M* malonate are from Vickery and Palmer (1957), and for 200 m*M* malonate from Vickery (1959).

TABLE 1-21

EFFECTS OF MALONATE ON THE FORMATION OF CITRATE FROM DIFFERENT SUBSTRATES IN KIDNEY AND TESTIS BREIS [a]

Substrate (20 m*M*)	Kidney			Testis			
	Control	% Inhibition by malonate		Control	% Inhibition by malonate		
		10 m*M*	20 m*M*		6.7 m*M*	10 m*M*	20 m*M*
None	0.5	—	Stim 20	0.8	50	75	87
Pyruvate	2.8	79	82	2.7	44	93	—
α-Ketoglutarate	11.3	50	57	3.5	16	8	16
Succinate	0.7	43	64	1.2	48	60	68
Fumarate	3.1	Stim 18	Stim 29	1.9	20	54	—
Malate	1.7	Stim 35	Stim 59	2.0	32	17	37
Oxalacetate	1.8	Stim 28	—	—	—	—	—
β-Hydroxybutyrate	1.1	73	90	1.6	81	90	—

[a] Incubation time 30 min for kidney and 60 min for testis. The figures for control citrate simply give the milligrams formed during the incubation. (From Hallman, 1940.)

TABLE 1-22

URINARY EXCRETION OF CITRATE FOLLOWING ADMINISTRATION OF THE SODIUM SALTS OF VARIOUS WEAK ACIDS

Substance	Urinary citrate [a] after dose [b] of:			
	4.35	26	26	39
None	0.47	16	—	—
NaCl	0.50	—	2.4	—
$NaHCO_3$	0.90	44	9-25	10-17
Malonate	14.76	583	54.2	72.4
Succinate	14.80	418	29.5	39.4
Pyruvate	—	—	10.2	42.3
α-Ketoglutarate	—	336	12.3	36.6
Fumarate	17.10	275	25.8	20.1
Malate	18.50	287	13.1	45.7
Oxalacetate	—	216	6.7	16.2
Glutarate	—	1150	44.3	65.8
Adipate	—	200	3.7	17.6
Maleate	61.30	210	11.4	45.0
Acetate	1.53	—	—	34.5
Citrate	115.2	—	—	52.5
Aconitate	—	413	26.3	42.0

[a] The second column shows the 24-hr urinary citrate (mg/kg) in dogs. From Orten and Smith, (1937.)

The third column shows the urinary concentration of citrate (mg%) in rats. (From Simola and Kosunen, 1938.)

The fourth and fifth columns show the 24-hr urinary citrate (mg) in rats. (From Krusius, 1940.)

[b] Dose in millimoles per kilogram.

buted to alkalosis. Crawford (1963) confirmed that the injection of 10 millimoles/kg of malonate in rats causes a marked rise in urinary citrate (1.7 → 47 μmoles/kg/hr), but found that succinate, malate, and sodium bicarbonate also have this effect. Serum citrate simultaneously rises very moderately. It was concluded that all the effects are due to an alkalosis induced by the administration of sodium. However, this cannot account for all of the actions of malonate, nor could it be responsible for the citrate accumulation in cell suspensions and isolated preparations.

Another possibility is that the citrate arises from the substances that are administered. This was favored by Orten and Smith (1937), but it was

difficult then with incomplete knowledge of the cycle and related pathways to understand how such conversions could take place; it still is. That is, if there is no significant impairment of the utilization of citrate by malonate, it is difficult to conceive of a pathway for the formation of citrate from malonate that would be so rapid as to lead to a large rise in the citrate level. A possibility that seems not to have been considered is that the substance determined as citrate may not have been citrate but a related tricarboxylic acid or some other compound giving a positive test. Although Hallman (1940) examined the specificity of the determination, there are many substances that have not been tested. For example, it is easy to formulate reactions in which malonyl-CoA could react with various carbonyl substances, such as glyoxylate or pyruvate, to form tricarboxylate anions which might be oxidized to pentabromacetone in the citrate test and be mistaken for citrate. Certain dicarboxylates, such as itaconate, also are determined in this test. Such substances may not be readily metabolized and hence would accumulate much more readily than citrate. Although this posibility may seem far-fetched, it would be well to make certain that it actually is citrate that is accumulating during the action of malonate. It would be necessary to convert only a small fraction of the administered malonate to such a compound, since a dose of 26 millimoles/kg (see column 3 of Table 1-22) would theoretically give rise to almost 1 g of a substance with a molecular weight near that of citrate, whereas actually only around one-twentieth of this was determined as citrate. Of course, such estimations depend on the degree of sensitivity of the test to the compound. If there is any validity in this suggestion, it may be that depressions of citrate levels may occur in those preparations or tissues where such reactions of malonate or its metabolic products do not occur, i.e., where the response to malonate is the one expected on the basis of its inhibition of the functioning of the cycle.

Accumulation of α-Ketoglutarate

Very large increases in urinary α-ketoglutarate following the administration of malonate to rabbits and rats were reported by Krebs *et al.* (1938) (Table 1-17) and this has been confirmed by El Hawary (1955), who found a 3.7-fold increase in serum α-ketogluterate 30 min after the intraperitoneal injection of 20 millimoles/kg malonate. As in the case of citrate, Krusius (1940) found that α-ketoglutarate excretion is increased not only by malonate but by many organic anions: malonate (46.4), maleate (42.0), malate (17.7), succinate (17.7), β-hydroxybutyrate (15.7), acetate (14.3), pyruvate (12.1), fumarate (9.3), and sodium bicarbonate (0.6–7.8) (the figures give urinary excretion in milligrams/day). He concluded that essentially all the substances that increase citrate also raise the α-ketoglutarate excretion. However, glutarate is a notable exception, for it potently augments citrate formation but has no effect on α-ketoglutarate excretion. This would seem

to disprove the theory that glutarate is active because it undergoes β-oxidative decarboxylation to malonate. Little has been done with isolated preparations, but in three cases accumulation of α-ketoglutarate has been observed in the presence of malonate: in locust sarcosomes from malate (Rees, 1954), in suspensions of ascites cells from fumarate (Kvamme, 1958 c), and in ascites cells and rat heart mitochondria from glutamate and malate (Borst, 1962), in all instances the malonate concentration being rather high (20–50 mM).

The mechanism of such accumulation is poorly understood. We have seen that in some tissues the α-ketoglutarate oxidase can be inhibited by malonate, especially at concentrations above 10 mM, so that the results can be partially explained in this way (at least for the ascites cells and locust particulate preparations). The moderate increases in α-ketoglutarate excretion brought about by the cycle intermediates and substrates might well be due to a greater rate of formation with unchanged utilization rate. However, the possibility of a formation of α-ketoglutarate from glutamate cannot be ignored. It will be recalled that in potato slices the succinate formed in the presence of malonate comes partly from such a source (Romberger and Norton, 1961). Permeability effects causing a leakage of α-ketoglutarate and other anions from the tissues must also be considered. El Hawary (1955) found that several inhibitors (arsenite, maleate, iodoacetate, alloxan, and fluoride) increase the serum α-ketoglutarate levels in rats, and simultaneously raise pyruvate levels. It may well be that any severe metabolic disturbance causes a release of cycle substrates from the tissues and an increased excretion, as well as secondary changes such as hyperglycemia.

Effects on the Levels of Other Cycle Substrates

The tissue concentrations of all the cycle substrates are probably altered by malonate and it is sufficient here to mention the results with potato tuber slices (Table 1-19) and tobacco leaves (Table 1-20). The reduction in the incorporation of C^{14} from glucose into malate in the former and the marked falls in malate level in the latter would be anticipated from a block of succinate oxidation. Fumarate and oxalacetate levels probably are changed similarly. In this connection, one wishes that more information were available on the factors that control the tissue pools of cycle intermediates, since it is evident that these substances do not occur only in the mitochondria in kinetic equilibria depending on the relative rates of the cycle reactions, but must also be present in cellular compartments. The transfer of the substances between these compartments and the active cycle regions must depend on processes that could be affected by inhibitors. Such compartments are well known in plant cells but it is probable that similar situations are applicable to animal cells.

Sequential Inhibition with Malonate and Fluoroacetate on Citrate Levels

Before leaving the subject of accumulation of cycle intermediates, a few words must be said on the effects of malonate on the increases in citrate brought about by fluoroacetate. This was discussed in Volume I (page 502) and the results obtained by Potter (1951) presented (Fig. I-10-5). The principle of the experiments is simply that fluoroacetate blocks the utilization of citrate so that the tissue citrate levels rise at different rates and to different degrees. If malonate is administered to the animals prior to the fluoroacetate, the accumulation of citrate may be modified. These studies thus provide information on the effects of malonate on the rates of formation of citrate and supplement the results discussed above.

The tissues differ greatly in their response to malonate in the presence of fluoroacetate. In thymus, for example, malonate blocks the formation of citrate completely and no citrate accumulation at all occurs. The kidney behaves similarly but some citrate begins to accumulate an hour after the fluoroacetate is injected. In spleen and brain the inhibition of citrate formation is around 50%. Heart responds quite differently from the other tissues. Here malonate actually increases the citrate formation somewhat. Potter believed these results to indicate that in heart there are pathways other than the cycle for the synthesis of oxalacetate, and suggested the conversion of ketone bodies (malonate induces ketonemia) to citrate by a pathway involving oxalacetate, the ketone bodies arising from fatty acids. Liver was not studied but if similar reactions occur in this tissue, they could,,at least in part, account for the increases in serum citrate and urinary excretion of citrate. In any event, these experiments illustrate very well the inherent differences between tissues with respect to their metabolic pathways. The stimulation of citrate formation in the heart in the presence of fluoroacetate was confirmed by Fawaz and Fawaz (1954), whereas in the kidney only a depression was observed. The use of fluoroacetate to block the utilization of citrate is a useful technique by which to study the effects of malonate uncomplicated by possible effects on the rate of disappearance of citrate.

ANTAGONISM OF MALONATE INHIBITION WITH FUMARATE

The overcoming of an inhibition by the addition of an intermediate normally arising distal to the site of the block is often excellent evidence for the locus of action of the inhibitor and for the specificity of the inhibition. For this reason, fumarate has frequently been used in malonate-inhibited preparations and a reversal of the inhibition taken as proof for the specific action of malonate on succinate oxidase. The first acceptable

data for fumarate reversal were reported by Quastel and Wheatley (1935), who showed that the inhibition of acetoacetate utilization by rat liver in the presence of malonate is mainly abolished when fumarate is added, and they used this as evidence that malonate does not act directly on the enzyme involved in the breakdown of acetoacetate. The use of fumarate immediately became popular and many studies since 1935 have included its addition for the purpose of demonstrating that the observed effect of malonate is indeed due to its block of succinate oxidation.

Most of the tests for fumarate reversal, it must be admitted, have not been done properly and the results have been evaluated uncritically, so that little of value has been demonstrated. There are several points that must be considered in the planning and interpretation of such experiments.

(*a*) An increase in the oxygen uptake upon addition of fumarate to a malonate-inhibited preparation is not, by itself, very meaningful. Let us take a typical experiment similar to many reported in the literature. The normal Q_{O_2} of a tissue preparation is 10 and this is decreased to 4.5 by malonate. When malonate and fumarate are added together, the Q_{O_2} is 8.7. It has been generally stated in such cases that fumarate is capable of antagonizing the malonate inhibition. Actually, an increase in the respiration could have been brought about by the addition of any substrate that is oxidized by the preparation, including substrates completely unrelated to the cycle. One can say that fumarate has overcome the malonate inhibition but the results are of no particular significance with respect to malonate specificity or the site of the inhibition. What one has shown is that fumarate can be oxidized in the presence of malonate and this need not imply that the operation of the cycle has been even partially restored. If fumarate had been added to the uninhibited preparation and a Q_{O_2} of 14.2 found, the following might have been concluded: malonate inhibits the endogenous respiration 55% and the respiration in the presence of fumarate either 13% (with respect to the endogenous control) or 39% (with respect to the rate with fumarate alone present), in both cases the inhibition being less than that for the endogenous respiration. Actually, it has been shown that the oxidation of fumarate is unaffected by malonate, since the same absolute rise in the Q_{O_2} is obtained from fumarate in the uninhibited and inhibited preparations. If the oxygen uptake resulting from the addition of fumarate results mainly from the oxidation of malate, the results would have little bearing on the mechanism or selectivity of malonate inhibition.

(*b*) The addition of fumarate would not in any case restore the complete cycle, since oxidation of succinate would still be depressed and less energy would be available from this step. It is possible in some instances that the energy from succinate oxidation, rather than the over-all energy production from all oxidations, is important, and this would not be restored by fumarate.

(*c*) The accumulation of succinate due to malonate inhibition would, of course, not be reversed by fumarate; instead, it is usually increased. If some response to malonate is dependent on the rise in succinate concentration (e.g., a direct effect of succinate on other enzymes or some cell function, or the increased formation of some substances derived from the succinate), this would not be antagonized by fumarate.

(*d*) The response to fumarate will often depend on what is being measured. One example will be used here to illustrate this and others will be mentioned later. Malonate inhibits the oxidation of trilaurin and octanoate in liver and kidney slices, and also reduces the amount of $C^{14}O_2$ formed from labeled substrates (Geyer *et al.*, 1950 a). It was found that fumarate is very ineffective in counteracting the inhibition of $C^{14}O_2$ production, and this might be attributed to a direct effect of malonate on fatty acid oxidations. However, the results can be explained on the basis of an inhibition of succinate oxidase. Much of the C^{14} taken into the cycle from acetyl-CoA would accumulate in succinate and fumarate would have no effect on this. For the full release of CO_2, the operation of the entire cycle is required, and thus fumarate would increase the $C^{14}O_2$ formation only moderately in the presence of malonate. If the oxygen uptake had been determined, fumarate could well have shown a complete reversal of the malonate inhibition.

(*e*) Absence of a reversal by fumarate, or the failure to achieve a complete reversal, can be due to a variety of causes. In an experiment, such as the one shown in the following tabulation, done on *Aplysia* muscle slices:

Additions	Q_{O_2}
Endogenous	0.33
Succinate	1.80
Malonate	0.33
Succinate + malonate	0.96
Succinate + malonate + fumarate	0.90

although it was stated that fumarate was unable to relieve the malonate inhibition (Ghiretti *et al.*, 1959), it may simply be that fumarate would not have been oxidized if added to the uninhibited tissue. Certainly the direct inhibition of succinate oxidase would not be expected to be overcome by fumarate, but in any case a control with fumarate alone must be run to give any significance to the results. It is even possible for fumarate to increase the inhibition produced by malonate. This was observed for the respiration of *Helix* hepatopancreas (see accompanying tabulation) (Rees, 1953). Here the endogenous respiration apparently is little dependent on the cycle, whereas upon the addition of fumarate, through the formation of oxalace-

tate and its partial decarboxylation to pyruvate, the cycle presumably becomes activated. The high inhibition of fumarate oxidation by malonate cannot, of course, be attributed solely to an inhibition of succinate oxidase,

Additions	O_2 uptake	% Inhibition
Endogenous	30	—
Malonate	25	16.7
Fumarate	167	—
Malonate + fumarate	47	71.9

and an effect on oxalacetate decarboxylase or some other enzyme at the relatively high malonate concentration (33 m*M*) must be assumed. It is desired to point out that the effects with fumarate are unrelated to the inhibition of the endogenous respiration observed with malonate and throw no light on the mechanism of the inhibition.

Let us now turn to some experiments in which the addition of fumarate provides results indicative of the mechanism of malonate inhibition. The most significant studies usually have involved the determination of the utilization or formation of a particular substance. In the original work of Quastel and Wheatley (1935) mentioned above, the disappearance of acetoacetate in rat liver slices was determined, and fumarate was found to increase its utilization in the presence of malonate, whereas in the absence of malonate it had essentially no effect. Krebs and Eggleston (1940) likewise showed that fumarate would increase the utilization of pyruvate in pigeon muscle in the presence of malonate, at high malonate concentrations the amount of pyruvate utilized being equivalent to the fumarate added. The results obtained by Stare *et al.* (1941) with pigeon muscle show definitely that the block of pyruvate utilization produced by malonate is effectively overcome by fumarate (see accompanying tabulation). Results such as

Additions	Pyruvate utilized
Endogenous	16.3
Malonate	7.2
Fumarate	21.4
Malonate + fumarate	23.3

these demonstrate that fumarate can counteract the effect of malonate on a specific process and, from the nature of the experiments, it is likely that the cycle block is being overcome in a sense, that is, that oxalacetate is being made available for condensation with acetyl-CoA. They also provide

some information on the site and specificity of the malonate inhibition. Quite different results on acetate utilization by yeast were obtained by Stoppani *et al.* (1958 b), who found that fumarate is completely unable to reverse the inhibition by malonate (see accompanying tabulation). These

Additions	Acetate utilized
Acetate	30
Acetate + malonate	4.4
Acetate + fumarate	27
Acetate + malonate + fumarate	3.8

data would imply that the inhibition of acetate utilization by malonate is not mediated through a block of succinate oxidation but by another mechanism. It is difficult to explain this by a failure of fumarate to penetrate into the cells since malonate seems to enter readily. It was suggested that malonate might interfere with acetate activation by depleting the system of coenzyme A, due to the formation of relatively stable malonyl-CoA. Whatever the mechanism, these results point to an action of malonate other than on succinate oxidase.

An interesting illustration of the useful information that may be obtained from the use of fumarate is given in the inhibition of urea formation by malonate. The formation of both arginine and urea from citrulline and glutamate in liver homogenates is potently inhibited by malonate (Cohen and Hayano, 1946; Krebs and Eggleston, 1948). Fumarate is able to counteract this block completely. These results were difficult to understand initially, but it is now known that transamination must occur between glutamate and oxalacetate to form aspartate, which reacts with the citrulline to form arginosuccinate, from which arginine and urea are derived. The effect of malonate is to reduce the supply of oxalacetate for transamination and it is clear why fumarate will abolish this inhibition (Ratner, 1955). Krebs and Eggleston (1948) observed the formation of 3–5 molecules of urea for each molecule of fumarate added. This may be explained by the fact that the formation of arginine from arginosuccinate involves the release of fumarate, which can again go to oxalacetate.

The demonstration that fumarate will counteract the inhibitory action of malonate on some tissue function is indicative of a primary block of the succinate oxidase, but even complete reversal does not prove a specific action of malonate. One example would be the inhibition of Br^- uptake in barley roots by malonate (Machlis, 1944). Malonate (10 m*M*) inhibits the uptake 57% but if fumarate is present the uptake is 35% above the control. On the surface this would imply an effective reversal of the malonate inhi-

bition, but actually fumarate alone increases the uptake to 71% above the control. Thus malonate inhibits a significant amount even in the presence of fumarate, pointing to an action other than on succinate oxidation. Another example would be the malonate inhibition of cell division of *Arbacia* eggs (Barnett. 1953). Cleavage is inhibited almost completely by 60 m*M* malonate and an equimolar concentration of fumarate abolishes this. It is likely that fumarate overcomes the cycle block but one cannot conclude that the action of malonate is specific on succinate oxidase. Malonate, can also inhibit to some extent steps in the utilization of fumarate, but due to the high concentration of fumarate enough oxalacetate is formed to allow cleavage. Indeed, succinate at 60 m*M* also abolishes the inhibition, indicating that as a result of the competitive nature of the inhibition enough succinate has broken through the block to restore cleavage. It must be remembered that a complete reversal of a metabolic block is not always necessary for a cell function to proceed normally.

SPECIFICITY OF MALONATE INHIBITION IN THE CYCLE

At this point we may summarize some of the conclusions with respect to the specificity of action of malonate on the succinate dehydrogenase. Possible effects of malonate outside the cycle will be discussed in a later section. We have seen that malonate can inhibit enzymes other than succinate dehydrogenase rather potently (Table 1-12), that the oxidations of certain cycle substrates are suppressed more than predicted on the basis of a selective action on succinate oxidation (Table 1-14), that the accumulation patterns of cycle intermediates are sometimes distorted by malonate in ways implying inhibition at more than one site, and that fumarate is seldom able to reverse the actions of malonate completely. Of particular significance are the clear demonstrations of the inhibition of reactions related to the entry of acetyl-CoA into the cycle, particularly those of Pardee and Potter (1949) pointing to an inhibition of the condensation reaction, those of Stoppani *et al.* (1958 b) reporting a marked inhibition of acetate utilization apparently unrelated to an inhibition of succinate oxidase, and our own results on rat heart mitochondria where malonate inhibits pyruvate oxidation in the presence of malate and with the α-ketoglutarate oxidase completely blocked. Another susceptible site is α-ketoglutarate oxidation, especially in view of the clear proof by Price (1953) that specific inhibition can not even be obtained in the simple system,

$$\alpha\text{-Ketoglutarate} \rightarrow \text{succinate} \rightarrow \text{fumarate}$$

There is thus a large amount of evidence that malonate can at certain concentrations in various conditions inhibit other reactions than succinate oxidation.

It would be convenient if one could specify a malonate concentration, or range of concentrations, which would most likely be specific, but there are too many factors involved to do this with any confidence. Some of the factors may be listed: (a) the species, tissue, or preparation used, (b) the enzymes or metabolic pathways involved in what is measured, (c) the conditions of the experiment, e.g., the pH or the Mg^{++} concentration, (d) the degree to which succinate can accumulate and antagonize the inhibition, (e) the effective concentration of malonate within cells, and (f) the possibility of nonenzyme effects on cell membranes or other structures. Specificity for an inhibitor is not a constant to which can be given a value for all cases, but a characteristic that must be evaluated for each experiment. Aside from the direct actions of malonate on enzymes, there are the problems of metal cation depletion, the possible effects of Na^+ or K^+ added with the malonate, and the inactivation of the coenzyme A in some preparations through the formation of malonyl-CoA.

Bearing in mind these difficulties, a few general remarks may be made. It is probable that malonate usually does not inhibit any cycle enzyme more strongly than succinate dehydrogenase, so that the major effect will be related to the inhibition at this site, but it will be recalled that in certain species the succinate dehydrogenase is not very susceptible to malonate. It is impossible to achieve a nearly complete block of succinate oxidation without affecting other cycle reactions; if one wishes a specific effect, one must be satisfied with a moderate inhibition of succinate oxidation. If a single malonate concentration for general usefulness had to be chosen, 5 m*M* might be taken provisionally. Although in some cases rather incomplete inhibition will be obtained, this concentration will probably not inhibit other cycle reactions significantly. This applies to noncellular preparations where penetration is not a factor but otherwise higher external concentrations may have to be used. In any case, good evidence for a specific action should be obtained under the conditions of the investigation, and reliance should not be based on generalities.

EFFECTS OF MALONATE ON OXIDATIVE PHOSPHORYLATION

An uncoupling action on oxidative phosphorylation has been claimed for malonate several times and it is important to determine if this is actually so. This is quite difficult because pertinent or reliable data are generally lacking. There are four important ways in which malonate could alter the P : O ratio: (1) a direct effect on the coupling between oxidation and phosphorylation, (2) an alteration of the pattern of substrate oxidation, since different substrates may have different P : O ratios, (3) a differential inhibition of electron transport pathways for a single substrate but with differ-

ent P : O ratios, and (4) an effect on the hydrolysis of ATP or other high energy substances. Only the first mechanism should be considered as true uncoupling, although it is often difficult to determine the exact mechanism. Included in the first mechanism would be the chelation of malonate with Mg^{++} or Mn^{++}, since these cations are believed to be cofactors in phosphorylation.

Claims for an uncoupling action will be discussed first. Lehninger (1951) stated that malonate uncouples oxidative phosphorylation associated with the oxidation of β-hydroxybutyrate by rat liver mitochondria but no data were given. In a previous report (Lehninger, 1949) 7.5 m*M* malonate was shown to inhibit oxygen uptake 48%, the formation of acetoacetate from β-hydroxybutyrate 64.7%, and phosphorylation 37.3% (as determined by the incorporation of P^{32} into the ester fraction). Since phosphorylation is inhibited less than oxidation, no uncoupling is evident, and indeed the P:O ratio should increase. Berger and Harman (1954) claimed that malonate inhibits phosphorylation associated with the one-step oxidation of α-ketoglutarate and completely suppresses phosphorylation during the oxidation of L-glutamate by muscle mitochondria. However, the malonate concentration is not given and the absence of data prevents evaluation of the results. An inhibition of phosphorylation does not necessarily mean an uncoupling action. Malonate at 30 m*M* drops the P:O ratio from 1.6 to 0.5 in the oxidation of choline by rat liver mitochondria (Rothschild *et al.*, 1954). Although no control with malonate alone was reported, it would appear that this is the most valid instance of uncoupling by malonate. The malonate concentration was high and a reduction of Mg^{++} (total concentration was 5.7 m*M*) must be considered. Finally, the phosphorylation associated with succinate oxidation in lupine mitochondria was shown to be strongly depressed by 10 m*M* malonate (Conn and Young, 1957), but it may be observed that the oxygen uptake was inhibited even more (Table 1-23), so that no uncoupling occurred.

All of the reports in which P:O ratios were calculated are summarized in Table 1-23 and in all cases, except for *E. coli*, *Carcinus maenas*, and the oxidation of choline, it is seen that the P:O ratio is actually increased by malonate. However, most of these only illustrate mechanism (2) above, because in the oxidation of α-ketoglutarate a rise in the P:O ratio would be expected upon blocking succinate oxidase due to the fact that P:O for succinate oxidation is 2, whereas for the one-step oxidation of α-ketoglutarate to succinate usually it is experimentally between 3 and 4. All one can say from such data is that there is no evidence for an uncoupling action by malonate. Copenhaver and Lardy (1952) used 3–20 m*M* malonate in all their media in the study of the phosphorylation associated with α-ketoglutarate oxidation and obtained high P:O ratios, again providing evidence against any uncoupling activity. It was shown by Slater and Holton in

TABLE 1-23

EFFECTS OF MALONATE ON OXIDATIVE PHOSPHORYLATION IN PARTICULATE PREPARATIONS

Preparation	Substrate	Malonate (mM)	% Change of		P : O	Reference
			P_i uptake	O_2 uptake		
Escherichia coli	Malate	—			0.6	Kashket and Brodie (1963 a)
		67	−47	+17	0.4	
Phaseolus aureus (mung bean)	α-Ketoglutarate	—			0.79	Bonner and Millerd (1953)
		10	−25	−43	1.03	
	Citrate	—			0.81	
		10	− 3	−21	1.00	
Lupinus albus (lupine)	α-Ketoglutarate	—			1.5	Conn and Young (1957)
		10	−13	−63	3.6	
	Succinate	—			1.9	
		10	−71	−88	4.6	
Ipomea batatas (sweet potato)	α-Ketoglutarate	—			2.0	Lieberman and Biale (1956 a)
		10	—	—	2.5	
	Succinate	—			1.29	Hackett *et al.* (1960)
		1	−24	−35	1.50	
		3	−52	−65	1.74	
Malus sylvestris (apple)	α-Ketoglutarate	—			0.9	Lieberman (1961)
		1	−24	−47	1.3	
	α-Ketoglutarate	—			2.32	Jones *et al.* (1964)
		20	—	—	2.89	

Carcinus maenas (crab)	α-Ketoglutarate	—			2.72	Beechey (1961)
		10	−54	−49	2.45	
		30	−70	−59	1.98	
Calliphora erythrocephala (blowfly)	α-Ketoglutarate	—			0.73	Lewis and Slater (1954)
		10	+14	−10	0.92	
Carp liver	α-Ketoglutarate	—			2.22	Gumbmann and Tappel (1962 a)
		15	−24	−25	2.24	
Pigeon muscle	α-Ketoglutarate	—			2.92	Azzone and Carafoli (1960)
		3.3	− 9.5	−15	3.11	
		13.3	−17	−26	3.28	
		26.7	−19	−26	3.18	
Pigeon Muscle	α-Ketoglutarate	—			2.60	Marcus and Manery (1963)
		25	−15	−37	3.52	
Mouse liver	α-Ketoglutarate	—			2.61	Kielley and Kielley (1951)
		3.3	−23	−40	3.38	
Rat liver	Succinate	—			2.0	Chappell (1964 b)
		1	−64	−70	2.4	
Rat liver	Choline	—			1.60	Rothschild *et al.* (1954)
		30	−71	− 5	0.50	
Rat heart	α-Ketoglutarate	—			1.71	Slater and Holton (1954)
		10	−19	−33	2.06	
Beef heart	Succinate	—			0.39	Conover *et al.* (1963 b)
		2	−58	−62	0.43	
	Malate + NAD	—			0.56	
		2	+ 9	−10	0.68	

rat heart (1954) that malonate increases the P:O ratio with α-ketoglutarate as the substrate as the malonate concentration is increased up to 20 mM; at 40 mM the P:O ratio decreases somewhat, so that at this high concentration a small degree of uncoupling may occur. Azzone and Carafoli (1960) found the same in pigeon muscle. Evidence against uncoupling by malonate intracellularly is provided by the study on ascites carcinoma cells by Creaser and Scholefield (1960). Comparison of the changes brought about by malonate (20 mM) in the respiration and the sum of the concentrations of ADP and ATP led to the estimation of a 15% increase in the P:O ratio, whereas the classic uncoupler, 2,4-dinitrophenol, depressed the P:O ratio 70%. Malonate may actually stimulate the esterification of phosphate. In addition to the two examples in Table 1-23, Jackson *et al.* (1962) reported an 8% elevation in phosphate uptake by 0.1 mM malonate in barley root mitochondria oxidizing succinate, and Rosa and Zalik (1963) found such a stimulation in pea seedling mitochondria, which is maximal around 0.01 mM malonate, the oxygen uptake not being altered. Above this concentration, both phosphorylation and oxidation are depressed in a parallel fashion; respiration is hence always depressed somewhat more than is phosphate esterification, and no uncoupling is seen at any malonate concentration. The transfer of phosphate from phosphorylated coenzyme Q to ADP to form ATP in mitochondrial preparations is not altered by even 10 mM malonate (Gruber *et al.*, 1963), whereas dinitrophenol inhibits this readily.

It would therefore appear to be legitimate to conclude that malonate does not exhibit uncoupling activity except possibly at high concentrations (above 30 mM). The evidence for uncoupling at concentrations commonly used is considered to be inadequate and outweighed by the mass of indirect evidence that P:O ratios are not reduced in the oxidations of citrate, α-ketoglutarate, and succinate.

EFFECTS OF MALONATE ON GLUCOSE METABOLISM

The actions of malonate on carbohydrate, lipid, amino acid, porphyrin, and other types of metabolism will now be considered, after which it will be possible to evaluate the specificity of malonate more broadly. The effects on glucose metabolism are important but difficult to analyze. In the first place, the interrelationships between the cycle and the glycolytic pathways are complex and secondary effects on glucose utilization must be expected. In the second place, much of the work on the alteration of glucose utilization by malonate has not been adequate for the determination of mechanisms nor do the results even provide useful information in many cases, and for this reason only certain reports will be discussed. There are three basic ways by which malonate, might alter glucose oxidation. (1) Malonate will usually cause a depression of the oxygen uptake related to glucose metabo-

lism because in the total oxidation of glucose to CO_2 and water, five-sixths of the oxygen is taken up in cycle reactions. This effect would be due to a block of the cycle and would be roughly equivalent to the inhibition of of pyruvate oxidation via the cycle. (2) Malonate may cause an increased uptake and utilization of glucose as a result of inhibition of oxidative reactions in the cycle, the mechanism being essentially that of the Pasteur reaction. (3) Malonate may have direct actions on the cellular uptake of glucose or on the glycolytic pathways. Evidence for each of these mechanisms will be presented, and then a more detailed analysis of the possible shifts in metabolic patterns brought about by malonate will be given.

Effects on Oxygen Uptake Associated with Glucose Oxidation

There are many reports on the effects of malonate on respiration with glucose as the substrate, but in few have the data provided a correction for an effect on the endogenous respiration. Indeed, an endogenous correction to determine the oxygen uptake associated only with glucose oxidation is particularly unreliable, even when the data are available. This is because of the well-known effect of glucose in suppressing endogenous respiration and mitochondrial oxidations (Crabtree effect). Thus the nonglucose respiration may be significantly changed when glucose is added and, perhaps, completely suppressed in some cases.

Let us assume that the oxygen uptake measured derives only from glucose and that malonate acts only on the cycle. What inhibitions could one theoretically expect? The inhibition will depend, other than on the degree of cycle block, on the final products of glucose oxidation before and after the addition of malonate. The following equations give the molar oxygen uptakes for the oxidation of glucose to varying degrees of completeness:

To CO_2 and water

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$$

To pyruvate

$$C_6H_{12}O_6 + O_2 \rightarrow 2\ CH_3COCOOH + 2\ H_2O$$

To acetate

$$C_6H_{12}O_6 + 2\ O_2 \rightarrow 2\ CH_3COOH + 2\ CO_2 + 2\ H_2O$$

To succinate

$$C_6H_{12}O_6 + 5/2\ O_2 \rightarrow HOOCCH_2CH_2COOH + 2\ CO_2 + 3\ H_2O$$

$$C_6H_{12}O_6 + 2\ HOOCCH_2COCOOH + 4\ O_2 \rightarrow 2\ HOOCCH_2CH_2COOH + 6\ CO_2 + 4\ H_2O$$

To lactate

$$C_6H_{12}O_6 + 2\ H \rightarrow 2\ CH_3CHOHCOOH$$

The second equation for the formation of succinate might express the situation in which there is a source of oxalacetate other than from pyruvate

carboxylation, whereas the first equation assumes no external oxalacetate source. If the inhibition on succinate oxidase is complete and glucose is oxidized completely in the control, we may calculate the inhibitions for the situations where in the presence of malonate the glucose is transformed into the various oxidation products: to succinate with external oxalacetate source (33%), to succinate in a closed system (58%), to acetate (67%), to pyruvate (83%), and to lactate (100%). Of course, experimentally it is probably impossible to achieve a complete cycle block with malonate, so that the inhibitions will usually be less than the theoretical. These calculations also assume that the rate of glucose disappearance is not altered by the inhibition. The degree of inhibition, therefore, even under these simple conditions, will vary a good deal, depending on the terminal product of glucose metabolism and thus on the enzymes and pathways occurring in the cells under consideration. Actually, it is likely that various substances will accumulate during glucose metabolism, and not only those given above but others into which these substances can be metabolized.

A strong inhibition of the oxygen uptake related to glucose metabolism probably indicates the participation of the cycle in the oxidation, at least when the malonate concentration is not unreasonably high. Thus, in the instances shown in the tabulation and selected at random of inhibition of

Preparation	Malonate (mM)	% Inhibition	Reference
Sarcina lutea	10	50	Dawes and Holms (1958)
Yeast	12.5	73	Krebs *et al.* (1952)
Bull sperm	10	57	Lardy and Phillips (1943 a)
Malarial parasitized RBC	20	69	Speck *et al.* (1946)
Brain mince	17	100	Huszák (1940)
K^+–Stimulated brain slices	10	62	Kimura and Niwa (1953)

glucose respiration, one would conclude that the role of the cycle is important, but no information on the exact mechanism or on the fate of glucose in the presence of malonate may be obtained. However, in the cases of brain mince and yeast, fumarate is unable to overcome the inhibition, so that some doubt is introduced even into this interpretation. The inhibition of the glucose oxygen uptake in parasitized erythrocytes is very similar to that for the oxygen uptake associated with pyruvate oxidation, as expected if the cycle is the terminal pathway for glucose metabolism. On the other hand, there have been many reports that malonate, even at high concentrations, does not inhibit the glucose respiration at all, and in these instances little can be concluded because of the possibility that malonate does not penetrate and that an adaptation of the glucose utilization occurs. The

endogenous respiration of Ehrlich ascites cells is inhibited around 35% by 30 m*M* malonate, but when glucose is present there is very little effect of malonate (Seelich and Letnansky, 1960). Put in another way, in the presence of malonate the addition of glucose increases the O_2 uptake somewhat instead of depressing it as it does in uninhibited cells. Another possible reason for a failure of malonate to depress glucose respiration is the occurrence of oxidative pathways unassociated with the cycle. *Caldariomyces fumago* has no hexokinase and the usual Embden-Meyerhof glycolytic pathway is absent; the oxidation of glucose occurs more directly through glucose and gluconate oxidases (Ramachandran and Gottlieb, 1963). The respiration in this organism is not affected by malonate at 10 m*M*.

Effects on Glucose Utilization

A depression of cycle oxidations by malonate would be expected to cause an increased utilization of glucose, as do hypoxic conditions, in cells capable of exhibiting a Pasteur reaction. This is a manifestation of one interrelationship between the cycle and the glycolytic pathway, and is partially mediated through changes in the concentrations of inorganic phosphate and phosphate acceptors, such as ADP. For such a response to occur, the utilization of glucose must have been initially limited by the coupled phosphorylation at the 3-phosphoglyceraldehyde oxidation step. Another mechanism for the influence of cycle activity on glucose utilization involves the membrane transport of glucose and its phosphorylation. The inward transport of glucose under certain conditions may limit glucose utilization, and it has been shown that anoxia accelerates this transport in rat heart (Morgan *et al.*, 1961 a, b). It is quite possible that malonate by its depression of cycle activity, can alter these transport processes. A third mechanism might involve the rate of oxidation of NADH. The addition of pyruvate to ascites cells stimulates the formation of $C^{14}O_2$ from labeled glucose (Wenner and Paigen, 1961) Initially the rate of pyruvate oxidation is limited by the rate of NADH oxidation and the exogenous pyruvate acts as an electron acceptor, 1 mole of lactate appearing for each mole of pyruvate oxidized through the cycle. The accumulation of pyruvate as a result of the cycle block by malonate could initiate this dismutation so that more glucose would be utilized than otherwise. Indeed, lactate formation is often increased during malonate inhibition. In all these ways, and perhaps others, a cycle block might affect glucose uptake and utilization.

A stimulation of glycolysis by malonate was first observed by Kutscher in Heidelberg (Kutscher and Sarreither, 1940; Kutscher and Hasenfuss, 1940). Malonate was injected into guinea pigs, the muscle removed later, and the formation of lactate determined in a brei. In some cases, glucose, succinate, or fumarate was also injected. Malonate accelerates lactate formation and this is overcome by both succinate (see accompanying tabula-

tion) and fumarate. It was also shown that the rate of glycogen disappearance in muscle brei is stimulated some 35% by malonate. These responses were equated with the Pasteur reaction. Similar effects have been found in

Injection	Lactate formation
Control	2.95
Malonate	9.2
Glucose	5.1
Glucose + malonate	9.4
Succinate + malonate	−1.0
Glucose + succinate + malonate	−0.1

other tissues more recently. The rates of glucose utilization and of lactate formation in brain slices are stimulated 13% and 44%, respectively, by 10 m*M* malonate (Takagaki *et al.*, 1958), an effect much like that produced by azide. Simultaneously less glucose is oxidized, the increased utilization being diverted to lactate. Ehrlich ascites tumor cells exhibit a more rapid rate of glycolysis when the oxygen tension is reduced and a similar response is seen with malonate (Kvamme, 1957, 1958 d). When fumarate is added to the malonate-blocked cells, glucose utilization and lactate formation are suppressed. Strictly speaking, it is fumarate that gives rise to a Pasteur reaction in the presence of malonate, just as the addition of oxygen does in preparations previously anaerobic. Kvamme obtained data which led him to conclude that this effect is mediated through changes in the concentrations of inorganic phosphate and phosphate esters. The relationships between glycolysis and mitochondrial oxidations are particularly well seen in the reconstructed systems of Aisenberg *et al.* (1957). A supernatant fraction from rat liver forms lactate from glucose and the addition of a mitochondrial suspension suppresses this markedly. Malonate partially prevents this suppression; that is, added to the complete system, lactate formation is increased. This stimulation of glycolysis occurs despite the fact that malonate inhibits the glycolytic rate 15.7% in the supernatant fraction. This is a good illustration of how malonate can produce different effects on glucose metabolism, depending on the conditions and the factors controlling glycolysis. Stimulation of aerobic glycolysis by malonate, and a variety of other inhibitors, is particularly well seen in thymocytes; malonate accelerates lactate formation sigmoidally from 3 m*M* to 100 m*M* (Araki and Myers, 1963).

Malonate may have no effect on glucose uptake, or may inhibit it, in other tissues. Chick chorioallantoic membrane infected with influenza virus exhibits a 47.5% reduction in the endogenous respiration in the presence of 6 m*M* malonate, and the final virus titer drops to zero, but the

uptake of glucose is unaffected (Ackermann, 1951). An example of a marked inhibition was reported for guinea pig brain slices, glucose utilization being reduced by 10 m*M* malonate in both normal and K^+-stimulated slices (see accompanying tabulation) (Tsukada and Takagaki, 1955). It may be ob-

KCl (70 m*M*)	Malonate (10 m*M*)	Glucose utilization (μmole/g)	Lactate formation (μmole/g)	O_2 uptake (μmole/g)
−	−	35.9	34.9	54.1
−	+	4.71	28.0	61.5
+	−	112	99.0	104
+	+	63.7	117	44.9

served that the amount of lactate formed per glucose consumed is increased by malonate, but some of the lactate must be derived from other sources than glucose. It is difficult to understand the discrepancy between these results and those obtained later on the same tissue (Takagaki *et al.*, 1958), where a slight stimulation of glucose utilization by 10 m*M* malonate was reported. There are so many different results obtained relative to the utilization of glucose that it often appears each organism or tissue exhibits a characteristic pattern of response. Malonate at 40 m*M* has relatively little effect on the uptake of glucose by brain and kidney slices, depressing it slightly in the former and perhaps accelerating it in the latter, but reduces the $C^{14}O_2$ formed from uniformly labeled glucose 79% and 52%, respectively, the O_2 uptake being suppressed comparably (Cremer, 1962). Since much less of the glucose goes to amino acids and proteins in the presence of malonate (Cremer, 1964), it is likely that here there is an accumulation of certain cycle intermediates, such as succinate, and of lactate. Succinate is normally formed and released into the medium by *Trypanosoma cruzi*. It is formed aerobically through both the glycolytic pathway and the cycle, and by CO_2 fixation; some must be metabolized through the cycle since malonate elevates the succinate level even further (Bowman *et al.*, 1963). The uptake of glucose is increased almost 20% by malonate but there is no change in $C^{14}O_2$ formation, most of the excess glucose probably appearing as succinate, acetate, and related anions.

Direct Effects on the Glycolytic Pathways

There is no evidence that any enzyme of the Embden-Meyerhof glycolytic pathway is significantly inhibited by malonate at concentrations below 20 m*M* (Table 1-12), although some of the enzymes have never been investigated. Several of the enzymes in this pathway require Mg^{++}, or a related cation,

and under certain conditions malonate could inhibit through the chelation of these ions. A possibly sensitive enzyme is lactate dehydrogenase, which functions in anaerobic glycolysis, although insufficient quantitative work has been done, and it is likely that the enzyme from different sources would be inhibited to different degrees by malonate. It is unfortunate that so little work on the effects of malonate on anaerobic glycolysis has been done, since studies under aerobic conditions are always complicated by the secondary reactions discussed above. In a number of cases, an inhibition of aerobic glycolysis has been observed, i.e., a decreased formation of lactate in air or 95% oxygen, and these inhibitions are usually small. In bull sperm (Lardy and Phillips, 1943 b), 10 m*M* malonate inhibits 6.7%; in beef thyroid homogenates (Weiss, 1951), 33 m*M* malonate inhibits 8%; and in rat liver supernate (Aisenberg *et al.*, 1957), 25 m*M* malonate inhibits 15.7%. Although rises in lactate can be explained on an indirect basis, a decrease in the rate of lactate formation must usually be attributed to some inhibition along the glycolytic pathway, since it is not very likely that malonate would shift the fate of pyruvate from lactate to the cycle. Greater inhibitions have been observed: in mouse brain, 40 m*M* malonate inhibits lactate formation 57% and in mouse liver mitochondria 100%, this being taken as evidence of some direct effect on glycolysis (du Buy and Hesselbach, 1956). This concentration is, of course, rather high and could have depleted the Mg^{++} from either the 3-phosphoglyceraldehyde dehydrogenase or enolase systems, or could have inhibited lactate dehydrogenase. Since the substrate in these cases was 3-phosphoglyceraldehyde, an action earlier in the pathway is impossible. There are also miscellaneous reports which might be interpreted as indicating an inhibition of glycolytic pathways, for example the results of Greville (1936) on rat brain, in which 20 m*M* malonate inhibits glucose oxidation around 50% and pyruvate oxidation only around 15%. Only one instance of a direct test on anaerobic glycolysis has come to my attention, that of Covin (1961), who found that 5 m*M* malonate inhibits lactate formation in rat ventricle slices, but the rate of lactate formation in this tissue is so slow, that Covin expressed some doubts as to the reliability of the measurements. One must conclude from the incomplete data, that there is some evidence for a minor inhibition of glycolysis by malonate, especially at the higher concentrations.

The problem of the direct inhibition of glycolysis by malonate has been studied particularly well by Eva and George Fawaz at the American University of Beirut. They had observed that 30 m*M* malonate almost completely blocks the cycle, leads to the accumulation of succinate, and yet does not depress the dog heart significantly (Fawaz *et al.*, 1958). However, 60 m*M* malonate causes rapid reduction in cardiac frequency and contractile failure, although no more succinate accumulates than with 30 m*M*. This depression of cardiac function must be related to an action other than on the

cycle. The formation of lactate from glucose in extracts of rat muscle is inhibited over 90% by 60 m*M* malonate; simultaneously there is a decrease in inorganic phosphate, creatine-P, and readily hydrolyzable phosphate, accompanied by an increase in nonhydrolyzable phosphate (Fawaz and Fawaz, 1962). It was concluded that the acid-resistant phosphate fraction occurring in the presence of malonate must be made up of glycolytic intermediates, and it was then shown by analyses of the incubated extracts at various times that there is accumulation of 3-P-glycerate and glycerol-1-P particularly, with smaller contributions from phosphoenolpyruvate and 2-P-glycerate (see accompanying tabulation). Furthermore, the addition

Incubation time (min)	Accumulation of intermediates (mg P/100 g tissue)			
	P-enolpyruvate	2-P-glycerate	3-P-glycerate	Glycerol-1-P
6	6.12	1.84	19.00	22.30
30	8.60	2.45	29.10	30.80
120	10.90	3.18	45.60	33.40

of these intermediates to a malonate-treated extract resulted in the appearance of a major fraction as 3-P-glycerate, whereas in control incubations they break down to inorganic phosphate and pyruvate or lactate. This might imply a block at the pyruvate kinase and some reversal of the glycolytic pathway which cannot proceed beyond 3-P-glycerate due to the lack of ATP. The addition of pyruvate to an inhibited extract leads mainly to the formation of lactate. Pyruvate kinase from rabbit muscle is inhibited 86% by 60 m*M* malonate and this correlates quite well with the results seen in the extracts. Malonate at 30 m*M* only partly inhibits glycolysis, causes less accumulation of the phosphorylated intermediates, and inhibits pyruvate kinase 67%. The relative lack of affect of 30 m*M* malonate on cardiac function is probably due to a combination of two factors: the intracellular malonate concentration is undoubtedly less than 30 m*M*, and it is likely that glycolysis in the heart can be depressed to a certain degree before failure occurs, the myocardium having other sources of energy available.* Glycolysis in dog muscle extracts proceeds somewhat differently than in rat muscle extracts (e.g. accumulation of hexose phosphates occurs) and the response to malonate is consequently different (Fawaz *et al.*, 1963). High concentrations of malonate cause the accumulation of the same

* Since the heart is generally considered to obtain much of its energy from fatty acid oxidation under certain circumstances, it would be important to know the effects of these high concentrations of malonate on this pathway. However, with the cycle inhibited, the generation of energy from the oxidation of fatty acids should be reduced.

intermediates in dog muscle as described above for rat muscle, but in addition there is an inhibition of the accumulation of fructose-1,6-diP, so that some inhibition of a proximal step in glycolysis seems likely. Of the four enzymes involved previous to fructose-1,6-diP, only phosphoglucomutase is sensitive to malonate, 40% inhibition being produced by 60 m*M* malonate and 76% inhibition by 120 m*M* malonate. Thus a secondary site for the inhibition of glycolysis is likely. Other mechanisms may be involved in intact muscle cells.

Effects on the Distribution of C^{14} from Labeled Glucose

If glucose is metabolized exclusively by the Embden-Meyerhof glycolytic pathway and no initial decarboxylation of glucose or the hexose phosphates occurs (as it would if the pentose-P pathway were operative), malonate should depress the formation of $C^{14}O_2$ from glucose-1-C^{14} and glucose-6-C^{14} equally. However, if the pentose-P pathway is important, malonate should decrease the formation of $C^{14}O_2$ from glucose-6-C^{14} relatively more than from glucose-1-C^{14}, and hence increase the C-1/C-6 ratio. The difference between the $C^{14}O_2$ formed from these precursors is often taken as a measure of the activity of the pentose-P pathway; this may not be strictly true because every hexosephosphate which is decarboxylated may not pass through the pentose-P pathway completely, but it is certainly the best evidence for the relative importance of these two pathways.

Experiments of this type were performed with slices of various tissues from the rat (van Vals *et al.*, 1956). The specific activities of the $C^{14}O_2$ formed from C-1 and C-6-labeled glucose are essentially the same in the controls, indicating the pentose-P pathway to be unimportant (see accompanying tabulation). Malonate increases the C-1/C-6 ratios, which was

Tissue	C-1/C-6 ratio	
	Control	Malonate 30 m*M*
Heart	0.95	2.38
Brain	0.93	2.18
Kidney	0.99	1.92
Diaphragm	1.02	1.32

taken as evidence for a malonate-induced appearance of the pentose-P pathway, although it would account for only a small fraction of the glucose oxidized. In the lung and various mouse tumors, in which the pentose-P pathway is operative normally (C-1/C-6 rations between 1.4 and 5.6), malonate further augments the importance of the pentose-P pathway as

determined by C-1/C-6 ratios, which are increased to values between 4 and 125. A similar situation has been encountered in sheep thyroid slices (the results of three experiments are averaged in the accompanying tabulation) (Dumont, 1961). The formation of $C^{14}O_2$ from glucose-6-C^{14} is inhibit-

$C^{14}O_2$	Control	Malonate 100 m*M*
From glucose-1-C^{14} (cpm)	10.7	9.72
From glucose-6-C^{14} (cpm)	4.1	0.47
C-1/C-6	2.58	20.5
(C-1) — (C-6)	6.6	9.25

ed strongly, whereas that from glucose-1-C^{14} is scarcely affected. This, of course, indicates an almost complete block of the cycle, which is not surprising at this high malonate concentration, but it also suggests a greater participation of the pentose-P pathway in the presence of malonate. These results are perhaps more understandable in the light of the glycolytic inhibitions by high malonate concentrations discussed in the previous section. Quite different results were obtained in electrically stimulated rat ventricle strips (see accompanying tabulation) in which the pentose-P

$C^{14}O_2$	Control	Malonate 5.6 m*M*
From glucose-1-C^{14} (cpm)	0.318	0.271
From glucose-6-C^{14} (cpm)	0.285	0.264
C-1/C-6	1.12	1.03
(C-1) — (C-6)	0.033	0.007

pathway is presumably not important, malonate at this concentration having little effect on glucose utilization (Rice and Berman, 1961). It is possible that higher concentrations of malonate would produce changes such as observed with other tissues. However, this concentration of malonate is quite effective in modifying ventricular function.

An indirect mechanism for the acceleration of the pentose-P pathway by malonate may involve the levels of NADP and ATP in the tissues. The oxidative decarboxylation of glucose-6-P to initiate this pathway requires NADP, the concentration of which may be changed due to the action of malonate on the cycle. Also the phosphorylation of fructose-6-P in the Embden-Meyerhof pathway requires ATP, the level of which may be reduced by high concentrations of malonate. However, little is known about the control of the pentose-P pathway and further experiments are needed to elucidate its

role during malonate inhibition. Nothing is known of the possible direct effects of malonate on the pentose-P pathway. The production of $C^{14}O_2$ from ribose-1-C^{14} is inhibited strongly by malonate in heart homogenates (Jolley *et al.*, 1958) and it is believed that ribose is metabolized in this pathway, but the inhibition may reflect an action on the cycle. It is interesting that fluoroacetate does not inhibit $C^{14}O_2$ formation very potently, except at very high concentrations, so that some direct effect on ribose metabolism is possible. The results of the experiments discussed above would argue against this. D-Xylose and D-ribose-5-P are oxidized through sedoheptulose-7-P in extracts of *Pseudomonas hydrophila* and this is not affected by malonate at 20 m*M* (Stone and Hochster, 1956).

The only investigation of the effects of malonate on the general distribution of C^{14} from labeled glucose is that of Romberger and Norton (1961) in potato tuber slices incubated with uniformly labeled glucose for 3 hr (Table 1-19). The situation in this tissue is complex inasmuch as fresh slices do not metabolize glucose appreciably, whereas 36-hr-old slices oxidize it quite rapidly. In the aged tissue, CO_2 production is inhibited 92% by 50 m*M* malonate at pH 5, while the formation of CO_2 in fresh tissue is stimulated 28%. In the fresh tissue, they suggest that CO_2 is formed mainly in the pentose-P pathway and little through the Embden-Meyerhof sequence, but glycolysis contributes more and more with time, so that the marked inhibition by malonate in aged tissue is not surprising. The synthesis of sucrose accounts for over half of the labeling from glucose-u-C^{14} and this is inhibited only 11% by malonate. After 3-hr incubation, however, one can deduce little about the initial attack on glucose. It may be mentioned in this connection that in *Acetobacter xylinum*, where the sole product of glucose assimilation is cellulose, malonate at 10 m*M* does not inhibit the formation of cellulose. Laties (1964) has investigated this problem in detail and found that different methods of aging result in metabolically different potato slices, in that some exhibit a malonate-sensitive and some a malonate-resistant respiration. In the malonate-sensitive slices the formation of $C^{14}O_2$ from labeled glucose is almost completely abolished by malonate, whereas in the malonate-resistant slices there is little effect by malonate on the production of $C^{14}O_2$. There is a similar correlation with respect to the effects of malonate on glucose uptake. Since dinitrophenol does not interfere with glucose uptake, one can eliminate depression of ATP formation by malonate as responsible for the inhibition of the uptake in sensitive slices. Laties considered the possibility that increased citrate levels might inhibit phosphofructokinase, but the mechanism is not yet well understood.

Effects of Tissue Age on the Response of Glucose Metabolism to Malonate

We have seen above that aging of potato slices increases their sensitivity to malonate with respect to glucose oxidation. It might be expected that

the metabolic characteristics of tissues would change with age and that this would be reflected in different susceptiblies to inhibitors. The altered response to inhibitors could provide some information on the nature of metabolic aging. The results obtained on rat brain are, however, discordant. Tyler (1942), using a minced preparation, found that malonate inhibition of the respiration in the presence of glucose increases up to a rat age of 10 days, after which it remains at the adult level (see accompanying tabulation). Although the control respiration rises, the malonate-resistant fraction

Rat age (days)	Control	Malonate 10 m*M*	% Inhibition
1	656	547	16.9
2	582	507	12.9
6	814	590	27.6
8	712	559	34.3
10	1080	530	50.9
16	1500	810	46.0
31	1900	960	49.5
Adult	1723	864	50.0

of the respiration remains constant up to 10 days, i.e., the increased respiration is all due to the development of activation of a system sensitive to malonate, presumably the cycle. On the other hand, Muir *et al.* (1959) reported that the glucose respiration of adult rat brain slices is less sensitive to 10 m*M* malonate (30% inhibition) than the respiration of tissue from young animals of 1–3 days (70%). In this case, the young brain respires almost 2.5 times as rapidly as adult brain. The differences in these observations may be related to the preparations used (mince or slice). There are several reasons why malonate sensitivity would change with age, for example, an alteration of cycle activity, the development or loss of pathways other than the cycle for the metabolism of acetyl-CoA, or a change in the ability to demonstrate a Pasteur effect. The effect of age should also be studied on the electrically stimulated or K^+-stimulated respiration of the brain, since it is more sensitive to malonate and the results might have more physiological pertinence.

The respiratory rates and patterns of fungus spores are altered during the initiation of germination and the subsequent development of the germ tube (Gottlieb, 1964). During the incubation of the spores of *Penicillium oxalicum* and *Ustilago maydis* and the progress of germination, the respiration in the presence of glucose rises markedly and this is accompanied by an increasing sensitivity to malonate (see accompanying tabulation) (Caltrider and Gottlieb, 1963). It is somewhat difficult to determine if this implies

an increase in cycle activity since the concentration of malonate was 100 m*M* and more than the cycle might be inhibited.

Incubation (hr)	Respiration	% Inhibition
0	1.3	0
6	6.0	16
9	11.1	22
12	23.5	32

Effects on Resting and Stimulated Glucose Metabolism in Brain

The effects of malonate on a tissue may depend on the activity of the tissue as well as the age. Stimulation of a tissue such as brain alters the metabolic pattern and this is exhibited in altered responses to inhibitors. The results obtained by Heald (1953) on guinea pig cerebral cortex slices are shown in Fig. 1-14. The resting respiration and aerobic glycolysis are

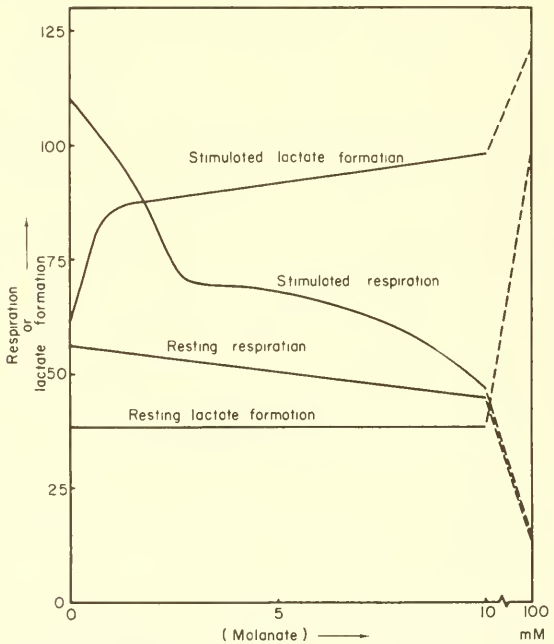


Fig. 1-14. Effects of malonate on guinea pig brain slices with glucose as the substrate. Electrical stimulation through grids. The respiration and aerobic glycolysis in μmoles/g wet weight/hr. (From Heald, 1953.)

little affected by malonate up to 10 m*M*, whereas the electrically stimulated respiration is readily depressed (down to the resting level at 10 m*M* malonate) and the stimulated lactate formation markedly increased. This means that the glucose metabolism appearing upon stimulation is quite sensitive to malonate and perhaps involves a greater participation of the cycle. These results were confirmed by Kimura and Niwa (1953) in guinea pig brain stimulated by K^+, and a stimulation of lactate formation by 10 m*M* malonate was observed by Tsukada and Takagaki (1955). An abolition of the inhibition of respiration upon addition of fumarate occurs (Takagaki *et al.*, 1958). Rat brain slices behave similarly, the resting respiration in the presence of glucose being unaffected by malonate up to 0.8 m*M*, while the stimulated respiration is readily suppressed (Wallgren, 1960). A malonate concentration as low as 0.2 m*M* inhibits the stimulated respiration 15%. Pyruvate utilization and the associated oxygen uptake are also inhibited more strongly in stimulated slices than in resting slices (Takagaki *et al.*, 1958). The $C^{14}O_2$ from labeled pyruvate is formed about twice as rapidly in high K^+ medium compared to the controls (Kini and Quastel, 1959), and this is inhibited more strongly by malonate in the K^+-stimulated slices, while the stimulated respiration is depressed to the endogenous level.

These results taken together clearly indicate a dependency of malonate inhibition on the metabolic activity of brain tissue, whether altered by electrical stimulation or K^+. A Pasteur effect is observed and it is possible that the inhibition by malonate would have been greater if it had not induced a greater utilization of glucose. The data do not necessarily imply a specific activation of the cycle; a greater uptake or utilization of glucose would impose a greater load on the cycle, and this might be inhibited more readily. Whatever the explanation for these effects, such results have important bearing on the actions of malonate on intact and functioning nervous tissue.

EFFECTS OF MALONATE ON LIPID METABOLISM

The major pathway for fatty acid oxidation is a helical degradation into acetyl-CoA, which normally enters the cycle by condensation with oxalacetate. Each turn of the helix, releasing one acetyl-CoA, takes up 2 atoms of oxygen, and the complete oxidation of acetyl-CoA through the cycle takes up 4 more atoms of oxygen. Thus, approximately two-thirds of the oxygen uptake due to fatty acid oxidation occurs in the cycle,* and one would expect malonate to depress this fraction in proportion to the cycle

* The term "cycle," as before, will indicate the tricarboxylate cycle only, and the pathway of degradation of fatty acids to acetyl-CoA and other terminal products will be designated the "helix" for convenience.

block it induces. The situation is vey similar to that of glucose oxidation in this respect. Generally speaking, malonate could act on either the cycle, or the helix, or both. Despite the extensive work that has been done on the effects of malonate on fatty acid oxidation, direct information on the actions on the helix is lacking, since the five reactions involved in each turn of the helix and the enzymes associated with these have not all been tested for susceptibility to malonate, nor has the operation of the helix dissociated from the cycle been studied. Our evidence on this point must be indirect.

Before considering this evidence, let us outline some of the possibilities for mechanisms of helix inhibition. Just as in the oxidation of glucose, ATP is required for the initiation of the helix reactions and Mg^{++} is a necessary cofactor (e.g., for the fatty acid thiokinase), so that malonate might depress the operation of the helix by depleting the system of either of these substances. The extent of such an inhibition will depend on the availablity of ATP or the presence of systems generating it, and on the concentration of Mg^{++}. Possibly a more important factor is the requirement for coenzyme A. Malonate could deplete conzyme A by at least two mechanisms. The formation of a relatively stable malonyl-CoA would remove some of the coenzyme A from participating in the helix. A block of the cycle would impede the entrance of acetyl-CoA into the cycle and the regeneration of coenzyme A will depend on the enzymes present for the metabolism of acetyl-CoA. The usual pathways for acetyl-CoA are (1) a simple splitting to form acetate, (2) a transfer of the coenzyme A to another acid, and (3) a condensation of two acetyl-CoA's to form acetoacetyl-CoA and eventually acetoacetate. As in the oxidation of pyruvate through the cycle, the fate of acetyl-CoA will depend also on the presence of reactions forming oxalacetate by pathways other than the cycle. The rate of fatty acid oxidation can thus be limited by the rate of regeneration of coenzyme A. These considerations lead one to predict that the effects of malonate on fatty acid oxidation would be variable and dependant on the metabolic characteristics of the tissue studied and the conditions of the experiment. This prediction is borne out.

There is a good deal of evidence that malonate in concentrations up to 20 m*M* does not directly inhibit the reactions of the helix. Although an inhibition of the oxygen uptake or the CO_2 production during fatty acid oxidation is not indicative of the site of inhibition when the helix and the cycle are operating together, the absence of inhibition implies a lack of action on the helix. Malonate at 16.8 m*M* has no effect on the $C^{14}O_2$ arising from palmitate-1-C^{14} in soluble extracts of peanut cotyledons (Castelfranco *et al.*, 1955), nor does 1 m*M* malonate have an effect on the oxygen uptake associated with palmitate oxidation in peanut microsomes (Humphreys *et al.*, 1954). The anaerobic dehydrogenation of C_4–C_{18} fatty acids in liver homogenates with methylene blue as an acceptor is not inhibited by

10 m*M* malonate (Blakley, 1952). The rate of oxidation of decanoate by *Serratia marcescens* is also not affected by 10 m*M* malonate, although 20 m*M* inhibits somewhat (Waltman and Rittenberg, 1954). Geyer and Cunningham (1950) stated that their data indicated no inhibition directly of octanoate oxidation in liver by 5 m*M* malonate (this work will be discussed in greater detail later).

On the other hand, Lehninger and Kennedy (1948) reported that 10 m*M* malonate strongly inhibits octanoate oxidation in particulate suspensions, from rat liver. Not only is the respiration from the oxidation inhibited but the utilization of octanoate is almost completely suppressed. The addition of malate or oxalacetate reduces the inhibition only partially, the utilization of octanoate still being inhibited around 70%. It may be noted that the total Mg^{++} concentration in these experiments was 0.25 m*M*, which is quite low, so that malonate could have inhibited by depletion of this cofactor. An interesting point is that the strain of rats used is very important, since 10 m*M* malonate inhibits octanoate oxidation 25% in preparations from livers of Sprague-Dawley rats, but in preparations made from a heterogeneous stock colony 2 m*M* malonate inhibits 50–75%. Such differences in strain behavior may explain some of the discrepancies in the reports on malonate inhibition. Weinhouse *et al.* (1949) found that 20 m*M* malonate almost completely blocks the oxidation of octanoate in rat liver slices and that fumarate only partially overcomes this, suggesting to them that malonate must have some action other than on succinate oxidase. In several instances malonate has been found to inhibit the oxygen uptake from fatty acid oxidation very potently. Butyrate respiration in peanut mitochondria is inhibited 75% by 6 m*M* malonate and the formation of $C^{14}O_2$ from butyrate-1-C^{14} is depressed even more strongly (Stumpf and Barber, 1956). Malonate at 10 m*M* inhibits the oxidation of octanoate by carp liver mitochondria 80% (Brown and Tappel, 1959). In suspensions of particulates from desert locust thorax, butyrate oxidation is inhibited 70% by malonate at concentrations as low as 1 m*M* and maximally 85% (Meyer *et al.*, 1960). The oxidation of octanoate-1-C^{14} and myristate-1-C^{14} by subcellular particles from the lateral line of the rainbow trout, as measured by the $C^{14}O_2$ released, is reduced 95% by 10 m*M* malonate (Bilinski and Jonas, 1964). One of the most sensitive systems is found in the oxidation of linolenate in liver mitochondria of vitamin E-deficient chicks, 0.25 m*M* malonate inhibiting 40% (Kimura and Kummerow, 1963). These examples must be interpreted as indicating either a direct or an indirect inhibition of the helix by malonate. Finally, it was stated by Mudge (1951), on the basis of unpublished experiments, that malonate inhibits fatty acid oxidation more strongly than succinate dehydrogenase in kidney particulate preparations. The possibility of effects on the helix must therefore be entertained on the basis of our present knowledge.

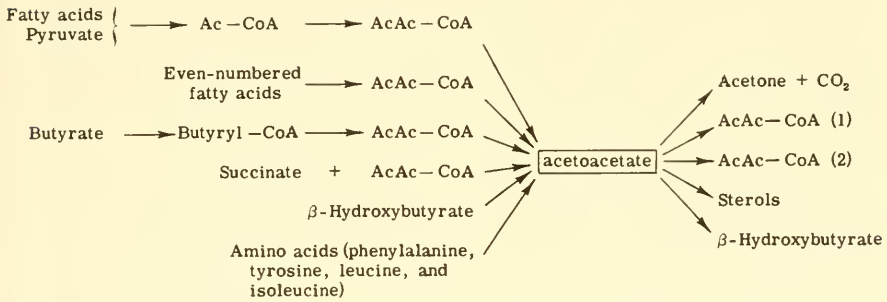
Effects on the Formation of Acetoacetate and Other Ketones

It was known before 1912 that acetate, oxalate, and maleate can either be metabolized to acetoacetate or so alter metabolism that acetoacetate accumulates, and for this reason Momose (1914) in Berlin studied the effects of malonate perfused through starved dog livers at a concentration of approximately 13 m*M*. He found that acetone appears and detected a substance which he only later, after returning to Japan (Momose, 1925), proved was acetoacetate. However, he postulated that malonate → acetate → acetoacetate → acetone, which was not unreasonable considering the inhibitory action of malonate was unknown. The appearance of acetone in the urine of rabbits fed malonate or rats injected subcutaneously with malonate was observed by Huszàk (1935), and simultaneously Annau (1935) demonstrated that malonate causes the formation of acetone in slices and breis of rabbit kidney. Acetoacetate has been shown to accumulate in tissues as a response to malonate (see accompanying tabulation). Since acetoacetate and acetone are the most important ketonic substances appearing in the tissues, these results clearly show that malonate is ketogenic.

Preparation	Substrate	Malonate	Reference
Whole rabbits (blood)	Endogenous	1.6 g/kg	Handler (1945)
Whole rats (blood)	Endogenous	0.8 g/kg	Mookerjea and Sadhu (1955)
Rat liver slices	Acetate	40 m*M*	Jowett and Quastel (1935 c)
	Butyrate	40 m*M*	Jowett and Quastel (1935 c)
Guinea pig liver slices	Propionate	40 m*M*	Jowett and Quastel (1935 c)
	Butyrate	40 m*M*	Jowett and Quastel (1935 c)
Rat liver slices	Endogenous	10 m*M*	Edson (1936)
Rat liver slices	Fatty acids	5 m*M*	Geyer and Cunningham (1950)
Rat liver suspension	Fatty acids	10 m*M*	Lehninger (1946 a)
Rat liver homogenates	Pyruvate	4 m*M*	Recknagel and Potter (1951)
Rabbit liver mitochondria	Fatty acids	15 m*M*	Cheldelin and Beinert (1952)
Jensen rat sarcoma mince	Glucose	6.7 m*M*	Boyland and Boyland (1936)
Peanut mitochondria	Butyrate	6 m*M*	Stumpf and Barber (1956)

Acetoacetate is an important substance in intermediary metabolism and the pathways for its formation and utilization are often complex. The concentration of acetoacetate will depend on the relative rates of its formation and utilization. The accumulation of acetoacetate in the presence of malonate could result from either an acceleration of its formation or an inhibition of its utilization, or both. The earliest concept that malonate itself gives rise to the acetoacetate was soon abandoned, and several investigators assumed that malonate interferes with the disposal of acetoacetate, while

recently more emphasis has been placed on the diversion of carbohydrate and fatty acid metabolism to acetoacetate by malonate. We may summarize some of the major pathways of acetoacetate before discussing the mechanisms for the action of malonate (Ac-CoA = acetyl-CoA, and AcAc-CoA = acetoacetyl-CoA). Reaction (1) for the formation of AcAc-CoA from aceto-



acetate is catalyzed by an activating enzyme in the presence of CoA and ATP, while reaction (2) is catalyzed by a CoA transferase in the presence of succinyl-CoA. All of these reactions do not occur in a single tissue and the response to malonate depends in part on which reactions are possible in any case.

A block of the cycle restricts the entrance of acetyl-CoA, derived from pyruvate and fatty acids, into the cycle, unless there is an adequate synthesis of oxalacetate from a noncycle source, which is seldom the case. If the acetyl-CoA accumulates, coenzyme A soon becomes tied up and the oxidation of pyruvate and fatty acids would cease. However, in most tissues 2 molecules of acetyl-CoA condense to form acetoacetate and coenzyme A is regenerated; in other situations, hydrolysis to acetate may occur. Malonate may thus divert acetyl-CoA from the cycle to acetoacetate. Quantitative conversion to acetoacetate has been observed (Recknagel and Potter, 1951). Another reaction possibly favoring acetoacetate formation during malonate inhibition results from the accumulation of succinate, which can now react more readily with acetoacetyl-CoA in a transfer of coenzyme A. The effectiveness of such a mechanism depends on the continued formation of succinate and, hence, usually on a noncycle source of oxalacetate. Acetoacetyl-CoA is also formed as the terminal product of the helical oxidation of even-numbered fatty acids. These relationships are illustrated in Fig. 1-15 where a block of succinate oxidation induces accumulation of acetoacetate by accelerating its formation through two mechanisms. The other pathways for the formation of acetoacetate are probably less important in most tissues and would not be accelerated by malonate. Accumulation of acetoacetate implies that its utilization must not be too rapid. Liver is notable in this respect because it lacks enzymes to metabolize acetoacetate rapidly,

especially the activating system for the formation of acetoacetyl-CoA, and possesses an active deacylase to split acetoacetyl-CoA. Therefore, acetoacetate accumulation is most readily observed in liver and most investigations have been on this tissue. The urinary acetoacetate found after the administration of malonate is probably derived mainly from liver. In heart, on the other hand, the enzyme balance is such as to favor the rapid metabolism of acetoacetate and it does not accumulate. Acetate rather than acetoacetate accumulates in some cells, for example in heart mitochondrial suspensions metabolizing pyruvate in the presence of 8.8 m*M* malonate (Fuld and Paul, 1952).

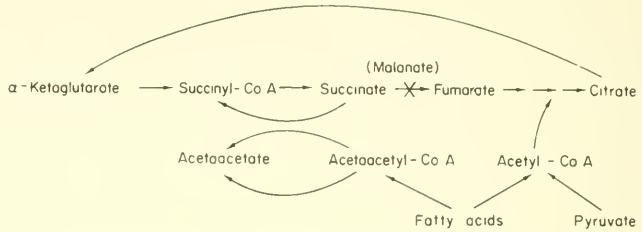


FIG. 1-15. Diagram of some pathways involved in the effects of malonate on the metabolism of acetoacetate.

One would predict that fumarate should counteract the ketogenic activity of malonate because, by supplying oxalacetate, acetyl-CoA will again be able to enter the cycle. However, it may be noted that fumarate may lead to an even greater accumulation of succinate and if the formation of acetoacetate by the transfer of coenzyme A from acetoacetyl-CoA to succinate is important, fumarate will only augment the malonate effect. Administration of fumarate with malonate to rabbits abolishes the appearance of acetone that arises with malonate alone (Huszàk, 1935). Addition of fumarate to malonate-inhibited minces of rat sarcoma likewise prevents the accumulation of acetone bodies (Boyland and Boyland, 1936). However, fumarate has very little effect on the appearance of acetoacetate in rat liver slices inhibited by malonate (Edson, 1936), and this might indicate a mechanism for malonate action other than the inhibition of succinate oxidation, or the importance of the coenzyme A transfer reaction.

It is now easy to see how malonate can reduce the oxygen uptake and the CO_2 production from fatty acid oxidation without necessarily decreasing the utilization of the fatty acids. A fraction that would normally be completely oxidized is diverted into acetoacetate (or acetate, acetone, and other products). One of the best indications that malonate does not inhibit the helix directly is the fact that the $C^{14}O_2$ appearing in the end products from labeled fatty acid is not reduced by malonate. To illustrate this it will be convenient to turn to the excellent studies of Geyer and his group at Harvard.

The basic procedure in these investigations was to incubate carboxyl-labeled fatty acids with rat liver and kidney slices, and determine the distribution of C^{14} in acetoacetate and CO_2. Malonate at 5 m*M* depresses the formation of $C^{14}O_2$ from octanoate-$C^{14}OO^-$ around 60% and fumarate is able to overcome this inhibition only partially (Geyer *et al.*, 1950 a). Fumarate and other cycle intermediates increase the total CO_2 formed but have little effect on the $C^{14}O_2$. This was explained by the accumulation of some of the C^{14} as succinate, this not being relieved by fumarate, and we have previously cited this as an example of the importance of considering what is measured in demonstrating a reversal by fumarate.

Where does the C^{14} go that does not appear as $C^{14}O_2$ in inhibited slices? They found that in the presence of malonate much of the C^{14} appears in acetoacetate (Table 1-24) (Geyer and Cunningham, 1950). The ratio AcAc-$C^{14}/C^{14}O_2$ is near 1.21 in the controls and is increased to around 4.51 by malonate, averaging the results from the five fatty acids used. It may also be noted that malonate generally increases the total C^{14} recovered, even though succinate was not determined, showing that malonate does not inhibit the fatty acid oxidation directly. Later they determined both the carboxyl and carbonyl C^{14} in acetoacetate and the more complete results are summarized in Table 1-25, where I have taken the liberty of averaging the data for all the fatty acids used, inasmuch as the effects are always in the same direction although differences between the different fatty acids are evident. These results show clearly the diversion of fatty acid metabolism into acetoacetate by malonate. Weinhouse *et al.* (1949) reported that in rat liver slices 10 m*M* malonate inhibits CO_2 formation and no acetoacetate appears, which was so contradictory to the results obtained by Geyer that the latter studied malonate in concentrations up to 20 m*M*, but found only that even more acetoacetate accumulates. Also they tested three different strains of rat and the results were the same. The reason for this discrepancy could not be explained.

The differential labeling in the carboxyl and carbonyl groups of acetoacetate is difficult to explain. If acetoacetate arises by a condensation of acetyl-CoA units, the labeling in these positions should be uniform. However, the ratio is seldom unity as may be seen in the results summarized by Chaikoff and Brown (1954). In the work of Geyer with rat liver slices, the ratio $CH_3C^{14}O—/—CH_2C^{14}OO^-$ is less than 1 in the controls and increases with the length of the fatty acid chain. Malonate increases this ratio, that is, it increases relatively the labeling in the carbonyl group. Chaikoff and Brown have given a detailed analysis of the possible factors determining this ratio, and the explanation is based on the existence of two types of 2-carbon fragment formed from fatty acids, one designated the $CH_3CO—$ fragment and the other the $—CH_2CO—$ fragment. These fragments are assumed to arise from different portions of the fatty acid chain and only the $—CH_2$

TABLE 1-24

EFFECTS OF 5 m*M* MALONATE ON THE DISTRIBUTION OF C^{14} FROM CARBOXYL-LABELED FATTY ACIDS IN RAT LIVER SLICES [a]

Substrate	$C^{14}O_2$		AcAc–C^{14}		$C^{14}O_2$ + AcAc–C^{14}		AcAc–C^{14}/$C^{14}O_2$	
	Control	Malonate	Control	Malonate	Control	Malonate	Control	Malonate
Pentanoate (valerate)	14,100	660	10,000	21,400	24,100	28,000	0.71	3.24
Hexanoate (caproate)	4,600	1,810	9,150	14,900	13,750	16,710	1.99	8.24
Heptanoate (oenanthate)	10,900	3,820	5,400	9,880	16,300	13,700	0.50	2.59
Octanoate (caprylate)	9,800	3,910	11,200	15,100	21,000	19,010	1.14	3.86
Nonanoate (pelargonate)	4,290	3,080	7,350	14,200	11,640	17,280	1.72	4.61

[a] The liver slices were incubated at 38° and pH 7.1 for 90 min (hexanoate 60 min). (From Geyer and Cunningham, 1950).

TABLE 1-25

EFFECTS OF 5 m*M* MALONATE ON THE OXIDATION OF FATTY ACIDS IN RAT LIVER SLICES [a]

	Control	Malonate	Change
$C^{14}O_2$ produced	10,694	4,536	− 6,158
AcAc-carbonyl-C^{14} formed	3,572	8,488	+ 4,916
AcAc-carboxyl-C^{14} formed	7,268	13,438	+ 6,170
$CH_3C^{14}O—/—CH_2C^{14}OO^-$	0.47	0.63	
Total AcAc-C^{14} formed	10,840	21,926	+11,086
$C^{14}O_2$ + AcAc-C^{14} formed	21,534	26,462	+ 4,928
AcAc-$C^{14}/C^{14}O_2$	0.83	4.55	

[a] Slices incubated 1 hr with carboxyl-labeled fatty acids shown in Table 1-24 at 38° and pH 7.1. The data from the five fatty acids were averaged to indicate the general effects of malonate. (From Geyer *et al.*, 1950 b.)

CO— fragments are believed to enter the cycle. The $CH_3C^{14}O—/—CH_2C^{14}OO^-$ ratio in acetoacetate will depend on the rates of production and utilization of these two fragments. As pointed out, these two types of 2-carbon fragment may be only convenient designations for two reactive forms of acetyl-CoA. Malonate is assumed to increase the formation of acetoacetate by condensation of randomized fragments of the $—CH_2C^{14}O—$ type, so that the ratio rises. The extra acetoacetate formed over the control when malonate is present does indeed exhibit a ratio of unity for hexanoate and octanoate oxidation (Geyer *et al.*, 1950 b). If malonate does this by blocking the cycle, a preferential accumulation of $—CH_2CO—$ fragments would occur, and a greater proportion of the acetoacetate would be formed from them.

The effect of malonate on acetoacetate accumulation will depend on the pathway of fatty acid oxidation in the uninhibited tissue and, hence, on the experimental conditions. For example, Witter *et al.* (1950) found that 3 m*M* malonate inhibits acetoacetate formation from hexanoate 4% and 10 m*M* malonate inhibits 9% in suspensions of washed particles from rat liver. However, hexanoate is quantitatively converted to acetoacetate in the controls, presumably because no cycle intermediates, are present to form oxalacetate for condensation of the acetyl-CoA units. Under such circumstances malonate would not be expected to increase acetoacetate and the small inhibitions must be attributed to actions directly on the helix. The relationship between acetoacetate formation in malonate-inhibited systems and the presence of cycle intermediates was illustrated and discussed by Cheldelin and Beinert (1952).

We have seen that the accumulation of acetoacetate in the presence of malonate can be attributed to an increased rate of formation of the acetoacetate. Is the accumulation due entirely to this or can malonate also inhibit the utilization of acetoacetate in some tissues? The rise in the acetoacetate in liver slices in the presence of malonate was believed by Jowett and Quastel (1935 c) to be due to the inhibition of the decomposition of acetoacetate, since at that time the pathways for the formation of acetoacetate were not understood. However, Quastel and Wheatley (1935) soon provided evidence that malonate can interfere with the disappearance of acetoacetate in rat liver and kidney slices. In kidney slices, malonate at 8 m*M* inhibits around 42% and at 16 m*M* 64%, and in liver slices an inhibition of 74% was observed with 40 m*M* malonate. Fumarate is able to counteract this inhibition partially and it was concluded that acetoacetate oxidation must be coupled with other oxidations inhibited by malonate. Very similar results were reported by Edson and Leloir (1936); indeed, 20 m*M* malonate inhibits disappearance of acetoacetate in rat kidney slices 93% and it was stated, "Malonate is a powerful and relatively specific inhibitor of respiration and of aerobic disappearance of acetoacetic acid in kidney." Both Handler (1945) and Mookerjea and Sadhu (1955) in their work with whole animals, favored the concept that malonate interfered with acetoacetate metabolism accounting for the rises in blood acetoacetate. Inasmuch as several different pathways are open to acetoacetate and these vary with the tissue used, it is difficult to interpret accurately these results. In some tissues, acetoacetate can be split into acetyl-CoA fragments that enter the cycle and here malonate might inhibit by blocking the cycle and the formation of oxalacetate, which is, of course, esentially the same mechanism adduced to explain the increased formation of acetoacetate. There is evidence that malonate does not inhibit the reduction of acetoacetate to β-hydroxybutyrate (Edson and Leloir, 1936), nor does it seem to interfere with the formation of sterols from acetoacetate (Mookerjea and Sadhu, 1955). It is probably best in the present state of our knowledge to attribute the accumulation of acetoacetate in the presence of malonate primarily to a diversion of 2-carbon units away from oxidation through the cycle, without eliminating the possibility that malonate may interfere in other pathways for the utilization of acetoacetate.

Effects on Propionate Metabolism

Propionate arises terminally from the β-oxidation of odd-numbered fatty acids and in certain tissues, such as the liver, can be oxidized completely through the cycle. However, the oxidation of propionate differs from that of other fatty acids. The following sequence of reactions has been suggested:

$$\text{Propionate} \xrightarrow{\text{ATP}} \text{propionyl-CoA} \xrightarrow[\text{ATP}]{\text{CO}_2} \text{methylmalonyl-CoA} \xrightarrow{B_{12}} \text{succinyl-CoA}$$

The over-all reaction is the carboxylation of propionate to succinate. Other pathways occur in bacteria, e.g.

Propionate → propionyl-CoA → acrylyl-CoA → lactyl-CoA → pyruvate → $CO_2 + H_2O$

Such a sequence may also operate in animal tissues, since lactate was identified chromatographically after incubation of mouse liver slices with propionate (Daus *et al.*, 1952). If the principal pathway of propionate is via succinate, malonate would be expected to inhibit its oxidation readily but, if acetyl-CoA is formed, the inhibition will vary with the conditions as discussed for the effects of malonate on pyruvate oxidation.

Malonate has been shown to inhibit strongly the $C^{14}O_2$ formation from labeled propionate in mouse liver slices (Daus *et al.*, 1952), rat liver slices (Katz and Chaikoff, 1955), suspensions of rabbit liver particles (Wolfe, 1955), and peanut mitochondria (Giovanelli and Stumpf, 1958), as anticipated. In the rabbit liver particulate preparation, 10 m*M* malonate suppresses the formation of $C^{14}O_2$ from both propionate-1-C^{14} and propionate-2-C^{14} almost completely, and at the same time leads to the accumulation of succinate, and in rat liver slices malonate also causes succinate accumulation. From these data alone it is impossible to say whether the succinate arises directly from propionate or is formed via the cycle, but the marked inhibition of $C^{14}O_2$ formation would indicate the former. This is substantiated by the demonstration of labeled methylmalonate in the rat liver slices. Another possible site for malonate inhibition is suggested by the work of Flavin *et al.* (1955) on rat tissues. The interconversion of methylmalonate and succinate was shown to be inhibited completely by 5 m*M* malonate and thus malonate leads to the accumulation of methylmalonate during propionate metabolism. However, it is not known if malonate can inhibit the methylmalonyl-CoA isomerase, which catalyzes the interconversion in the normal pathway, or if malonate only inhibits the formation of methylmalonyl-CoA from methylmalonate. The latter is reasonable because malonate could compete with methylmalonate for the active site on the enzyme.

In peanut mitochondria the situation may well be different. Malonate at 6 m*M* inhibits the formation of $C^{14}O_2$ from propionate-1-C^{14} 41% (Giovanelli and Stumpf, 1958). It was felt that this inhibition is not as much as would be expected if the pathway from propionate leads to succinate. Furthermore, fluoride, which inhibits the carboxylation of propionyl-CoA, does not depress the $C^{14}O_2$ significantly. The pathway through methylmalonyl-CoA to succinate may not be operative here, and the following pathway was proposed:

Propionate → propionyl-CoA → acrylyl-CoA → β-hydroxypropionyl-CoA → β-hydroxypropionate → malonic semialdehyde → malonyl-CoA → acetyl-CoA

The CO_2 formed in the last step derives from the carboxyl group of propion-

ate so that an inhibition of $C^{14}O_2$ formation from proprionate-1-C^{14} would imply an action of malonate somewhere along this pathway. It is possible that malonate, or malonyl-CoA formed from it, could compete with the malonyl-CoA from propionate and in this way reduces the formation of $C^{14}O_2$. It is known that the addition of methylmalonate simultaneously with propionate depresses the propionate utilization strongly (Feller and Feist, 1957). Lactating rat mammary gland preparations convert propionate to fatty acids in part, the principal pathway being direct condensation with malonyl-CoA to form the odd-chain fatty acids. Malonate at 10 m*M* inhibits this incorporation 50–65%, whatever the position of C^{14} in propionate, and simultaneously $C^{14}O_2$ is depressed around 30% from proprionate-1-C^{14} and propionate-2-C^{14}, and nearly 50% from priopionate-3-C^{14} (Cady *et al.*, 1963). This was interpreted not as a direct action on the propionate pathway but as a reduction of ATP or NAD(P)H, these being necessary for fatty acid synthesis, as a consequence of inhibition of the cycle.

In *Rhodospirillum rubrum*, both the oxidation (Clayton *et al.*, 1957) and the photosynthetic dissimilation (Clayton, 1957) of propionate are inhibited by malonate to approximately the same extent as are the similar reactions of succinate, and this was given as evidence to support the metabolism of propionate to succinate in these organisms.

Effects on Synthesis of Fatty Acids

There are at least three systems for the synthesis of fatty acids; one is the reversal of the β-oxidation in the helix and the other two involve the formation of malonyl-CoA from acetyl-CoA, one mitochondrial and the other nonmitochondrial (Green and Wakil, 1960). There are obvious relationships between fatty acid synthesis and oxidative metabolism of various substrates. The controls that establish the rates of fatty acid synthesis, or the balance between oxidation and synthesis, have not been elucidated and it is difficult to determine in a particular case what the effect of a cycle block would probably be. The level of acetyl-CoA and the availability of the various pathways for its metabolism must be an important factor, but the concentrations of coenzyme A, ATP, NADH and NADPH could also play a significant role.

Malonate has been found to produce a variety of effects. Most of the studies have involved the incubation of tissue preparations with acetate-1-C^{14} and the subsequent determination of labeled fatty acids formed from the acetate. In some cases a marked stimulation of fatty acid synthesis in the presence of malonate has been observed. Malonate at 50 m*M* inhibits the O_2 uptake of nonparticulate extracts of rat mammary gland and yet increases the formation of fatty acids, sometimes as much as 10-fold (Popják and Tietz, 1955). The addition of oxalacetate or α-ketoglutarate with the malonate increases the synthesis aven more (see accompanying

tabulation). The stimulating action of α-ketoglutarate was attributed to the generation of NADH by which hydrogen atoms are provided for fatty acid synthesis. Dils and Popják (1962) claimed that malonyl-CoA is not

Additions	Incorporation of acetate into fatty acids (mμ moles/100 mg)
Control	18.2
Malonate	118
Oxalacetate	72.7
Malonate + oxalacetate	241
α-Ketoglutarate	51.7
Malonate + α-ketoglutarate	517

formed from malonate in these extracts and that the stimulation of fatty acid synthesis must be an indirect effect, possibly the suppression of the deacylation of malonyl-CoA formed from acetyl-CoA, or the inhibition of the decarboxylation of malonyl-CoA. Kallen and Lowenstein (1962) pointed out that if this were the mechanism by which malonate acts, it should also stimulate the synthesis of fatty acids from malonyl-CoA, which it does not; indeed, malonate at 10 m*M* inhibits the conversion of malonyl-CoA into fatty acids 33%. There is actually a stimulation of the formation of fatty acids from acetyl-CoA. Furthermore, Spencer and Lowenstein (1962) found that malonate is incorporated into fatty acids in an extramitochondrial extract from rat mammary gland; acetate stimulates malonate incorporation just as malonate stimulates acetate incorporation. All of the stimulation by malonate, however, cannot be explained by its conversion to malonyl-CoA since several times more acetate than malonate is incorporated. The varying effects of malonate on different preparations from a single tissue are well illustrated in the studies of Abraham *et al.* (1961) with rat mammary gland, where malonate stimulates fatty acid synthesis markedly in cell-free systems (maximal stimulation around 130% at 17 m*M* malonate), inhibits the synthesis 63% in slices, and has very little affect when glucose is present. Glucose was assumed to provide NAD(P)H by forming cycle substrates and also to augment the ATP level, which in the absence of glucose might have been reduced by malonate. In the homogenates ATP was added so that this aspect of the action of malonate could not be exhibited. The response to malonate is markedly dependent on the experimental conditions, as shown by Hosoya and Kawada (1961) with human placental slices, additions of estradiol, ATP, NAD, or bicarbonate altering the fatty acid synthesis and its modification by malonate. It may be noted that fatty acid synthesis in particulate preparations from the locust fat body occurs rapidly only in the presence of malonate (Tietz, 1961).

So far we have considered total fatty acid synthesis. Separation of the different fatty acids from animal tissues in malonate experiments has not been done, but in mycobacteria malonate shifts the incorporation of acetate into the higher fatty acids (Kusunose *et al.*, 1960). The synthesis of total fatty acid is moderately increased and this was attributed to the formation of malonyl-CoA (see accompanying tabulation). Differential effects of mal-

Fatty acid	Acetate-1-C^{14} incorporation		% Change
	Control	Malonate 3.3 m*M*	
Palmitate	7627	3488	− 54
Stearate	2887	1497	− 48
Arachidate	1144	1332	+ 16
Behenate	1089	2110	+ 94
Lignocerate	1862	8807	+373
Total acids	14609	17234	+ 18

onate on the synthesis of short-chain and long-chain fatty acids are also seen in rat liver slices metabolizing octanoate-1-C^{14} (Lyon and Geyer, 1954). Although the over all effect of malonate on lipid synthesis in a particulate preparation from avocado mesocarp is not marked, the incorporation of acetate is shifted from stearate to oleate (see accompanying tabulation)

Malonate (m*M*)	Acetate incorporation into lipid	Distribution of label		
		Palmitate	Stearate	Oleate
0	8.20	26	33	41
5	8.50	23	12	65
10	8.15	24	14	62
30	7.10	21	9	70

(Mudd and Stumpf, 1961). Although malonate may inhibit the cycle, this may be counteracted by the formation of malonyl-CoA, which dilutes the labeled malonyl-CoA formed from labeled acetate. It is interesting that malonate is formed from acetate in avocado and this may be one regulatory factor in fatty acid synthesis. Malonate has been found in three instances to exert only inhibitory effects on fatty acid synthesis: in cell-free preparations from pigeon liver, 20 m*M* malonate inhibits the incorporation of acetate into fatty acids 32% (Brady and Gurin, 1952); in various tumor tissues (mammary and testicular carcinomata, and a sarcomatoid ovarian

tumor), 30 m*M* malonate inhibits 8–73% (van Vals and Emmelot, 1957); and in rat liver extracts containing mitochondria, 10 m*M* malonate inhibits 51% (Iliffe and Myant, 1964).

These divergent observations are difficult to explain satisfactorily and one must conclude that the final effects of malonate must depend on many factors. In cellular preparations malonate may alter the levels of ATP, NAD(P)H, and coenzyme A, as well as divert the metabolism of acetyl-CoA by its inhibition of the cycle. In nonmitochondrial soluble enzyme systems these actions would be minimized or absent, and the most important factors might be the facilitation of fatty acid synthesis through the formation of malonyl-CoA or direct effects on the enzymes involved, although there is no evidence for such direct affects at present. When malonate is itself incorporated into fatty acids, as in several examples above and in spinach chloroplasts, where malonate incorporation occurs at about half the rate for acetate (Mudd and McManus, 1964), additional complications must be considered. Since the incorporation of acetate-1-C^{14} into lipid in chloroplasts is reduced 71% by 0.67 m*M* malonate (Mudd and McManus, 1962), it would appear that malonate also inhibits some step or steps in the pathway. The compartmentalization of the pools of acetyl-CoA, malonyl-CoA, acetoacetate, and the various enzymes and cofactors within the cell must be borne in mind in trying to explain certain differential affects of malonate.

Effects on the Metabolism of Fats, Phospholipids, and Sterols

Several observations on total lipid response to malonate are interesting even though it is impossible to assign a mechanism. In rat liver homogenates incubated with palmitate-1-C^{14}, the lipids other than phospholipids increase 22% in the presence of 10 m*M* malonate compared to controls (Jedeikin and Weinhouse, 1954). Whether this is direct utilization of palmitate or lipid synthesis with the $C^{14}O_2$ formed from palmitate is difficult to say. Malonate at 50 m*M* also increases the total lipid content of potato tuber slices some 230% (Table 1-19) (Romberger and Norton, 1961) and this could be due mainly to an increased synthesis of fatty acids. On the other hand, lipid synthesis from glucose-C^{14} in human placenta is depressed 75% by 20 m*M* malonate (Hosoya *et al.*, 1960). These results again show that the action of malonate on lipid metabolism is quite variable. It will be more profitable to turn to the synthesis of particular lipid fractions.

Injections of malonate lead to elevation of the free and esterified cholesterol in the liver, kidney, and blood of the rat (see tabulation) (Mookerjea and Sadhu, 1955). Injections of 800 mg/kg of sodium malonate were made daily for 3–4 weeks, some toxic effects being noted, and the animals then sacrificed. Simultaneously, the blood glucose rose from 92 mg% to 196 mg% and the blood acetoacetate from 0.8 mg% to 3.6 mg%. Kidney

	Free cholesterol (mg/100 g wet wt.)			Esterified cholesterol (mg/100 g wet wt.)		
	Control	Malonate	% Change	Control	Malonate	% Change
Liver	205	426	+107	73	104	+ 42
Kidney	360	724	+101	72	228	+216
Blood	43	83	+ 93	68	80	+ 17

and liver slices showed impaired respiration with succinate, acetate, and acetoacetate as substrates. This augmentation of tissue cholesterol is clear and is reasonable on the basis of diversion of acetyl-CoA metabolism by a block of succinate oxidase. However, *in vitro* work has shown only inhibition of cholesterol synthesis. The formation of labeled cholesterol from octanoate-1-C^{14} in rat liver slices is consistently depressed by 5.84 m*M* malonate, and fumarate was very ineffective in counteracting this inhibition (Lyon and Geyer, 1954). The total lipids rise and this is partly attributable to the increased synthesis of short-chain fatty acids. The formation of labeled cholesterol from acetate-1-C^{14} in the same tissue is inhibited 73% by 50 m*M* malonate (Kline and DeLuca, 1956) and 78% by 30 m*M* malonate (van Vals and Emmelot, 1957). Cholesterol synthesis in rat tumors is even more strongly depressed. The discrepancy between the *in vivo* and *in vitro* results might be due to several factors. In the intact animal many secondary effects may occur, e.g. as a result of the marked rise in blood glucose. Also the malonate concentration in the tissues of the rats is undoubtedly less than in the work with slices. It is unfortunate that most of the studies have been made with unreasonably high malonate concentrations so that a specific inhibition of succinate oxidation is doubtful. The catabolism of cholesterol, as determined by the formation of $C^{14}O_2$ from the labeled terminal methyl groups of cholesterol, in suspensions of rat liver mitochondria is inhibited 78% by 10 m*M* malonate (Whitehouse *et al.*, 1959), so that this factor must also be considered in explaining changes in tissue levels over longer periods of time. The synthesis of other sterols has been studied very little. Pieces of rat adrenal form corticosteroids in the presence of glucose and this is markedly stimulated by the addition of ACTH. Malonate at 10 m*M* stimulates the formation of sterols in the absence of ACTH from 17 to 22 μg/100 mg/2 hr (+29%) but depresses the synthesis in the ACTH-activated preparations from 81 to 72 μg/100 mg/2 hr (−11%) (Schönbaum *et al.*, 1956). Fluoroacetate also inhibits very little and it was concluded that the cycle does not play a major role in sterol synthesis, glucose metabolism and particularly the pentose phosphate pathway being of more importance. The bearing of such studies on the metabolic basis of cholesterol and hormonal sterol levels in animals, especially the relationship

to the activity of the cycle and the other pathways for the utilization of acetyl-CoA, warrants further investigations of the actions of malonate and other cycle inhibitors both *in vitro* and *in vivo*. One approach to the metabolic defect in hypercholesteremia could be made in this way.

The incorporation of inorganic P^{32} into phospholipids is almost invariably inhibited strongly by malonate. This has been shown in peanut mitochondria (Mazelis and Stumpf, 1955), mycobacteria (Tanaka, 1960), guinea pig brain dispersions (R. M. C. Dawson, 1953), rat liver mitochondria (Marinetti *et al.*, 1957), and other tissues. In cat brain slices, the effects of malonate are very slight and it is possible that malonate does not penetrate well (Strickland, 1954). Yet 3 m*M* malonate inhibits such incorporation 87% in K^+-stimulated rat brain slices, although this may be due to a more active cycle participation in the active tissue, inasmuch as respiration is 93% inhibited (Yoshida and Quastel, 1962). The phosphorylation of phospholipid precursors probably involves the formation of high-energy phosphate compounds and malonate could depress this as the result of a block of the cycle. A direct effect on the phosphorylation is unlikely. On the other hand, the incorporation of activity into phospholipids from palmitate-1-C^{14} in rat liver homogenates (Jedeikin and Weinhouse, 1954) or from acetate-1-C^{14} in rat liver slices (Kline and DeLuca, 1956) is affected scarcely at all by malonate. The phospholipids comprise a very heterogenous group and the response to malonate probably depends on which type of phospholipid is under investigation.

EFFECTS OF MALONATE ON AMINO ACID AND PROTEIN METABOLISM

The pathways of amino acid metabolism often lead to or from the cycle so that malonate would be expected to influence amino acid utilization and formation by its inhibition of succinate oxidase. The intracellular accumulation of amino acids and their incorporation into proteins are processes requiring energy and consequently malonate could depress these important reactions involved in cellular growth by a depletion of high-energy phosphate derived from the cycle. Finally, malonate might act directly on the enzymes catalyzing amino acid transformations. Information on these matters is fragmentary but enough work has been done to demonstrate some interesting effects of malonate on this phase of metabolism.

Effects on Amino Acid Metabolism

None of the enzymes involved directly in amino acid metabolism seems to be very sensitive to malonate (Table 1-12) but a number of important reactions have never been studied. Enzymes catalyzing the reactions of

the dicarboxylic amino acids particularly might be expected to bind malonate to some extent, but there is only indirect evidence for this. The dehydrogenation of glutamate with methylene blue as an acceptor in toluene-treated *E. coli* is inhibited about 20% by 71 m*M* malonate (Quastel and Wooldridge, 1928), but it may be that all of the hydrogen atoms do not arise from glutamate here and that some other reaction is inhibited. In Walker carcinosarcoma, kidney, and liver, glutamate is metabolized readily to succinate in the presence of 6.3 m*M* malonate (Nyhan and Busch, 1957), but no controls are avilable so that some inhibition is possible. Aspartate and glutamate are metabolized by *Hemophilus parainfluenzae*; malonate does not interfere with the oxidation of the former but does inhibit glutamate oxidation (Klein, 1940). Contrary to these results, malonate completely inhibits aspartate oxidation in rat liver homogenate (Nakada and Weinhouse, 1950). It was believed rightly that this could not be entirely attributed to an inhibition of succinate oxidase.

Glutamate may be converted to α-ketoglutarate by either glutamate dehydrogenase or transamination, or it may be decarboxylated to γ-aminobutyrate; the decarboxylase is limited mainly to certain bacteria and the nervous system of animals, so the major product is usually α-ketoglutarate, which can be oxidized through the cycle or participate in transaminations whereby it is reconverted to glutamate (this occurs also with γ-aminobutyrate so that the net reaction forms succinic semialdehyde, ammonia, and CO_2 from glutamate). The pattern of glutamate metabolism will depend on the relative activities of these various enzymes, the availability of other amino acids for transamination, and the supply of NAD for the glutamate and α-ketoglutarate dehydrogenases; likewise, the response to malonate inhibition will depend on these factors. If malonate selectively inhibits succinate oxidation, the O_2 uptake due to glutamate should be reduced moderately (perhaps around 25–50%) unless much of the α-ketoglutarate formed is transaminated and does not enter the cycle. Malonate, however, occasionally inhibits the formation of ammonia from glutamate, indicating some effect on the oxidative deamination. Malonate also inhibits the oxidation of glutamate by guinea pig mammary gland mitochondria completely (Jones and Gutfreund, 1961), which would not be the case if only succinate oxidation were blocked. The glutamate respiration of rat brain mitochondria is depressed 88% by 17.3 m*M* malonate (see note in Table 1-14) (Løvtrup and Svennerholm, 1963), which would indicate that glutamate is being converted mainly to α-ketoglutarate by transamination (glutamate decarboxylase is not present in brain mitochondria). Similar high inhibitions by 20 m*M* malonate are observed in the mitochondria from pigeon muscle, rat heart, rat liver, and ascites cells (64-99%) (Borst, 1962). Conclusions as to the pathway of glutamate catabolism based on the results with malonate depend on the assumption that the inhibition is specifically

on succinate oxidation, and at these high concentrations this may not be true. On the other hand, Das and Roy (1961, 1962) claim that transamination contributes little to the metabolism of glutamate in mitochondria from *Vigna sinensis*, and since the decarboxylase is absent, oxidation by glutamate dehydrogenase would seem to be the major route. Glutamate is converted primarily to aspartate in rat brain homogenate via the pathway glutamate → α-ketoglutarate → succinate → oxalacetate → aspartate (Haslam and Krebs, 1963). The addition of fumarate removes this inhibition, as expected.

Certain amino acids appear to be involved in the functioning of nerve tissue and the effects of inhibitors on the metabolism of these substances are of particular interest in this connection. Glutamate is accumulated in brain and plays a role in the active transport of ions, while γ-aminobutyrate and *N*-acetylaspartate have recently attracted attention because of their ability to modify central nervous system activity. Glutamate and K^+ are taken up by retina and brain slices in approximately equivalent amounts. Malonate at 20 m*M* depresses the formation of glutamate + glutamine only 12% while it reduces K^+ uptake 40% (Terner *et al.*, 1950), indicating that the major effect of malonate is not mediated through interference with glutamate. When guinea pig brain slices are incubated with glucose-u-C^{14}, a good deal of the C^{14} appears in amino acids, the most important of which is glutamate (Tsukada *et al.*, 1958). Malonate at 10 m*M* inhibits glutamate formation around 25%, γ-aminobutyrate formation around 75%, and the formation of aspartate appreciably. The total C^{14} incorporation into amino acids from glucose-u-C^{14} in rat brain slices is inhibited 64% by 10 m*M* malonate at normal K^+ concentration but 83% in the presence of 105 m*M* K^+, which produces an activation of brain metabolism (Kini and Quastel, 1959). Such results can be readily explained on the basis of a malonate-reduced pool of amino acid precursors due to the reduction in cycle activity. Glutamate is a central substance in amino acid formation through transaminations and anything which decreases the formation of α-ketoglutarate would be expected to impair these pathways. Cremer (1964) has recently found that 40 m*M* malonate not only reduces drastically the incorporation of glucose-u-C^{14} into glutamate, aspartate, γ-aminobutyrate, and protein in brain slices, but also causes a loss of amino acids from the cells. This concentration, of course, is probably not specifically inhibiting succinate oxidation. A disputation type of reaction occurs in certain tissues:

$$2\ \alpha\text{-Ketoglutarate} + NH_3 + ADP + P_i \rightarrow \text{glutamate} + \text{succinate} + CO_2 + ATP$$

Tager (1963) used malonate to block succinate dehydrogenase and surprisingly found that it augments the formation of glutamate in suspensions of rat liver mitochondria (see accompanying tabulation). It was suggested

	Control	Malonate 20 m*M*
Δ O_2 (μ atoms)	−1.2	− 1.2
Δ α-Ketoglutarate (μ moles)	−3.9	−10.4
Δ Glutamate (μ moles)	+2.0	+ 4.9
Δ Esterified phosphate (μ moles)	+1.9	+ 4.8

that malonate is converted to oxalosuccinate via malonyl-CoA. The oxalosuccinate might function in the NAD- and NADP-dependent isocitrate dehydrogenase systems to form a transhydrogenase so that NADPH is the eventual donor for the formation of glutamate, oxalosuccinate acting catalytically. Such studies show how complex the effects of malonate on amino acid metabolism can be. The oxidation of certain amino acids proceeds via an initial transamination followed by degradation of the deaminated acids. The oxidation of γ-aminobutyrate is completely inhibited by 1 m*M* malonate in rat brain mitochondria (Sacktor *et al.*, 1959) but in *Bacillus pumilus* is not affected even by 40 m*M* malonate (Tsunoda and Shiio, 1959). Whether the former inhibition is the result of an indirect suppression of transamination by cycle block or a direct effect on the oxidative pathway of this amino acid is not known.

N-Acetylaspartate occurs at a relatively high concentration in mammalian and avian brain, increasing rapidly after birth. Its formation involves direct acetylation of aspartate and the brain has little ability to metabolize this substance. When acetate-1-C^{14} is injected intracerebrally, some of the C^{14} is later found in *N*-acetylaspartate (Jacobson, 1959). The injection of malonate with the acetate reduces the incorporation of the C^{14} about 50%. The injection of acetate depresses the level of total *N*-acetylaspartate and malonate counteracts this. These effects are quite complex and difficult to interpret. The concentration of malonate injected was high (1.34 *M*) and could have caused a severe fall in ATP so that acetate activation prior to acetylation would be depressed. The rise in *N*-acetylaspartate seen with malonate might have been due to a cycle block counteracting the effect of the injected acetate, whereby cycle intermediates involved in transaminations would be decreased, the level of aspartate being maintained with more aspartate available for acetylation. There are so many pathways associated with aspartate metabolism and acetylation reactions that the final effects of a cycle block are difficult to predict. A good example of the complex effects of malonate on amino acid metabolism is seen in Table 1-19, where certain types of amino acid in potato slices increase and other types decrease during incubation with malonate (Romberger and Norton, 1961).

Effects on Protein Synthesis

The intracellular synthesis of protein requires the simultaneous operation of many metabolic pathways and thus is susceptible to inhibition on a variety of reactions. Some of the processes involved in protein synthesis are: (1) the active uptake or accumulation of exogenous amino acids, (2) the production of high-energy substances such as ATP from the oxidative reactions of the cycle (except in anaerobes), (3) the formation of amino acid precursors, again mainly by the operation of the cycle, and (4) all the complex reactions for the activation and assemblage of the amino acids into proteins. There are thus a multitude of possible sites for malonate action but, at reasonable concentrations, the most important mechanism must be a cycle block leading to both depletion of energy supplies and decrease in amino acid precursors. There is no evidence that malonate can interfere significantly either with the proteases or peptidases involved in the breakdown of proteins to amino acids or with the terminal assembling reactions for the formation of protein.

The effects of malonate on the uptake and accumulation of amino acids by cells have been studied in three types of tissue. Excised diaphragm maintains the same tissue/medium ratio for glycine as in the whole animal, and the marked effects of 2,4-dinitrophenol indicate that glycine is concentrated actively (Christensen and Streicher, 1949). Malonate, however, at concentrations of 3–55 m*M* does not uniformly alter the tissue/medium ratio. It is possible that malonate does not penetrate adequately, because muscle is often rather impermeable to anions. The situation is different in Ehrlich mouse ascites carcinoma cells. Glycine is accumulated so that tissue/medium ratios are often 10–15. In two experiments, malonate at 37 m*M* decreased this ratio from 13.0 to 5.1 and at 40 m*M* from 13.9 to 4.0 (Christensen and Riggs, 1952). This occurred despite the fact that malonate increased the synthesis of glycine. In cell suspensions of Gardner lymphosarcoma the uptake of labeled glycine is inhibited 73% and the uptake of alanine 56% by 10 m*M* malonate (Kit and Greenberg, 1951). These studies demonstrate that malonate can interfere with protein synthesis, at least in some cells, by inhibiting the initial process of amino acid uptake.

The synthesis of protein is usually strongly inhibited by malonate, but no analyses of the block have been made and the mechanisms are unknown (see accompanying tabulation). The formation of adaptive enzymes has often been taken as indicative of the synthesis of general cell proteins, but this is not necessarily so, as pointed out by Mandelstam (1961). In *E. coli* any substance acting as a substrate and source of energy represses enzyme synthesis, whereas inhibitors, such as malonate and 2,4-dinitrophenol, counteract such effects and stimulate the synthesis. Furthermore, under conditions in which β-galactosidase synthesis is inhibited, the incorporation of leucine-C^{14} into cell protein is not affected. The lack of inhibition in

Process	Malonate (mM)	% Change	Reference
Formation of induced β-galactosidase in *E. coli*	33.5	+ 31	Mandelstam (1961)
	100	− 8	
	134	− 42	
	167	− 79	
Incorporation of leucine-C^{14} into tobacco leaf proteins	10	0	Stephenson *et al.* (1956)
Protein formation in chick embryo tissue culture	10	−100	Gerarde *et al.* (1952)
Incorporation of glycine-2-C^{14} into rat liver homogenates	45	− 86	Peterson and Greenberg (1952)
Incorporation of glycine-C^{14} into antibody in rabbit lymph nodes	1.2	− 67	Ogata *et al.* (1956)
Incorporation of acetate-1-C^{14} into rat liver slices	30	− 50	van Vals and Emmelot (1957)
Incorporation of acetate-1-C^{14} into various tumor slices	30	−74 to −90	van Vals and Emmelot (1957)
Incorporation of glutamate-u-C^{14} into Walker carcinosarcoma	6.25	−23 to −56	Nyhan and Busch (1957)
Incorporation of glycine-1-C^{14} into chick embryo proteins	20	− 57	Quastel and Bickis (1959)
Incorporation of glycine-1-C^{14} into ascites protein	20	− 92	Quastel and Bickis (1959)

tobacco leaves was attributed to the presence of preformed precursors or energy donors, so that interference with metabolism during the 2-hr incubation does not modify the assembling of the proteins (Stephenson *et al.*, 1956). In two instances, glucose is able to partially reverse the effects of malonate. Glucose addition to the Walker carcinosarcoma slices reduces the inhibition by malonate, sometimes restoring the normal rate of protein synthesis (Nyhan and Busch, 1957), and in ascites cell suspensions glucose decreases the malonate inhibition from 92% to 14% (Quastel and Bickis, 1959), although the inhibition, is even increased slightly in chick embryo. The marked glycolytic activities of tumor tissue may be responsible for this phenomenon, sufficient energy for protein synthesis being obtained from noncycle pathways.

The inhibition of amino acid uptake and the synthesis of proteins and enzymes by malonate must be considered in long-term experiments or in whole animal experiments, since this could secondarily affect many other

metabolic systems. Most enzymes are probably in a state of simultaneous formation and degradation, so that an inhibitor of synthesis would induce a steady fall of the enzyme level in the cells. This could apply, of course, to all inhibitors of protein synthesis.

Effects on Urea Formation

The terminal product of much protein and amino acid metabolism is urea and it has been found that under certain circumstances malonate inhibits the formation of urea quite potently. The inhibition has been mentioned in connection with its antagonism by fumarate (page 116). The most important reactions of the urea cycle comprise the following, assuming that glutamate is the immediate amino-group donor:

(1) Glutamate + oxalacetate → aspartate + α-ketoglutarate (*transaminase*)

(2) Aspartate + citrulline + ATP → argininosuccinate + ADP + P_i (*argininosuccinate synthetase*)

(3) Argininosuccinate → arginine + fumarate (*argininosuccinase*)

(4) Fumarate → oxalacetate (*fumarase and malate dehydrogenase*)

(5) Arginine → ornithine + urea (*arginase*)

(6) Ornithine + NH_3 + CO_2 → citrulline (*citrulline synthetase*)

Glutamate + NH_3 + CO_2 + ATP → α-ketoglutarate + urea + ADP + P_i

This urea cycle thus makes contact with the tricarboxylate cycle at several points. The α-ketoglutarate formed in the over all reaction can be oxidized through succinate to oxalacetate or can be transaminated to regenerate glutamate. The operation of the urea cycle thus requires sources for oxalacetate and ATP, both of which may be blocked by malonate.

Cohen and Hayano (1946) found that 5.7 m*M* malonate inhibits the conversion of citrulline to arginine 90% in liver homogenates when glutamate is the amino donor. The mechanism of the inhibition was not apparent at that time. These results were confirmed by Fahrländer *et al.*, (1947) and, in addition, they showed that fumarate or malate, can counteact the inhibition, indicating a block of succinate oxidation. They interpreted the mechanism as a depletion of ATP and a consequent inhibition of reaction (2). Subsequently, they showed that low malonate concentrations (1–2 m*M*) inhibit urea formation as much as 75% and felt this was evidence for a specific action on succinate dehydrogenase (Fahrländer *et al.*, 1948). The ATP level in the homogenates drops from 130 to 49.6 in the presence of 2.5 m*M* malonate and fumarate restores the ATP level to normal. It was believed that glutamate not only furnishes the amino group but also cycle substrates from which the energy is derived; a block by malonate at the succinate level would reduce the amount of ATP formed. Krebs and

Eggleston (1948) then demonstrated a differential effect of malonate on the formation of urea depending on whether glutamate or aspartate is used as the amino donor, the inhibition being less in the latter case. An elucidation of the true mechanism of the inhibition was presented by Ratner and Pappas (1949), who showed a very definite differential effect of malonate when glutamate and aspartate are used (see tabulation). The

Substrate	% Inhibition by malonate 20 m*M*			
	Aspartate		Glutamate	
	Arginine synthesis	O_2 uptake	Arginine synthesis	O_2 uptake
None	6	27	73	38
Pyruvate	11	32	57	37
Oxalacetate	8	20	Stim 22	5
Fumarate	1	7	Stim 2	9
α-Ketoglutarate	Stim 6	16	66	40

transamination forming aspartate from glutamate is not inhibited by malonate so the mechanism must be sought elsewhere. It was proposed that malonate prevents the formation of oxalacetate and thus indirectly blocks the formation of aspartate; fumarate would, of course, overcome this block. They opposed the idea that ATP depletion is important and felt that the ATP derived from α-ketoglutarate oxidation would be sufficient. However, they did not by any means disprove the ATP depletion hypothesis and it is quite possible that it also plays a role in assigning an over all mechanism for the inhibition. Müller and Leuthardt (1950) extended these observations by showing chromatographically that malonate inhibits the formation of aspartate by reducing the formation of oxalacetate from α-ketoglutarate, and also demonstrated conclusively that the transamination reaction itself is not sensitive to malonate. It should be noted that in the reactions written above, oxalacetate appears to be regenerated in the arginosuccinase reaction followed by the hydration and oxidation of fumarate, but this is apparently not sufficient to maintain the cycle, probably because much of the oxalacetate disappears in other reactions. This is why an external source of oxalacetate is necessary.

EFFECTS OF MALONATE ON PORPHYRIN SYNTHESIS

The pathway for the synthesis of porphyrins in both animals and plants originates in the cycle in the condensation of succinyl-CoA with glycine (Fig. 1-16). The succinyl-CoA can be formed either from α-ketoglutarate

or from succinate; the latter reaction requires ATP and is catalyzed by succinyl-CoA synthetase (P-enzyme). A total of 8 molecules of succinate and 8 molecules of glycine is required for the synthesis of a molecule of protoporphyrin. The close connection between this pathway and the succinate steps of the cycle, and the great importance of porphyrin synthesis in all tissues, make the study of the action of malonate on this system interesting. We may speculate on the various ways in which malonate could modify porphyrin synthesis. (1) If the succinyl-CoA is formed in the cycle through α-ketoglutarate, malonate could restrict its formation by blocking the cycle

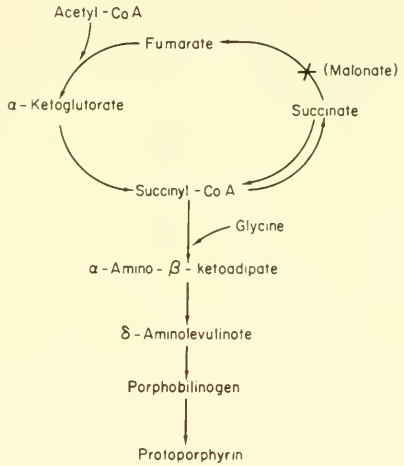


FIG. 1-16. The pathways involved in porphyrin biosynthesis.

and reducing the rate of acetyl-CoA entry, especially if no noncycle source of oxalacetate is available. (2) If succinyl-CoA can be formed through the cycle readily in spite of a malonate block, malonate might divert more succinate into the synthesis of porphyrin by inhibiting succinate oxidation. (3) If the succinyl-CoA arises from succinate, this requires ATP and malonate could deplete the system of ATP. (4) Malonate might deplete the system of coenzyme A by the formation of malonyl-CoA. (5) It is possible in some way that malonate might inhibit the formation of glycine, although this is rather unlikely because there are usually several pathways available for glycine synthesis. The effects of malonate will thus depend on the type of preparation used and the conditions of the experiment.

Duck erythrocytes (intact or hemolyzed) incubated with succinate and glycine form porphyrin. Succinyl-CoA could be formed from succinate either directly or through the cycle and the relative importance of these pathways may be demonstrated by the use of succinate-C^{14} with subsequent deter-

mination of the porphyrin labeling (Shemin and Kumin, 1952). Succinate-$C^{14}OO^-$ when oxidized through the cycle gives rise to α-carboxyl-labeled α-ketoglutarate and hence to unlabeled succinyl-CoA; therefore, no porphyrin labeling should result from this pathway. However, succinate-$C^{14}OO^-$ could also directly form succinyl-CoA, which in this case would be labeled and C^{14} would be found in porphyrin. On the other hand, succinate-$C^{14}H_2$ would form labeled succinyl-CoA by both pathways. If it is assumed that malonate inhibits the oxidation of succinate and the cycle pathway only, malonate should not inhibit any porphyrin labeling after incubation with succinate-$C^{14}OO^-$, but should inhibit appreciably the porphyrin labeling from succinate-$C^{14}H_2$. This was found by Shemin and Kumin, as the averaged results in the accompanying tabulation show (figures are counts/

Hemin	Control	Malonate 20 m*M*	% Change
From succinate-$C^{14}OO^-$	215	210	− 2
From succinate-$C^{14}H_2$	1025	421	−59

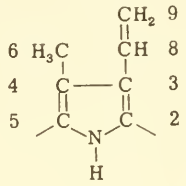
minute for intact erythrocytes). It would appear that both pathways are operative in these cells. The failure of malonate to increase the porphyrin labeling from succinate-$C^{14}OO^-$ is rather surprising because one might expect malonate to divert some of the succinate from the cycle into the formation of succinyl-CoA. It is possible that this effect is somewhat counteracted by an inhibition on succinyl-CoA synthetase.

Further information on porphyrin synthesis and the effects of malonate were obtained by Wriston *et al.* (1955) by the use of labeled acetate. Different malonate effects were obtained when methyl-labeled and carboxyl-labeled acetate were incubated with glycine, the inhibition of porphyrin labeling being much greater with the former (see tabulation). This is the expected

Hemin	Control	Malonate 20 m*M*	% Change
From acetate-$C^{14}H_3$	376	174	−54
From acetate-$C^{14}OO^-$	72.5	67	− 8

result, because the formation of labeled succinyl-CoA from acetate-$C^{14}OO^-$ does not involve the complete cycle and the C^{14} pathway does not go through the succinate oxidation step, whereas porphyrin labeling from acetate-$C^{14}H_3$ depends on the operation of the entire cycle (except for the contribution from the γ-C atom of α-ketoglutarate). Furthermore, the labeling

in the porphyrin from acetate-$C^{14}H_3$ should be altered by malonate. Labeling in the carbon atoms of the A and B pyrrole rings of protoporphyrin occurs after incubation of duck erythrocytes with acetate-$C^{14}H_3$ and glycine.



	Control	Malonate 10 m*M*	% Inhibition
Total porphyrin	186,000	54,000	71
Pyrroles A and B	88,000	27,500	69
Carbon 4	13,000	3,700	72
Carbon 5	12,000	900	93
Carbon 6	20,000	9,400	53

Acetate-$C^{14}H_3$ will lead directly to $^{-}OOC-C^{14}H_2CH_2-CO-CoA$ and if the cycle is blocked completely by malonate, carbons 6 and 9 only will be labeled, except for some labeling of carbons 4 and 8 due to the reversible reaction succinyl-CoA $\rightleftarrows$ succinate (as long as ATP is available). Carbons 2, 3, and 5 should not be labeled. This is essentially seen in the tabulation. The cycle, of course, is not blocked completely so that some labeling in carbon 5 occurs. The over all inhibition is due to a depression of the entry of acetate into the cycle. These experiments not only show the variable effects of malonate on a pathway associated with the cycle but well illustrate the use of an inhibitor to elucidate a metabolic pathway.

The analysis of the action of malonate on porphyrin synthesis was extended by Granick (1958) in his work with chicken erythrocytes. The formation of protoporphyrin is innibited 90% by 10 m*M* malonate when only glycine is present, 85% when succinate is added, and 80% when α-ketoglutarate is added. The effects of different concentrations of malonate are shown in Fig. 1-17. Malonate could decrease the incorporation of succinate into porphyrin by blocking the cycle and reducing the ATP level, and thus inhibit both pathways of succinate-CoA formation from succinate. However, the quite strong inhibition of protoporphyrin formation from glycine + α-ketoglutarate is surprising. Inhibition of the step α-ketoglutarate $\rightarrow$ succinyl-CoA is not likely as the only explanation, because 1 m*M* malonate inhibits protoporphyrin synthesis 32% and there is no reason for thinking that this low concentration would inhibit α-ketoglutarate oxidase. The for-

mation of protoporphyrin from δ-aminolevulinate is not inhibited by malonate so that an action on this part of the pathway is excluded. Granick suggested that malonate reacts with coenzyme A and thus depletes the system so that succinyl-CoA cannot be so readily formed. These effects were confirmed in lysed chicken erythrocytes by Brown (1958). Porphyrins are not formed in these preparations but δ-aminolevulinate is formed from glycine and succinyl-CoA derived from a variety of cycle substrates. Malonate at 10 m*M* inhibits this reaction 30% when the incubation is with glycine and citrate, substantiating the action on this region of the pathway.

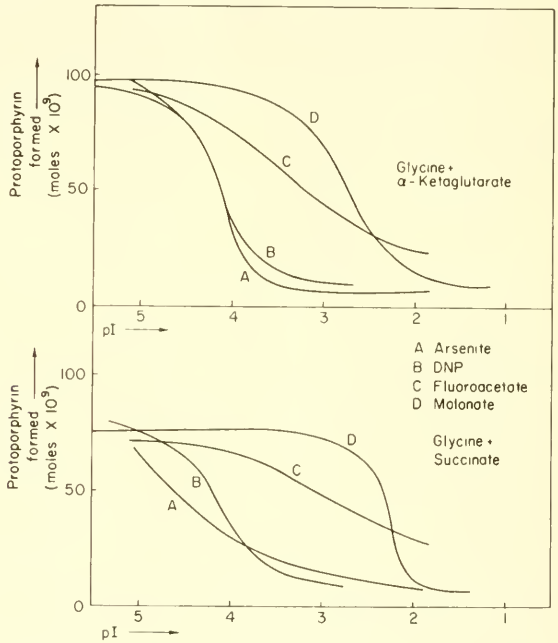


FIG. 1-17. Effects of four inhibitors on the synthesis of protoporphyrin in chicken erythrocytes with the substrates as indicated. (From Granick, 1958.)

It is interesting that the addition of succinate to glycine and citrate in the incubation medium leads to an inhibition of δ-aminolevulinate synthesis. This was shown to be due to the formation of oxalacetate, which inhibits α-ketoglutarate oxidase. Malonate is able to overcome this inhibition by succinate through the prevention of oxalacetate formation. This indicates another minor mechanism for the effect of malonate on porphyrin synthesis, namely, the reduction in oxalacetate concentration and a consequent release from any inhibition on the oxidation of α-ketoglutarate. Finally, we may note that coproporphyrin synthesis from glycine and α-ketoglutarate in

Rhodopseudomonas spheroides is inhibited 50% by 20 m*M* malonate and 75% by 40 m*M* malonate (Lascelles, 1956), indicating again some inhibition of the formation of succinyl-CoA.

The incorporation of iron into heme, as demonstrated with Fe^{59}, is not inhibited by 10 m*M* malonate in canine reticulocytes (Yoshiba *et al.*, 1958) but is inhibited 26% in chicken erythrocytes (Kagawa *et al.*, 1959). It is possible in the latter case that the inhibition is due to the chelation of part of the Fe^{59}, making it unavailable for incorporation.

EFFECTS OF MALONATE ON MISCELLANEOUS METABOLIC PATHWAYS

There have been many reports on the actions or lack of action of malonate on enzyme reactions or metabolic pathways of varying degrees of importance. Some of these are worth mentioning, either because they indicate areas where further study might be profitable or because they provide some evidence for noncycle actions of malonate.

One might expect very little effect of malonate on *photosynthesis* but, although very little work has been done, in every case some effect has been observed. Even the Hill reaction is susceptible to inhibition (Ehrmantraut and Rabinowitch, 1952). This reaction is the photochemical oxidation of water with the production of oxygen and the reduction of a substance, usually quinone, other than CO_2. In *Chlorella* this reaction is inhibited 30% by 6 m*M* malonate and 50% by 60 m*M* malonate. The inhibition is, surprisingly, prevented by fumarate, indicating that the site of action is succinate dehydrogenase and that this enzyme takes part in the transport of hydrogen in the Hill reaction, which would not be the case if quinone were serving as the immediate hydrogen acceptor. It may also be that malonate does not inhibit the Hill reaction directly, but depletes the cells of cycle intermediates or other cycle products necessary for the Hill reaction to proceed. Malonate not only inhibits the total incorporation of $C^{14}O_2$ in *Scenedesmus* by about 20%, but almost completely blocks the formation of labeled malate (Bassham *et al.*, 1950). This was taken as evidence that malate is not on the direct line of phosphoglycerate synthesis, but it also demonstrates that by some mechanism malonate can inhibit CO_2 incorporation. An inhibition of glucose formation in *Chlorella* by malonate has also been reported (Kandler, 1955), although there is less inhibition in the light than in the dark. The synthesis of glucose was believed to be closely related to the formation of high energy phosphate intermediates, and it is thus interesting that malonate inhibits the photosynthetic phosphorylation of ADP in *Rhodospirillum*, although the inhibition is only 17% at the very high concentration of 100 m*M* (Smith and Baltscheffsky, 1959). With these limited observations on the effects of malonate, it must be admitted that it

is difficult to fit the data into the modern concepts of the carbon pathway in photosynthesis, which does not directly involve the cycle, and particularly to understand the mechanism whereby the Hill reaction is inhibited.

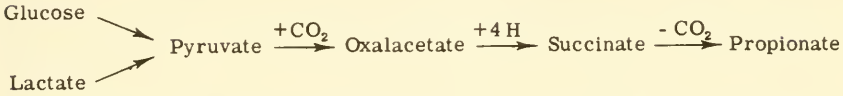
Malonate is also able to interfere in the *metabolism of glycerol.* Glycerol is fermented to succinate, accompanied by the uptake of CO_2, in *Propionibacterium pentosaceum*, and 30 m*M* malonate inhibits both the glycerol fermentation and the CO_2 uptake around 10% (Wood and Werkman, 1940). It is impossible to attribute this to an action on succinate dehydrogenase. The oxidation of glycerol may involve an initial phosphorylation with subsequent formation of pyruvate and entry into the cycle:

$$\text{Glycerol} + \text{ATP} \rightarrow \text{glycerophosphate} \rightarrow \text{pyruvate} \rightarrow \text{cycle}$$

The phosphorylation is inhibited in rat liver homogenates (Ruffo and D'Abramo, 1952), but the total oxidation is not markedly affected in the mycobacteria (G. J. E. Hunter, 1953). Malonate at 10 m*M* inhibits 5% in *M. stercoris* and stimulates 4–6% in *M. smegmatis* and *M. butyricum.* It is surprising that greater inhibition is not observed if the oxidation does involve the cycle. In castor bean cotyledons, malonate at high concentrations has very marked effects on the utilization of glycerol (Beevers, 1956). At 70 m*M* the $C^{14}O_2$ formation from labeled glycerol is inhibited 97% and the sucrose formation is inhibited 82%, while at 130 m*M* the oxygen uptake is inhibited 60%. Such high concentrations may interfere with the formation of ATP and hence depress phosphorylation and also block the cycle CO_2 release.

The *stimulation of muscle respiration by insulin* is inhibited potently by malonate (Stare and Baumann, 1940). Insulin almost doubles the respiration of minced breast muscle from depancreatized pigeons and 1 m*M* malonate inhibits this increase 92%. Fumarate is able to overcome both this inhibition and the inhibition of nonstimulated respiration completely. This interesting action *in vitro* led to a study of the antagonism in the whole animal. Solutions of sodium malonate were injected subcutaneously in rabbits either before or with insulin and the drop in blood glucose was much less than with insulin alone. Insulin (4 units) decreases the blood glucose 65% in 4 hr, whereas with malonate present the reduction is only 13% and none of the rabbits goes into convulsions. Malonate alone increases the blood glucose 26%. The respiratory inhibition in the muscle mince could be explained on the basis of a typical cycle block (although the degree of inhibition is surprising for a concentration of 1 m*M*), but the inhibition of glucose utilization in the animal is more complicated. The initial phosphorylation of glucose and its uptake could have been inhibited indirectly by a reduction of the available ATP, or it could have resulted, at least in part, from a hyperglycemic action of malonate unrelated directly to the insulin stimulation.

Succinate and propionate are formed anaerobically in *Ascaris* muscle from glucose and lactate, presumably by the following pathway:



Malonate at 20 m*M* does not appreciably inhibit the *decarboxylation of succinate to propionate* (about a 13% reduction in total radioactivity) but the small inhibition indicates a possible competition with succinate for the enzyme. However, the *incorporation of lactate-2-C^{14} into succinate* is inhibited almost 90%. If succinate is formed by reduction of fumarate derived from oxalacetate, malonate would be expected to inhibit well, not only because of the effect on the succinate dehydrogenase but also by an inhibition of oxalacetate formation. Malonate inhibits the formation of labeled propionate from lactate-2-C^{14} 65%. The smaller inhibition compared to that for succinate formation implies another less important pathway for the formation of propionate, perhaps by direct reduction, as shown in several bacteria.

The *metabolism of glyoxylate* by liver mitochondria is rather complex; it is decarboxylated to formate by a devious route, it may be oxidized to oxalate, or it may be aminated to glycine (Crawhall and Watts, 1962). Malonate inhibits the decarboxylation competitively but does not interfere with the formation of oxalate or glycine; indeed, the latter may be stimulated slightly due to diversion in a branched chain. The decarboxylation reaction, which requires glutamate, is quite sensitive to malonate, around 50% inhibition occurring at 0.15 m*M*, both substrates being at 3 m*M*. This would certainly appear to be one system in which a marked effect can be exerted by malonate at low concentrations and which is unrelated to succinate oxidation.

The *synthesis of acetylcholine* is an endergonic process and is related to the cycle both for the supply of energy and with respect to the utilization of acetyl-CoA. The effects of inhibitors on acetylcholine synthesis and hydrolysis are particularly important when considering the mechanisms by which malonate can alter nerve and muscle function. Unfortunately, only one study of the action of malonate has been made (Torda and Wolff, 1944 a). The formation of free acetylcholine in minced frog brain, in the presence of physostigmine to prevent hydrolysis, is inhibited 32% by 0.08 m*M*, 46% by 0.8 m*M*, and 49% by 8 m*M*; the inhibition of total acetylcholine is about the same. Succinate, fumarate, and citrate increase acetylcholine formation. It would be interesting to investigate the effects of malonate on the purer enzyme systems now available for acetylcholine synthesis to determine if the inhibition is a direct effect or secondary through ATP depletion. The effects of malonate in the intact cell may be quite complex, because malonate might suppress the incorporation of acetyl-CoA

into the cycle and thereby lead to a greater availability of acetyl-CoA for choline acetylation. However, it is not known if the acetyl-CoA pool is common to both the cycle and the synthesis of acetylcholine. In many cases the effects of malonate are due only to a depression of the cycle operation and a decreased formation of ATP. For example, in the *synthesis of chondroitin sulfate* in tibial condyles of chick embryos, the fixation of sulfate is inhibited by malonate in a parallel fashion to the inhibition of respiration (Boyd and Neuman, 1954). The fixation of sulfate requires ATP, as shown by the marked inhibition with 2,4-dinitrophenol, so that here the mechanism of malonate action is simply an inhibition of energy formation. Other processes, such as calcium deposition in tibial cartilage (Hiatt *et al.*, 1953), do not require energy and are not inhibited by malonate.

EFFECTS OF MALONATE ON THE ENDOGENOUS RESPIRATION

The alterations of the most important metabolic pathways by malonate have been discussed, and we shall now conclude this aspect of the subject with a survey of the effects on the total oxygen uptake of cells respiring in the absence of any external substrate. Although the interpretation of the results of such studies is very difficult, the changes in the endogenous respiration have been examined more frequently than any other response to malonate. There is, thus, a vast and variable mass of data, some of which is summarized in Table 1-26. The aim of most of these investigations has been to demonstrate the absence or presence of the cycle in the types of cells tested, and we must attempt to assess the validity of conclusions based on the response to malonate. The most unsatisfactory work has been done, and the most unjustified conclusions have been drawn, in studies of this type, inasmuch as the inherent complexities of the situations have seldom been appreciated. Although the cycle has a wide distribution in the cells of microorganisms, plants, and animals, its operation during the metabolism of endogenous substrates is quite variable and dependent on the state and past history of the cells.

Factors That May Determine the Degree of Malonate Inhibition

Certain basic factors should be considered in every investigation of the susceptibility of the endogenous respiration to malonate. Although some of these have been mentioned previously and some will be taken up in greater detail later, it may be convenient to enumerate here the most important.

(a) *Intrinsic susceptibility of succinate dehydrogenase to malonate.* This enzyme from different species varies a good deal in its ability to bind mal-

onate, as is evident from the range of K_i values observed (page 33). It is, perhaps, too often assumed that in every organism the succinate dehydrogenase will be readily blocked by malonate and that the inhibition of the endogenous respiration depends only on the importance of the enzyme in the total oxygen uptake. It is mandatory to demonstrate the sensitivity of succinate dehydrogenase to malonate in the preparation being studied.

(b) *Degree to which intracellular succinate dehydrogenase is inhibited.* In addition to the intrinsic susceptibility, there are other factors which can alter the inhibition occurring within the cell. The concentration of succinate, both initially and following the accumulation resulting from the inhibition, may be high enough to oppose the malonate effect appreciably. The relative stability of plant respiration to malonate has been attributed to the high concentrations of succinate and other organic anions in plant cells. It is probably very seldom that an inhibition even approaching completeness can be achieved in cells at concentrations likely to be specific.

(c) *Specificity of malonate inhibition.* Inhibition of the endogenous respiration can, of course, arise from actions other than on succinate dehydrogenase. If the object of the study is to evaluate the contribution of the cycle to the oxygen uptake, inhibitions on noncycle pathways must be eliminated. At the high malonate concentrations often used (see Table 1-26), there is certainly no assurance that the inhibition is specific.

(d) *Intracellular concentration of malonate.* Malonate does not penetrate readily into most cells, especially at physiological external pH values, so that the internal concentrations of malonate may be far below those in the surrounding medium (see page 190). The degree of respiratory inhibition observed is probably often more of a measure of malonate penetrability than of the nature or susceptibility of the metabolic systems. There are several instances in Table 1-26 in which the inhibition rises with destruction of the normal tissue structure or the removal of permeability barriers. In general, the inhibition is greater in homogenates than in minces, and greater in minces than in slices or intact cells. The results of Bonner (1948) on *Avena* coleoptiles are interesting in this regard. Soaking in water for 24 hr increases the susceptibility to malonate and removal of the endosperm further increases the inhibition. The many observations that a lowering of the pH augments the inhibition also provide evidence of the importance of permeability.

(e) *Metabolism of malonate.* Many tissues and organisms can metabolize malonate to acetyl-CoA, the oxidation of which contributes to the oxygen uptake (see page 228). Some of the respiratory stimulations noted with malonate must be due to this, and it is likely that the experimentally determined inhibition in other cases is reduced from that which would be observed if malonate were not metabolized. The best way to test for and

TABLE 1-26

EFFECTS OF MALONATE ON THE ENDOGENOUS RESPIRATION [a]

Species	Preparation	pH	Malonate (m*M*)	% Inhibition	Reference
Brucella abortus	Suspension	6.8	3.3	0	Gerhardt *et al.* (1950)
Achromobacter guttatum	Suspension	5.8	4	6	Sguros and Hartsell (1952 b)
			8	8	
			32	20	
Thiobacillus thiooxidans	Suspension	4.7	100	0	Vogler *et al.* (1942)
Micrococcus lysodeikticus	Extract	6.1	2	26	Saz and Krampitz (1955)
			6	61	
			12	85	
Leptospira ictero-haemorrhagiae	Suspension	7.4	10	5	Fulton and Spooner (1956 a)
			400	3	
Mycobacterium phlei	Suspension	6.8	1	Stim 43	Müller *et al.* (1960)
			10	Stim 71	
			100	Stim 76	
Rhodospirillum rubrum	Suspension	7.4	100	Stim 59	Smith and Baltscheffsky (1959)
Ashbya gossypii	Mycelia	5.0	4	0	Mickelson and Schuler (1953)
			40	5	
Allomyces macrogynus	Mycelia	4.8	5	41	Bonner and Machlis (1957)
			10	69	
			50	67	
Microsporum audouini	Suspension	4.0	55	14	Chattaway *et al.* (1956)
		6.4	55	12	

Microsporum canis	Suspension	4.0	55	17	Chattaway *et al.* (1956)
		6.4	55	10	
Trichophyton schoenleini	Suspension	4.0	55	12	Chattaway *et al.* (1956)
		6.4	55	8	
Trichophyton rubrum	Suspension	4.0	55	14	Chattaway *et al.* (1956)
		6.4	55	12	
	Suspension	4.6	10	20	Nickerson and Chadwick (1946)
Epidermophyton floccosum	Suspension	4.0	55	15	Chattaway *et al.* (1956)
		6.4	55	17	
Puccinia (stem rust)	Uredospores	4.8	20	0	Farkas and Ledingham (1959)
Mycorrhizal fungus (Fagus roots)	Suspension	4.2	28.6	27	Harley and Ap Rees (1959)
Blastocladiella emersonii	Suspension	5.5	0.4	48	McCurdy and Cantino (1960)
Penicillium chrysogenum	Suspension	6.1	200	0	Rolinson (1954)
Yeast	Suspension	2.8	44	70	Stoppani *et al.* (1958 b)
Chlorella vulgaris	Suspension	—	10	0	Bach (1961)
Gymnodinium nelsoni	Suspension	8.1	1	65	Hochachka and Teal (1964)
Porphyridium cruentum	Suspension	5.0	150	0	Speer and Jones (1964)
Ascophyllum nodosum (brown alga)	Slices	4.5	5	16	Kelly (1953)
			10	47	
			25	87	
			50	81	
Oat (*Avena sativum*)	Coleoptile (normal)	4.5	50	12	Bonner (1948)
			100	61	
			200	73	
	Coleoptile (soaked)	4.5	50	57	Bonner (1948)
	Coleoptile (no endosperm)	4.5	50	93	Bonner (1948)
	Coleoptile	6.0	5	0	Albaum and Eichel (1943)
	Coleoptile	4.5	13.8	29	Bonner (1949)

TABLE 1-26 (*continued*)

Species	Preparation	pH	Malonate (m*M*)	% Inhibition	Reference
Barley (*Hordeum vulgare*)	Roots	4.5	10	56	Laties (1949 a)
	Roots	5.0	1	7	Machlis (1944)
			10	50	
			100	88	
	Roots	5.8	2	18	Honda (1957)
	Roots	6.0	5	30	Ordin and Jacobson (1955)
			10	55	
	Seedlings	7.0	50	74	Rubin and Ladygina (1960)
Wheat	Field-grown seedlings	4.5	20	30	Farkas *et al.* (1957 b)
	Greenhouse-grown seedlings	4.5	20	55	Farkas *et al.* (1957 b)
	Etiolated seedlings	4.5	20	25	Farkas *et al.* (1957 b)
Pea (*Pisum sativum*)	Seed homogenate	6.4	0.01	4	Davison (1949)
			0.1	5	
			1	8	
			10	9	
	Seedling homogenate	6.4	0.01	3	Davison (1949)
			0.1	7	
			1	5	
			10	10	
Spinach	Leaf cytoplasm	4.5	48	80	Bonner and Wildman (1946)
		5.5	48	15	
		7.0	48	0	

	Leaves	4.5	48	49	Laties (1949 b)
			96	67	
			144	72	
Tomato	Root slices	—	16	0	Henderson and Stauffer (1944)
	Stem slices	3.5	5	41	Link *et al.* (1952)
		4.0	5	25	
		4.5	5	35	
		5.5	5	10	
		6.0	5	2	
		7.0	5	0	
	Stem slices	5.5	5	4	Eberts *et al.* (1951)
Carrot	Root slices	4.0	50	73	Turner and Hanly (1947)
		6.0	50	0	
	Root slices	4.0	50	80	Hanly *et al.* (1952)
		5.0	50	77	
		6.0	50	44	
		7.0	50	Stim 50	
	Root slices	5.0	20	26	Dalgarno and Birt (1962)
			50	48	
			100	81	
			300	95	
			800	100	
Rhubarb	Leaves	4.5	6.8	42	Morrison (1950)
			34	70	
		5.3	6.8	16	
			34	55	
		6.3	6.8	15	
			34	37	
Cuckoopint (*Arum maculatum*)	Spadix slices	6.0	50	14	James and Beevers (1950)

TABLE 1-26 (*continued*)

Species	Preparation	pH	Malonate (m*M*)	% Inhibition	Reference
Corn (maize)	Roots	3.2	1	25	Beevers (1952)
			10	75	
			100	100	
		4.0	1	10	
			10	41	
			100	82	
		4.8	1	2	
			10	31	
			100	63	
		5.3	10	12	
			100	62	
		6.3	1	0	
			10	0	
			100	33	
Jerusalem artichoke (*Helianthus tuberosus*)	Tuber disks	4.5	20	26	Bonner *et al.* (1953)
Beech (Fagus)	Roots	4.2	28.6	45	Harley and Ap Rees (1959)
	Mycorrhiza	4.2	28.6	22	Harley and Ap Rees (1959)
Potato	Tuber slices (fresh)	5.0	50	15	Romberger and Norton (1961)
	Tuber slices (aged)	5.0	50	50	Romberger and Norton (1961)
Tobacco	Leaves	5.0	20	0	Vickery and Palmer (1957)
			100	22	

Chicory	Root slices (fresh)	5.0	30	13	Laties (1959 a)
	Root slices (aged)	5.0	30	64	
Apple	Fruit mitochondria	7.1	20	48	Hatch *et al.* (1959)
Rose (*Rosa hybrida*)	Petals	4.5	40	12	Siegelman *et al.* (1958)
			60	30	
			80	39	
			100	45	
Strigomonas oncopelti	Suspension	7.3	40	0	Ryley (1955 a)
Trichomonas foetus	Suspension	7.6	10	3	Suzuoki and Suzuoki (1951)
			100	8	
	Suspension	7.3	40	0	Ryley (1955 b)
	Suspension	6.4	50	2	Doran (1957)
Trichomonas suis	Suspension	6.4	50	0	Doran (1957)
Trypanosoma hippicum	Suspension	7.4	10	0	Harvey (1949)
Balantidium coli	Suspension	7.2	10	42	Agosin and von Brand (1953)
Paramecium caudatum	Suspension	7.0	20	31	Holland and Humphrey (1953)
Colpidium campylum	Suspension	5.6	20	41	Seaman (1949)
Myxomycete	Plasmodium	6.0	1	Stim 4	Ohta (1954)
			10	Stim 1	
Hymenolepis diminuta (tapeworm)	Whole	7.2	10	Stim 39	Read (1956)
	Homogenate	7.4	50	68	Read (1952)
			100	82	
Echinococcus granulosus (tapeworm)	Scolices	7.4	10	7	Agosin *et al.* (1957)
Fascicola hepatica (trematode)	Pulp	—	10	35	Van Grembergen (1949)
Gynaecotyla adunca (trematode)	Excysted worms	6.9	10	67	Vernberg and Hunter (1960)

TABLE 1-26 (*continued*)

Species	Preparation	pH	Malonate (m*M*)	% Inhibition	Reference
Nematodirus filicollis (nematode)	Mince	7.3	1	1	Massey and Rogers (1950)
			10	41	
			20	50	
Ascaridia galli (nematode)	Mince	7.3	1	10	Massey and Rogers (1950)
			10	30	
			20	40	
Neoaplectana glaseri (nematode)	Mince	7.3	1	0	Massey and Rogers (1950)
			10	15	
Peloscolex velutinus (oligochete)	Homogenate	—	25	12	Petrucci (1954 a)
Aplysia depilans	Gizzard muscle slices	7.3	10	0	Ghiretti *et al.* (1959)
Australorbis glabratus	Mince	7.4	50	17	Weinbach (1953)
Helix pomatia	Hepatopancreas homogenate	7.4	33	16	Rees (1953)
	Hepatopancreas slices	7.7	100	23	Baldwin (1938)
Oyster (*Saxostrea commercialis*)	Muscle homogenate	7.3	1	22	Humphrey (1947)
			10	24	
			100	40	
	Egg suspension	7.5	—	0	Cleland (1949)
	Egg homogenate	7.5	100	35	Cleland (1949)
Oyster (*Crassostrea virginica*)	Mantle	8-9	25	0	Jodrey and Wilbur (1955)
Sea urchin (*Arbacia*?)	Fertilized eggs	8.2	30	8	Barron and Goldinger (1941)

Sea urchin (*Echinus esculentus*)	Egg homogenate	7.5	10	44	Cleland and Rothschild (1952 b)
Sea urchin (*Strongylocentrotus purpuratus*)	Egg homogenate	7.1	1	8	Yeas (1954)
			2	29	
			10	35	
Locust (*Locusta migratoria*)	Muscle homogenate	7.4	33	95	Rees (1954)
Toadfish (*Opsanus tau*)	Pancreatic islets	7.4	10	43	Friz *et al.* (1960)
Frog	Muscle mince	—	10	13	Thunberg (1909)
			40	29	
			100	32	
			200	34	
Chick	Kidney homogenate	7.4	34	64	Potter and Elvehjem (1937)
	Chorioallantoic membrane	—	20	3	Ackermann (1951)
			40	18	
			60	47	
	Embryo condyles	7.4	10	50	Boyd and Neuman (1954)
			100	75	
	Embryo yolk sac	7.4	10	28	Moulder *et al.* (1953)
Pigeon	Brain brei	—	24	24	Banga *et al.* (1939)
	Brain homogenate		24	42	Banga *et al.* (1939)
	Stomach brei	—	100	90	Breusch (1942)
	Muscle brei		100	80	Breusch (1942)
	Muscle slices	—	10	73	Greville (1936)
	Muscle mince	7.4	5	70	Baumann and Stare (1940)
		7.4	20	77	Stare and Baumann (1936)
		7.4	1	31	Stare and Baumann (1939)
			10	75	
Porpoise	Testis brei	—	100	60	Breusch (1942)

TABLE 1-26 (*continued*)

Species	Preparation	pH	Malonate (m*M*)	% Inhibition	Reference
Guinea pig	Brain slices	7.4	10	0	Heald (1953)
	(unstimulated)		100	77	
	Brain slices (stimulated)	7.4	1	12	Heald (1953)
			3	35	
			10	56	
	Brain slices	7.4	10	0	Kimura and Niwa (1953)
	Brain slices	7.0	10	1	Takagaki *et al.* (1957)
	Mammary gland homogenate	7.4	10	61	Terner (1954)
	Liver slices	7.4	40–50	Stim 11	Jowett and Quastel (1935 c)
	Lung slices	—	40	33	Moussatché and Prouvost-Danon (1957)
	Seminal vesicle	7.4	3.3	0	Levey and Szego (1955)
Pig	Heart mince	7.4	6.6	39	Stare and Baumann (1936)
Sheep	Heart mince	—	1	32	Smyth (1940)
			25	83	
Ox	Sperm suspension	7.0	10	75	Lardy and Phillips (1943 a)
	Sperm suspension	7.0	10	55	Lardy and Phillips (1945)
	Retina homogenate	—		81	Burgess *et al.* (1960)
	No bicarbonate			0	
	With bicarbonate				
	Kidney culture cells	7.5	100	23	Polatnick and Bachrach (1960)
	Thyroid slices	7.4	33	0	Weiss (1951)

Cat	Brain mince	7.3	17	23	Huszàk (1940)
	Nerve mince	7.3	17	16	Huszàk (1940)
	Muscle mince	7.3	17	61	Huszàk (1940)
	Brain brei	—	100	20	Breusch (1942)
	Muscle brei		100	80	Breusch (1942)
	Heart brei		100	90	Breusch (1942)
	Kidney brei		100	85	Breusch (1942)
	Liver brei		100	70	Breusch (1942)
	Pancreas brei		100	30	Breusch (1942)
	Lung brei		100	10	Breusch (1942)
	Embryo muscle brei		100	80	Breusch (1942)
	Embryo liver brei		100	60	Breusch (1942)
	Embryo lung brei		100	50	Breusch (1942)
Mouse	Brain mitochondria	7.4	40	55	du Buy and Hesselbach (1956)
	Liver mitochondria	7.4	40	39	du Buy and Hesselbach (1956)
	Crocker 180 sarcoma mince	—	6.7	33	Boyland and Boyland (1936)
	Earle sarcoma cells	—	50	40	Packer *et al.* (1959)
	Ehrlich ascites cells	7.4	20	50	Creaser and Scholefield (1960)
		7.4	12.5	35	Maizels *et al.* (1958)
Rat	Brain slices	—	10	58	Greville (1936)
	Brain mince	7.4	10	4	Cohen and Gerard (1937)
	Brain mince	—	125	84	Das and Roy (1943)
	Brain homogenate	7.2	4	60	Pardee and Potter (1949)
			20	73	
	Liver slices	7.4	40–50	24	Jowett and Quastel (1935 c)
	Liver slices	7.4	10	13	Edson (1936)
			20	32	
	Liver mince	—	125	46	Das and Roy (1943)
	Liver homogenate	7.4	27	50	Holtkamp and Hill (1951)

TABLE 1-26 (*continued*)

Species	Preparation	pH	Malonate (m*M*)	% Inhibition	Reference
	Liver homogenate	7.2	4	44	Pardee and Potter (1949)
			20	52	
	Liver homogenate	7.7	10	33	Nakada and Weinhouse (1950)
	Heart slices	7.4	2	11	Webb *et al.* (1949)
			5	21	
			10	32	
			20	42	
			50	62	
			100	70	
	Heart mince	—	125	23	Das and Roy (1943)
	Heart homogenate	7.4	1	70	Lehninger (1946 b)
	Heart homogenate	7.2	4	33	Pardee and Potter (1949)
			20	33	
	Kidney slices	7.4	20	55	Elliott and Greig (1937)
	Kidney slices	7.4	2	37	Saffran and Prado (1949)
			20	86	
	Kidney mince	—	125	48	Das and Roy (1943)
	Kidney homogenate	7.2	4	33	Pardee and Potter (1949)
			20	83	
	Muscle mince	—	125	48	Das and Roy (1943)
	Muscle mitochondria	7.2	4	90	Klingenberg and Schollmeyer (1960)
	Adipose tissue	7.4	13	27	Haugaard and Marsh (1952)

	Thoracic aorta	7.4	20	10	Briggs *et al.* (1949)
	Reticulocytes	7.4	1	47	Fulton and Spooner (1956 a)
			20	64	
	Gastric mucosa	7.4	10	0	Lutwak-Mann (1947)
	Jensen sarcoma slices	—	6.7	28	Boyland and Boyland (1936)
	Jensen sarcoma mince		6.7	43	Boyland and Boyland (1936)
	Yoshida sarcoma suspension	7.4	10	17	Tanaka *et al.* (1952)
Rabbit	Liver slices	—	6.8	18	Annau (1935)
	Liver brei		6.8	39	Annau (1935)
	Kidney slices		6.8	25	Annau (1935)
	Kidney slices	7.4	50	46	Mudge (1951)
	Kidney brei	—	6.8	21	Annau (1935)
	Soleus (tonic) muscle	7.3	10	68	Domonkos and Latzkovits (1961)
	Semimembranosus (tetanic) muscle	7.3	10	37	Domonkos and Latzkovits (1961)
	Reticulocytes	7.4	25	76	Rubinstein *et al.* (1956)
	Fertilized ova (blastocysts)	7.4	10	10	Fridhandler *et al.* (1957)
Man	Brain slices	7.35	5	25	Elliott and Sutherland (1952)
	Atrial slices	7.4	100	40	Burdette (1952)
	Prostate slices	7.2	100	Stim 15	Andrews and Taylor (1955)
	Placenta slices	7.4	20	3	Hosoya *et al.* (1960)
	Placenta homogenate	6.9	20	Stim 26	Hosoya *et al.* (1960)

[a] In some instances it is difficult to determine if the respiration is truly endogenous since the experimental conditions are not stated explicitly. Hence, in a few case, glucose may have been present, but in no instance were cycle substrates added. The values for the pH have been included where they have been given, because the degree of inhibition is usually dependent on the penetration of malonate into the cells.

correct for this phenomenon is to determine the $C^{14}O_2$ formed from labeled malonate.

(f) *Nature of the cycle operation and the presence of alternate pathways.* The inhibition of the oxygen uptake associated with the cycle will depend on a number of factors in addition to the inhibition of succinate dehydrogenase. The availability of a large pool of organic acids to form oxalacetate, or the presence of pathways from which oxalacetate may arise (e.g., by carboxylation of pyruvate, or from aspartate by transamination), will reduce the inhibition of oxygen uptake from that which would be observed if all the oxalacetate had to be derived from the cycle. Other pathways for the metabolism of succinate may circumvent the block to some extent. These matters have been discussed in some detail (see pages 72-88).

(g) *Adaptive changes in the presence of malonate.* Inhibition of the cycle may accelerate other pathways. The increased uptake and metabolism of glucose brought about by malonate have been noted in several types of cells, and such a phenomenon will tend to counteract the malonate inhibition on the oxygen uptake. Adaptive changes in enzyme concentrations probably are seldom important in short-term experiments but cannot be completely ignored in work with certain microorganisms. Inhibitions by malonate have occasionally been noted to decrease with time, and adaptive changes are the most obvious explanation.

These and other more subtle factors determine the effect of malonate on the total oxygen uptake of a preparation, and it should be apparent that deductions based exclusively on the inhibitions of endogenous respiration are frequently untenable. A definite inhibition with a reasonable malonate concentration is more significant than a negative result, because there are many factors which can reduce or abolish the action of malonate even though the cycle is present and active.

The Time Course of Malonate Inhibition

The inhibition of respiration by malonate may occur fairly rapidly and remain constant, or it may increase slowly to a level at which it is maintained, or it may gradually disappear, or it may vary in quite complex fashion with time. A slowly developing inhibition would not be unexpected with a substance which does not penetrate readily. The inhibition of succinate dehydrogenase is essentially instantaneous so that an approximately linear increase in the inhibition to a constant level would imply that the rate of inhibition is determined by the penetration. On the other hand, secondary effects, such as would result from the depletion of ATP, may also contribute to a progressive inhibition. Greville (1936) noted that malonate does not immediately inhibit the respiration of rat diaphragm but

that the inhibition developes over 1–2 hr. A very similar time course was observed in barley roots by Laties (1949 a). After the addition of 10 m*M* malonate, the respiration drops linearly for 60–90 min and then becomes relatively constant at about 40%. However, over the next 5 hr, the inhibition lessens somewhat. This was postulated to be due to increasing succinate concentration, but actually such changes in succinate take place much more rapidly in most cases. It must be admitted that in spinach leaves the maximal succinate accumulation occurs at 4 hr (Laties, 1949 b), so that this explanation can by no means be eliminated. The inhibition of the spinach leaf respiration by malonate is greater at 6 hr than at 3 hr but not enough data are available to correlate the changes in inhibition with succinate levels. Malonate requires about 1 hr to produce its maximal inhibition of sea-urchin egg homogenate respiration (Ycas, 1950). This is the only report of such a slowly developing inhibition in subcellular preparations and no explanation is evident. Another situation seems to exist in bovine kidney culture cells, where the inhibition by 100 m*M* malonate slowly increases from 23% at 1 hr to 35% at 5 hr (Polatnick and Bachrach, 1960). It is more likely here that secondary changes are responsible.

Definite decrease in the respiratory inhibition of pigeon brain dispersions (probably similar to homogenates) with time was reported by Banga *et al.* (1939). The inhibition is 41.7% at 10 min, 29.5% at 30 min, and 20.5% at 50 min. Since the malonate concentration was 24 m*M*, it seems unlikely that metabolism of malonate could have reduced its concentration significantly. Adaptive changes in homogenates are improbable and sufficient accumulation of succinate from the relatively limited substrate supply is scarcely possible. Besides, the inhibition of pyruvate oxidation increased over this interval. Sometimes changes in tissue metabolism occur during the course of an experiment independently of malonate action. When malonate is added to human brain slices immediately, the endogenous respiration is inhibited around 25% at 5 m*M*, but if malonate is added after 90 min incubation of the slices, there is no inhibition (Elliott and Sutherland, 1952). The role of succinate oxidase in the respiration must change as a result of the slicing or the abnormal medium.

The inhibition of the respiration of rat ventricle slices by malonate 5–20 m*M* follows a more complex course (Webb *et al.*, 1949). Following an initial inhibition, the respiration rises for approximately 1 hr and then begins to fall again. There is thus a maximum or hump in the respiration curve. After 1 hr, the respiratory level with malonate is higher than in the controls. The reversal of the inhibition is inhibited by fluoride, which would indicate that the hump is due to augmented glucose oxidation or to the metabolism of malonate. Calcium is also necessary for the typical response to malonate. Very complex effects of malonate were also found by Turner and Hanly (1947) and Hanly *et al.* (1952) in carrot slices. The var-

iation of the inhibition depends on the pH. At pH 4, the inhibition develops over 1 hr and remains constant, but at higher pH's the inhibition may disappear or only stimulation may be seen. The value of these interesting experiments is greatly reduced by the unaccountable use of potassium malonate rather than the sodium salt. Potassium at 50 m*M* (which is the concentration of malonate generally used by them) stimulates the respiration and alters its character, so that all the results must be the summation of two usually opposing actions. This illustrates how the incorrect choice of an inhibitor salt can vitiate the results of an otherwise excellent investigation.

Most of the work on the malonate inhibition of endogenous respiration has been done without regard for possible alterations in the inhibition with time. The inhibitions have simply been determined over an arbitrary interval. Inasmuch as changes in the inhibition by malonate occur quite frequently, it is likely that over all inhibitions, such as are presented in Table 1-26, are often mean values and do not reflect either the initial inhibition or the maximal inhibition. As was pointed out in Chapter I-12, the value of many studies on inhibitors would be increased by determinations of the variation of the inhibitions with time.

Effects of Different Conditions on the Inhibition of Endogenous Respiration

One of the most important variables affecting the response of the endogenous respiration to malonate is the *age of the tissue*, particularly as it relates to the stage of development or the interval between the preparation of the tissue and the experimental testing. The respiration of plant tissues usually becomes more sensitive to malonate with time. This indicates a progressive change in the metabolic pattern in the direction of a greater participation of the cycle. The changes in the inhibition during malonate inhibition, discussed in the previous section, can be due to the effects of the malonate or to inherent metabolic alterations. It is thus important in such studies to determine both the changing inhibition in the presence of malonate and the changing susceptibility as malonate is added at various intervals. The malonate inhibition rises with time in the *Avena* coleoptile (Bonner, 1948), rose petals (Siegelman *et al.*, 1958), chicory root slices (Laties, 1959 a), *Arum* spadix slices (Simon, 1959), and potato tuber slices (Romberger and Norton, 1961). These changes are usually associated with an increase in the total uninhibited respiration. For example, in potato slices there is a 4-fold rise in the respiration during incubation for 30 hr; the malonate resistant fraction doubles and the malonate-sensitive fraction increases 10- to 15-fold. Carrot slice inhibition by malonate, on the other hand, decreases steadily up to 376 hr after cutting the sections (Hanly *et al.*, 1952), although the uninhibited respiration first rises and then falls. The results obtained with animal tissues are less striking and more variable.

The inhibition of rabbit ova respiration at various times postcoitum does not change significantly (Fridhandler *et al.*, 1957), while the inhibition of trematode respiration decreases with time from excystment (Vernberg and Hunter, 1960). When 20 m*M* malonate is added to rat ventricle slices 1 hr after slicing, the inhibition of the respiration is only 25%, whereas initially the inhibition is near 50% (Webb *et al.*, 1949), and this relationship holds for all malonate concentrations up to 100 m*M*. We have seen that in the presence of malonate the inhibition has been replaced by stimulation at 1 hr. Therefore the metabolism changes differently during the action of malonate and the maxima in the time curves cannot be explained by inherent alterations of the metabolic pattern. All of these results point to the importance of considering the time factor in studies of malonate inhibition.

Another apparently important factor is the *ion and buffer composition of the medium*, although no thorough studies have been done and the mechanisms are not understood. Many years ago Annau (1935) observed that the inhibition of the respiration of both rabbit liver and kidney slices is less in Ringer than in phosphate medium. The results were quite variable but on the average the inhibition is 30% in phosphate and 15–20% in Ringer medium. Unfortunately, Annau did not state what form of malonate was used nor did he mention pH control, so the results are perhaps unreliable. The presence of bicarbonate abolishes the inhibition by malonate in ox retina homogenates (Burgess *et al.*, 1960), and it is probable that inhibition in intact cells would also vary with the bicarbonate concentration. Bicarbonate can, of course, facilitate the formation of oxalacetate through carboxylation reactions. When Ca^{++}, Mg^{++}, or K^+ is removed from the medium, the inhibition of rat brain slice respiration by 10 m*M* malonate is not altered, but in the presence of fumarate the inhibition becomes progressively greater as these ions are successively removed (Greville, 1936). The addition of Ca^{++} to nematode minces increases both the respiratory inhibition and the inhibition of succinate oxidation (Massey and Rogers, 1950). The sensitivity of chicory root slice respiration to malonate is markedly affected by K^+ and Li^+ (Laties, 1959 b). Slices incubated with 50 m*M* K^+ are inhibited more and with 50 m*M* Li^+ inhibited less than the fresh slices. It is also interesting that increase in CO_2 tension results in progressive disappearance of the malonate inhibition, whereas increase in O_2 tension augments the malonate-sensitive fraction of the respiration. It is thus clear that the medium can play an important role in the response to malonate. Much work has been done in quite nonphysiological media and the results are thus difficult to apply to the actions of malonate *in situ*. Much more effort should be directed at creating approximately physiological conditions.

The *functional activity of the tissue* determines the level and type of respiration, and therefore is often a major factor in the sensitivity to mal-

onate. This has been shown particularly clearly in brain slices, stimulated both electrically (Heald, 1953) and by K^+ (Kimura and Niwa, 1953; Yoshida and Quastel, 1962). The stimulated respiration is readily inhibited by malonate (Fig. 1-14) whereas the resting respiration is insensitive. This behavior is probably exhibited by many tissues. It is often very difficult to determine exactly the functional state of isolated tissues, such as slices, but where possible this should be attempted. We shall find later that active tissues are more easily functionally depressed by malonate and this may have a metabolic basis. Indoleacetate stimulates the growth of *Avena* coleoptiles and increases the respiration simultaneously. This additional respiration brought about by indoleacetate is readily inhibited by malonate (Bonner, 1949), and it is likely that the respiration of rapidly growing tissue is generally inhibited more strongly by malonate than that of resting or slowly proliferating tissue.

Consideration must also be given to the *history of the tissue.* Bonner (1948) has shown that the nutritional state of the *Avena* coleoptile determines the inhibition by malonate, and it is probable that the same applies to animal tissues. The inhibition of wheat seedling respiration by malonate depends on a number of factors, including the type and duration of irradiation, the nutrition, and the region from which the plants come (Farkas *et al.*, 1957 a, b). This is a field that has been very little explored. The changes in the respiratory inhibition of animal tissues with nutrition might not only provide information on the metabolic patterns under various conditions, but be important in the use of the inhibitor to selectively depress the metabolism and growth of neoplastic tissues.

Effects on the Respiratory Quotient

The effects of an inhibitor on the respiratory quotient (R.Q. = CO_2 formed/O_2 uptake) are often indicative of shifts in metabolic pathways. Let us first consider the theoretical values of the R.Q. for the metabolism of various substrates (see tabulation) in the presence and absence of malonate, assuming that malonate is able to block succinate oxidation completely. Cases in which oxalacetate is formed in the cycle and from noncycle sources must be separated. Summarizing these results, one would expect malonate to increase or decrease the R.Q., depending on the substrate and the nature of the cycle operation. Since a complete block of succinate oxidation would prevent the formation of oxalacetate through the cycle, malonate may shift the pathway from cycle oxalacetate to externally formed oxalacetate, if the latter reaction is possible. If this is so, the R.Q. should rise in every case.

This prediction is quite consistently borne out experimentally. The R.Q. of rat liver slices rises from 0.72 to 0.77 in the presence of 20 m*M* malonate, at which concentration the respiration is inhibited 14% (Elliott and Greig,

Substrate metabolism	R.Q.
Glucose → $CO_2 + H_2O$	1.0
Glucose → succinate + $CO_2 + H_2O$	0.8
Glucose + 2 oxalacetate → $CO_2 + H_2O$	1.27
Glucose + 2 oxalacetate → 2 succinate + $CO_2 + H_2O$	1.5
Pyruvate → $CO_2 + H_2O$	1.2
2 Pyruvate → succinate + $CO_2 + H_2O$	1.33
Pyruvate + oxalacetate → $CO_2 + H_2O$	1.4
Pyruvate + oxalacetate → succinate + $CO_2 + O_2$	2.0
Butyrate → $CO_2 + H_2O$	0.8
Butyrate + oxalacetate → $CO_2 + H_2O$	1.23
Butyrate + oxalacetate → succinate + $CO_2 + H_2O$	1.0
Butyrate + 2 oxalacetate → $CO_2 + H_2O$	1.2
Butyrate + 2 oxalacetate → 2 succinate + $CO_2 + H_2O$	1.33

1937). However, malonate decreases the R.Q. of kidney slices, both endogenous and with pyruvate as the substrate. Malonate elevates the R.Q. of rat adipose tissue from 1.0 to 1.13 endogenously and from 1.14 to 1.41 in the presence of glucose (Haugaard and Marsh, 1952). In frog muscle, the R.Q. first rises from 0.9 to 0.97 at 10 m*M* malonate, but then progressively decreases as the malonate concentration is raised so that at 200 m*M* malonate the R.Q. is 0.39 (Thunberg, 1909). In plant tissues, the effects are less variable. Malonate has been shown to increase the R.Q. of barley roots from 0.97 to 1.14 (Machlis, 1944), of maize roots (Beevers, 1952), of carrot roots up to values as high as 3 (Hanly *et al.*, 1952), of chicory roots from 1.03 to 1.14 (Laties, 1959 a), and of rhubarb leaves at pH 5.3 (Morrison, 1950).

Of course, there are many factors which must be taken into account, since malonate can secondarily alter several metabolic pathways. A stimulation of glucose uptake could change the R.Q. in either direction, depending on the nature of the substrates used in the uninhibited tissue; in the presence of a significant cycle block, this would usually depress the R.Q. and counteract the more direct effects described above. On the other hand, metabolism of malonate would tend to elevate the R.Q. since the complete oxidation would give R.Q.'s of 1.50–1.55 and the oxidation to succinate 3.0–4.0. A final factor of importance is the relative dependence of glucose and fatty acid metabolism on the operation of the cycle and the levels of ATP, since malonate could alter the oxidative contribution from these substrates secondarily.

Significance of Respiratory Inhibition

Does the degree of malonate inhibition indicate the contribution of the cycle to the total oxygen uptake? This must certainly be answered in the negative. Lack of inhibition can be due to a failure to penetrate, the metabolism of malonate, a source of oxalacetate external to the cycle, metabolic adaptations of the cells, and many other factors. Positive evidence of inhibition is more valuable than absence of inhibition, but even when definite inhibition is observed the possibility of actions other than in the cycle must be considered, especially when the malonate concentration must be high to achieve an effect. It is doubtful if anyone examining Table 1-26 would attempt to correlate the inhibitions with the importance of the cycle in the organisms and tissues. For example, in general there is greater inhibition of mammalian endogenous respiration than of the respiration of microorganisms or plants. This might indicate a greater role of the cycle in mammalian tissues, but it could also be attributed to a poorer penetration in the plants and microorganisms, or to a greater metabolic flexibility and adaptability in these more resistant forms. It must also be clear that the degree of respiratory inhibition bears no necessary relationship to the degree of inhibition of succinate dehydrogenase. A significant inhibition by a reasonable concentration of malonate is evidence for the operation of the cycle, but the quantitative aspects of the contribution cannot be derived from these data alone. The effects of malonate on the endogenous respiration are sometimes of greater physiological significance than effects on the oxygen uptake in the presence of high concentrations of often abnormal substrates, since the endogenous metabolism may be representative of a more normal balance of substrates. In this connection, studies of inhibitors would often be improved if the attempt were made to provide the cells with a mixture of physiologically pertinent substrates at the concentrations normally occurring in the cellular environment.

PERMEABILITY OF CELLS TO MALONATE

One of the major problems in the use of malonate has always been the degree of penetration of the inhibitor into the cells or tissues, and it has been frequently stated that this is the primary factor responsible for the low inhibitions observed in many cases. It is true that the plasma membrane is relatively impermeable to most ions, particularly anions and those carrying two or more charges, but if this is so how can one explain the marked respiratory stimulations usually seen with succinate or other dicarboxylate ions? Furthermore, malonate is often metabolized readily by tissues and this presupposes entrance into the cells. Since there are other possible reasons for a resistance to malonate, the permeability hypothesis must be examined critically.

Experimental Evidence Relating to the Penetration of Malonate

Malonic acid is more than 10 times as lethal on injection into frogs as is sodium malonate (Heymans, 1889). No explanation was offered for this observation but it could have been due to the greater permeability of the cells to the acid or, on the other hand, to a nonspecific acidification of the animals. Malonate administered to rabbits circulates initially in a volume equivalent to the extracellular compartment and the intracellular transfer occurs slowly (Wick *et al.*, 1956). The failure of malonate to alter the metabolism of labeled acetate was attributed to both the slow penetration into the tissues and the simultaneous metabolism of the malonate, both factors keeping the intracellular concentration at low levels. The inability of malonate to alter gastric acid secretion in frogs, even at lethal doses, was similarly attributed to these factors (Davenport and Chavré, 1956). Inasmuch as succinate oxidase is present in the secretory cells and the cycle is important in secretion (as shown by the inhibition with fluoroacetate), the lack of action must be due to an insufficient concentration within the cells.

Turning to isolated tissues and cell suspensions, the augmentation of malonate effects by procedures designed to reduce or abolish the permeability barriers has been demonstrated many times (Table 1-26). Rat diaphragm respiration is inhibited slowly by malonate, but if the diaphragm is cut into small pieces, and hence presumably damaged, the inhibition is immediate (Greville, 1936). The oxygen uptake of pigeon brain brei is inhibited rather poorly by 24 mM malonate, but when the brain is dispersed more completely in the form of a homogenate the inhibition is more marked (Banga *et al.*, 1939). The succinate dehydrogenase of yeast incubated for 5 hr in liquid nitrogen is much more susceptible to malonate than in normal cells (Lynen, 1943), and the same holds for *E. coli* treated with toluene (Ajl and Werkman, 1948). Sensitivity to malonate can be induced by liquid nitrogen treatment in the fungus *Zygorrhynchus* (Moses, 1955) and by drying *Pseudomonas* (Gray, 1952). Malonate does not inhibit glutamate oxidation in intact cells of *Pasteurella*, but inhibits well in sonic lysates (Kann and Mills, 1955). All of these phenomena have been interpreted in terms of permeability. This is certainly the most obvious explanation and it is probably generally correct, but it must be admitted that such drastic treatments could affect many other things; for example, alter the organized enzyme structure so that the attacked enzyme is more exposed, or reduce the ability of the cells to metabolize malonate.

Only one investigation of the relative permeabilities of the dicarboxylate anions has been made. Giebel and Passow (1960) determined the half-times for penetration of these ions into bovine erythrocytes and the results are given in Table 1-27. Giebel and Passow attempted to correlate the permeabilities with the ionic sizes and the acidic ionization constants. The ionic volumes and lengths presented in the table, which are somewhat

Table 1-27

Erythrocyte Permeabilities and Molecular Properties of Dicarboxylate Anions [a]

Anion	Relative permeability	Ionic volume (Å³)	Ionic length (Å)	(H_2B) (μM)	(HB^-) (μM)
Oxalate	100	54	5.1	0.00015	276
Malonate	17	66	7.5	0.112	6590
Maleate	14	75	7.9	0.051	252
Fumarate	1.4	75	7.6	0.014	445
Succinate	0.45	78	7.5	2.56	6410
Malate	0.31	91	7.5	0.135	1860
Glutarate	0.085	90	8.5	1.99	3780
Tartrate	0.045	104	7.5	0.0103	338

[a] The permeabilities were determined in bovine erythrocytes by Giebel and Passow (1960). The values given here for the relative permeabilities are the reciprocals of the entrance rate half-times ($t_{1/2}$) multiplied by 100. The calculations of the ionic volumes and lengths are approximate and usually depend on the configuration of the ions. The concentrations of H_2B and HB^- are given for a total concentration of 1 M.

different than those given by Giebel and Passow, must be considered as only relative values, neglecting hydration and special configurations of the ions. Their experiments were run at a pH of 7.35 so the concentrations of the undissociated and singly dissociated forms of the acids are given for this pH. The ionization constants are sometimes quite different from those assumed by Giebel and Passow and are those in Table 1-2. There is certainly little or no general correlation between permeabilities and the concentrations of either H_2B or HB^-. Succinate, for example, penetrates one-thirty eighth as rapidly as malonate and yet (H_2B) for succinate is 23 times higher than for malonate. This does not necessarily invalidate the assumption that for a single substance the unionized forms penetrate more rapidly than the ionized, but it shows that there are other factors which are quite important. There is also no correlation with the ionic length and it is unlikely that one would be expected. However, there is some correlation with ionic volume, leading Giebel and Passow to suggest that the dicarboxylate anions penetrate through pores in the membrane, whereas the monocarboxylates pass through the lipid phase of the membrane. They calculate the pore radius to be between 3.8 and 4.5 Å. If these ions pass through the pore channels in the extended form, which is likely, there are two major factors which may contribute to the permeability: the cross-sectional area perpen-

dicular to the direction of passage and the degree of interaction of the molecules with the walls of the pores. This interaction may be of various types and includes steric repulsion and attractive forces (such as van der Waals' forces or hydrogen bonds). The configuration of the ion must be important in this connection. Maleate penetrates 10 times faster than fumarate and this must be mainly due to the structure of maleate wherein the carboxylate groups are much closer than in fumarate. That both of these ions penetrate more rapidly than succinate may be due to the greater rigidity of the former, energy perhaps being required for succinate to change from its statistically most probable configuration to that necessary for penetration. It is evident, however, that none of these explanations satisfactorily fits the experimental data and that we need to know much more about the membrane before accurate interpretations can be made. It should be mentioned that these results on erythrocytes do not apply to other types of cells or tissues, inasmuch as erythrocyte permeability is in some senses unique.

Malonate inhibits the succinate dehydrogenase of calf thymus nuclei and yet at 10 m*M* has no effect on the respiration or ATP level of intact nuclei (McEwen *et al.*, 1963 b). This indicated a failure to penetrate and it was shown with labeled malonate that this is indeed the case, which is somewhat surprising in view of the usual concepts of the nuclear membrane.

Malonic acid does not enter organic solvents from water readily, due probably to the dipolar nature of the unionized carboxyl groups. The partition ratios for malonic acid are given as (concentration in solvent/concentration in water): oleyl alcohol 0.049 (Collander, 1951), ether 0.083, isobutanol 0.62, methylisobutylketone 0.15, and methylisobutylcarbinol 0.37 (Pearson and Levine, 1952). The partition ratios for succinic acid are always somewhat higher, as expected. These data would indicate that even the unionized malonic acid would not penetrate through the lipid phase of the membrane too readily. The fact that the un-ionized forms penetrate better than the ionized does not imply that passage through a lipid phase occurs. The negative charge on the ions could impede movement through pores, especially when it is considered that in most cells these ions must move up an electrical potential gradient to cross the membrane.

Variation of Malonate Inhibition with pH

One of the strongest arguments for the preferential uptake of the less ionized forms of malonic acid is the rise in the inhibition observed with a lowering of the pH. This has been examined particularly in plant tissues and the results are quite uniform. Such effects have been observed in tomato stem slices (Link *et al.*, 1952), maize roots (Beevers, 1952), rhubarb leaves (Morrison, 1950), barley roots (Laties, 1949 a), spinach leaves (Bonner and Wildman, 1946), carrot root slices (Hanly *et al.*, 1952), and *Avena*

coleoptile (Cooil, 1952). These results are plotted in Fig. I-14-19. On the other hand, rather insignificant effects of pH have been noted in fungi, such as *Microsporum*, *Trichophyton*, *Epidermophyton* (Chattaway *et al.*, 1956), and *Pullularia* (Clark and Wallace, 1958). In *Pullularia* malonate is readily oxidized; the oxygen uptake from malonate increases with a lowering of the pH along with the inhibition of the cycle and the effects tend to cancel one another. The effects of malonate in tobacco leaves are not changed greatly by lowering the pH from 7 to 4 (Vickery and Palmer, 1957), although down to pH 5 the uptake of malonate becomes progressively greater. The incubation here was very long (48 hr) and hence there was more opportunity for malonate to penetrate than in shorter experiments. It is unfortunate that no quantitative work on pH has been done with animal tissues.

It is also regrettable that in those instances in which malonate inhibits more strongly at low pH values the reversibility of the inhibition has not been adequately tested. Lowering the pH of the medium in the presence of a weak acid increases the amount of unionized acid entering the cells and can decrease the intracellular pH to a degree causing cell damage. It was noted in carrot root tissue that injury to the cells occurred, including loss of turgor and release of some of the cell contents, with malonate at a pH around 4 (Hanly *et al.*, 1952). It is very difficult in experiments of this type to distinguish between a specific malonate effect and a nonspecific acid damage. Examination of the reversibility of the inhibitions might provide some evidence on this point.

In Volume I it was shown that the intracellular concentration of a dicarboxylate anion is related to the total external concentration in the following manner (Eq. I-14-178):

$$(I^{=})_i = \frac{(H^+)_o^2}{{}_of_i''(H^+)_i^2}\,(I_t)_o \tag{1-4}$$

where the subscripts o and i refer to outside and inside the cells and ${}_of_i''$ is the appropriate pH function for the external inhibitor (see Eq. 1-14-12). Two assumptions are involved in this formulation: (1) only the H_2I form of the inhibitor penetrates, and (2) the cells are internally completely buffered. The variation of the intracellular active $I^{=}$ form with external pH is very marked, as shown in the accompanying tabulation, assuming an intracellular pH_i of 6.8. Of course, cells are not completely buffered and, as the internal pH_i drops, the entrance of the inhibitor is slowed, so that with decrease in the buffering capacity the ratios given will be lessened. Here one is presented with the dilemma that at low pH values one must either assume a high internal inhibitor concentration or a significant fall in pH_i. Since the inhibitions observed are not as high as would be predicted on the basis of the above equation and tabulation, one is forced to conclude

that the intracellular pH_i must fall. This may not only damage the metabolic systems but will tend to reduce the inhibition on succinate dehydrogenase by decreasing the concentration of dicarboxylate anion.

pH_o	$(I^-)_i/(I_t)_o$
8.2	0.0016
7.4	0.063
7.0	0.39
6.0	34.8
5.0	1610

If one assumes that the HI^- form can also penetrate, the internal concentration of the active $I^=$ form will not be so strongly dependent on the external pH_o, and the inhibition will increase significantly as the pH_o is lowered from 6 to 5, but otherwise the same behavior will be expected. It is usually difficult to distinguish between penetration by the H_2I form only and penetration by the HI^- form also. Some arguments have arisen on this point, Bonner and Wildman (1946) believing that the HI^- form penetrates and Beevers (1952) holding that only the H_2I penetrates. A Simon-Beevers plot of the data from maize roots (see Chapter I-14) shows that (H_2I) does not remain constant for 50% inhibition of respiration over the pH range 3–6.5, which would indicate a possible contribution from the HI^- form. Actually, it might be better to express the total entry rate of malonate as:

$$\text{Entry rate} = P_{H_2I}(H_2I)_o + P_{HI^-}(HI^-)_o + P_{I^=}(I^=)_o \qquad (1\text{-}5)$$

where the P's represent the permeabilities to the various forms of the inhibitor. Although $P_{H_2I} > P_{HI^-} > P_{I^=}$, above pH 4 (HI^-) is much greater than (H_2I) so that the contribution of the second term to the total rate may be significant. One would like to know the relative values of the P's for a particular tissue and these could be determined if the entry rate of malonate were determined (for example, with labeled malonate) at different pH values. One must also bear in mind that a change of pH could alter the permeability properties of the membrane.

The only data on the effects of pH on malonate action in animal tissues were obtained on rat ventricle strips by Masuoka *et al.* (1952). Malonate stimulates the amplitude of hypodynamic strips much more at pH 6.2 than at 7.4. This positive inotropic action may be unrelated to the inhibition of succinate dehydrogenase, since succinate at pH 6.2 gives essentially the same response, and could depend on the oxidation of malonate (see page 216). Whatever the mechanism, these results indicate that malonate penetrates more readily at the lower pH.

In comparing the actions and penetrations of malonate with succinate, or with other dicarboxylate anions, it is necessary to consider that the relative concentrations of the ionic species can be quite different. Thus the H_2B form of succinate is at a much higher concentration than the same form of malonate at the same total concentrations (Table 1-3). It is also possible that some cells possess active transport or carrier systems for the substrate dicarboxylate anions, allowing succinate to penetrate more readily than malonate. Permeability in some cases seems to be as specific as are enzyme reactions, and can be quite dependent on the configurations and charge distributions of the transported substances. This could be due to either the structure of the membrane pores or the nature of a carrier. For these reasons one must anticipate striking differences between different tissues with regard to the relative permeabilities to the various ions, and it is particularly important not to apply the results on plants unreservedly to animal tissues or microorganisms.

GROWTH, DEVELOPMENT, AND DIFFERENTIATION

The responses of growth, cleavage, and histogenesis to inhibitors are interesting because they often demonstrate the nature of the metabolic basis for these important biological processes. The results may also have bearing on the possible use of the inhibitors for the selective depression of the growth of organisms or abnormal cells which are detrimental to the host. These processes all require energy from the metabolism so that any reduction of either the exergonic reactions or their coupled phosphorylations would be expected to interfere in some manner. In addition, more specific effects may occasionally be observed. The selective inhibition of the growth of certain cells can result from different rates of growth of the cells involved, or from differences in the metabolic requirements for growth. It is generally true that rapidly proliferating cells are more readily affected by inhibitors than are the same or other cells growing or multiplying at slower rates. It has also been demonstrated that various types of cells may utilize different enzymes or metabolic pathways to support proliferation. With respect to malonate, one might anticipate that cells whose growth is in one way or another significantly dependent on the cycle would be inhibited more than cells not requiring the operation of the cycle. However, other factors, such as the degree of penetration of the malonate or the susceptibility of the succinate dehydrogenases to malonate, may be important.

Virus Multiplication

Malonate is able in some instances to suppress the intracellular formation of virus without permanently damaging the host cells. The results obtained

with influenza type A virus isolated from man and cultured in chick chorioallantoic membrane illustrate this well. Ackermann (1951) found that malonate inhibits the formation of virus and simultaneously reduces the oxygen uptake of the host cells (see accompanying tabulation). Malonate is not

Malonate (m*M*)	% Inhibition of respiration	Final virus titer	Infectivity titer
None	—	213	$10^{7.8}$
20	3	192	$10^{7.8}$
40	18	69	$10^{6.5}$
60	47	0	$10^{3.3}$

virucidal since the original virus can be recovered from the infected cultures. The major effect of malonate is not to prevent infection of the cells, since essentially the same results were obtained when malonate was added 4 hr after the inoculation of the virus. The inhibition is thus exerted on the synthesis of new virus material. Furthermore, the host cells are not damaged; if infected chorioallantoic tissue is exposed to 60 m*M* malonate for 24 hr, virus proliferation is completely inhibited but, if the tissue is washed free of malonate, becomes susceptible to infection and supports virus multiplication. These results were confirmed and extended by Eaton (1952), who reported that 42 m*M* malonate completely inhibits virus multiplication, inhibits respiration around 50%, and does not alter aerobic glycolysis. The formation of virus in minced chorioallantoic membrane is inhibited 85% by 27 m*M* malonate when glucose is the substrate and 95% when glutamate is the substrate (Eaton and Scala, 1957). The injection of 1 mg sodium malonate into chick embryos 1 hr after inoculation with virus reduces the amount of virus after 48 hr by 55% (Hannoun, 1952). The energy for the synthesis of new virus material must come mainly from the host cell tricarboxylate cycle. It is impossible to reduce the energy supply for virus synthesis without simultaneously restricting the energy for host cell activities. However, it appears that the virus propagation is selectively depressed because it is the most endergonic process occurring, the chorioallantoic cells otherwise not being very functionally active. As long as the low maintenance energy requirement is satisfied, the cells are not damaged (see Fig. I-9-6). If one were dealing with virus in an active tissue, such as nerve or muscle, it would be much more difficult, or impossible, to selectively block virus multiplication.

Other viruses growing in animal cells have been studied with various results. Malonate at 6.7 m*M* has a definite depressant effect on the proliferation of vaccinia virus in chick embryo cultures (R. L. Thompson, 1947)

and similar results were reported for psittacosis virus (Morgan, 1954). In neither case does malonate depress the growth of the tissue cultures, nor does it inhibit the cellular contractions of chick heart cultures. Very slight effects of malonate at concentrations from 10 to 100 m*M* were observed with foot-and-mouth disease virus in bovine kidney culture cells (Polatnick and Bachrach, 1960). Since there is little inhibition of the respiration, it is possible that malonate does not penetrate adequately. At 100 m*M*, respiration is inhibited 23%, virus yield perhaps 20%, and there is a 10 min delay in the appearance of virus. Finally, malonate was found to actually stimulate feline pneumonitis virus proliferation in the isolated chick embryo yolk sac, 10 m*M* increasing the virus titer about 33% (Moulder *et al.*, 1953). Thus a wide variety of effects have been observed with different viruses and host cells, no general conclusions being possible at this time. I am not aware of any studies on the effects of malonate on the course of virus infections in whole animals.

Plant viruses have been inadequately investigated and results on the tobacco mosaic virus only are available. Although malonate at 0.5 m*M* decreases the number of lesions/cm^2 in detached tobacco leaves from 28.9 to 21.9, Chiba *et al.* (1953) felt that this result is statistically insignificant. Ryzhkov and Marchenko (1954, 1955) reported that malonate inhibits multiplication of this virus and that this is reversed by fumarate, but Schlegel (1957) found only variable effects of 3 m*M* malonate on the yield of virus in leaf discs. The spraying of 10 m*M* malonic acid solutions (pH 2.7–3.6) onto the leaves of bean plants decreases the number of virus lesions 68% without leaf damage (Matthews and Proctor, 1956), but this may be unrelated to the action of malonate on the cycle inasmuch as succinic acid is even more inhibitory. This is probably a nonspecific acid effect because the cycle intermediates usually increase the virus yield when they are added to leaf cultures.

It is somewhat surprising that malonate has no demonstrable effect on the multiplication of *E. coli* phage. Spizizen (1943) found no effect at 10 m*M* under any conditions of virus growth, and Czekalowski (1952) reported no actions on either T2 phage or host cells at concentrations from 0.1 to 100 m*M*. It may well be that phage proliferation is not directly dependent on the energy derived from the cycle, but inhibition by 2,4-dinitrophenol, cyanide, and fluoride is observed. In fact, Czekalowski stated that all the inhibitors that depress phage selectively seem to act in some manner on the cytochrome system and are able to inhibit succinate oxidase; yet the most specific inhibitor for this enzyme is inactive. Lack of penetration is an unlikely explanation and this failure of malonate to inhibit deserves further study.

Multiplication of Bacteria, Fungi, and Other Microorganisms

Bacterial growth is apparently fairly resistant to malonate, despite the many observations of enzyme and metabolic inhibitions in these organisms. The absence of any effect on *E. coli* at malonate concentrations up to 100 m*M* reported by Czekalowski was mentioned above, but Loveless *et al.* (1954) found 50% inhibition of growth at 19.2 m*M* malonate, with no effect on cell size. Malonate at 300 m*M* produces somewhat elongated *E. coli* cells and at 800 m*M* they are markedly lengthened and often U-shaped: division is abolished but the cells continue to elongate slowly (Schweisfurth and Schwartz, 1959). The effects of malonate must depend on many factors, including the nutrient medium, the duration of the growth phase studied, and the pH. Although Rosenberg (1948) found the growth of *Clostridium saccharobutyricum* to be inhibited by malonate, a concentration of 100 m*M* was used, so that the mechanism of the effect is not clear. His observation that the inhibition is overcome by *meso*-inositol and borate must be interpreted as indicating a unique approach to the study of malonate inhibition. Malonate at 10 m*M* has a slightly depressant action on rate of germination of *Bacillus subtilis* spores but does not affect growth in culture (Hachisuka *et al.*, 1955). The bacteriostatic concentrations of malonate were given as 3.2 m*M* for *Pseudomonas fluorescens* and 7.7 m*M* for *B. aerogenes* (Cooper and Goddard, 1957) but the acid was used, the pH being 2.5 and 1.5, respectively, so that a nonspecific acid effect is the most likely explanation, especially as succinic acid is as inhibitory. It is clear that the investigations of the effects of malonate on bacterial growth leave everything to be desired.

The sporulation and growth of yeast are inhibited by malonate quite potently and in parallel fashion, 50% depression of each occurring at 5 m*M* (Miller and Halpern, 1956). On the other hand, *Hensenula ellipsoidospora*, a vellum-forming yeast, grows more rapidly in the presence of malonate (Luteraan, 1953). It would certainly not be too surprising to find certain microorganisms stimulated by malonate inasmuch as many can oxidize malonate readily (page 228). Malonate arrests sporulation of *Aspergillus niger* without suppressing growth (Behal, 1959) but germination of *Neurospora* ascospores is not blocked by 10 m*M* malonate even at pH 2.3 and after 24 hr (Sussman *et al.*, 1958), nor is the germination of *Puccinia* uredospores affected significantly by 20 m*M* malonate at pH 4.8, although the respiration is inhibited around 37% (Farkas and Ledingham, 1959). The formation of conidia is often essential for the spread of the scab disease of apple caused by *Venturia inaequalis* and so Kirkham and Flood (1963) investigated the effects of various respiratory inhibitors on ascosporulation. Malonate was found to inhibit at high concentrations, the inhibition surprisingly increasing with increase in the pH (see accompanying tabulation). This might imply an action on or within the membrane; this is supported by the relative lack of effect on the respiration and the rather potent inhi-

bition produced by *trans*-aconitate. The injection of 50 m*M* malonate into the leaf petioles, however, increases infectivity so that a more significant

Malonate (m*M*)	Initial pH	% Inhibition of sporulation
50	4.2	4
100		39
40	5.0	Stim 7
100		71
50	6.2	68
100		96

effect on the host tissue is evident. The development of *Puccinia* rust on wheat seedling leaves is inhibited by 10 m*M* malonate, but the leaf tissue is damaged (Samborski and Forsyth, 1960). In this particular case the phytotoxicity is greater than the rust suppression so that malonate could not be used commercially. Malonate esters have been tested for inhibition of mold growth in syrups, but 13 m*M* does not have much effect over 144 hr (Lord and Husa, 1954); however, these esters are used as fungistatic agents in soy sauce in Japan (Tsukamoto, 1951). Another instance of growth stimulation by malonate was reported for *Endamoeba histolytica* (Nakamura and Baker, 1956); the average cell count per field at the end of 3–4 days was 1 in the control and 16 in the presence of 12.8 m*M* malonate, indicating possible metabolism of the malonate by these organisms.

Plant Growth

Avena coleoptile growth is sometimes stimulated and sometimes depressed by malonate, the response depending on the strain of oats used, the pH, and whether the sodium or potassium salt of malonate is used. The marked inhibition (61%) reported by Commoner and Thimann (1941) for 10 m*M* potassium malonate over 24 hr has not been observed by others. Albaum and Eichel (1943) found only stimulation (around 30%) from 1–5 m*M* potassium malonate over a period of 160 hr, and it was felt that malonate was serving as a substrate, which was substantiated by the higher respiration in the presence of malonate. Thimann and Bonner (1948) provided further evidence for this by the finding that malonate at 1 m*M*, having little effect by itself, antagonizes the marked inhibition produced by iodoacetate. How much of this is due to malonate and how much to potassium is difficult to say. Cooil (1952) confirmed the counteraction of iodoacetate inhibition by potassium malonate, but found that the sodium salt is not nearly as potent, implicating the potassium ion. The failure of malonate to inhibit the growth is probably the result of poor penetration, as shown by the

effects of pH (see accompanying tabulation); the pH alone has little effect on growth. The malonate inhibition is satisfactorily reversed by fumarate.

pH	Mean growth (mm) in malonate 3 m*M*
6.5	1.56
6.0	1.70
5.5	1.55
5.0	0.22
4.5	0.16

The mitotic activity of the excised roots of the garden pea (*Pisum sativum*) is stimulated by glucose. This is very strongly blocked by malonate at pH 5.5. A concentration of 0.01 m*M* delays the action of glucose 2 hr but does not inhibit mitosis; 0.1 m*M* inhibits mitotic activity around 50%; 0.5 m*M* almost completely inhibits mitoses; and 1 m*M* not only inhibits completely but produces some toxic effects (Wilson *et al.*, 1959). It was postulated that the initiation of mitosis is dependent on the cycle, since once mitosis began it proceeded to telophase normally. The progression through mitosis may be coupled with a more anaerobic type of metabolism. The growth and cell proliferation in tissue cultures of the crown galls of various plants (marigold, Paris daisy, periwinkle, and sunflower) are inhibited to different degrees by malonate. Cultures from normal tobacco stem are inhibited similarly. At 10 m*M*, the following inhibitions may be estimated from the curves given by Hildebrandt *et al.* (1954): sunflower 0%, marigold 29%, tobacco 40%, Paris daisy 60%, and periwinkle 67%. At 80 m*M* malonate, all are inhibited completely. A question arises again as to whether these effects are related to cycle inhibition, since succinate, pyruvate, acetate, and other organic anions inhibit also. The pH was 6.0 so that acid effects should not be important.

Egg Cleavage and Embryogenesis

The best and most interesting work on the growth responses to malonate has been done with marine invertebrate eggs and embryos. Since this work was done in sea water, we must bear in mind that the concentration of free malonate is much less than the total concentration due to the high amounts of Ca^{++} and Mg^{++}. When malonate is added at a total concentration of 25 m*M*, it is likely that the free malonate is around 4 m*M* (see Table 1-5). In addition, the pH of sea water is near 8.2 and this is unfavorable to malonate penetration into the cells. Considering these factors, it is surprising that such definite and characteristic effects of malonate have been observed.

Egg cleavage is generally rather sensitive to malonate. Although *Arbacia* eggs divide normally in 1 m*M* malonate (Krahl and Clowes, 1940), *Dendraster* eggs are inhibited quite well at this concentration (Pease, 1941). The development of bilaterality in *Dendraster*, seen with many inhibitors, does not occur with malonate even at 10 m*M*, demonstrating a true differential effect on cleavage. Division of *Arbacia* and *Chaetopterus* eggs is inhibited completely by malonate at 70 m*M*, although 40 m*M* is essentially without action, and this is completely reversed by fumarate, indicating a block of the cycle (Brust and Barnett, 1952; Barnett, 1953). This is not a sodium effect since NaCl does not inhibit. Such high concentrations of malonate are not unreasonable in work in sea water and the inhibitions here may be quite specific. Egg cleavage seems to depend on the ATP generated in the cycle, because concentrations of malonate that completely inhibit the cleavage of *Chaetopterus* and *Lytechinus* eggs cause an immediate drop in the high-energy phosphate to the unfertilized levels, and inorganic phosphate is actually lost from the cells (Barnett and Downey, 1955).

The effects of malonate on the development of *Arbacia* eggs were thoroughly investigated by Rulon (1948), who demonstrated abnormal differentiation with low concentrations of malonate. If fertilized eggs in the 1–2-cell stage are placed in 1.2 m*M* malonate, a slight retardation of cleavage is observed, and at 13 hr (when the controls are swimming bilateral gastrulae) there is no evidence of gastrulation, development having progressed only to spherical blastulae with no ventral flattening. After 24 hr (when the controls are plutei), abnormal gastrulae with thickened apical ends and long active cilia are seen. At 48 hr some had developed into abnormal plutei with ciliated apical knobs rather than normal arms. When malonate is removed after 13 hr, quite normal plutei are formed, showing that the effect is readily reversible. It is interesting that eggs exposed to malonate for varying times, then washed free of malonate and fertilized, show abnormal development, demonstrating that malonate can so disturb egg metabolism that the effects are made evident later after the malonate is no longer present. Exposure of unfertilized eggs to 1.44 m*M* malonate for 12 hr, for example, leads to only 30–40% cleavage with irregular cleavage furrows and cells of unequal sizes, development not progressing beyond irregular blastulae. Rulon postulated a gradient of succinate dehydrogenase throughout the cells and embryos, paralleling the physiological activity gradient, but it is not necessary to assume this to explain effects on differentiation. In connection with our work on parapyruvate (Montgomery and Bamberger, 1955), we examined the development of *Strongylocentrotus purpuratus* in 25 m*M* malonate. Up to 24 hr no discernible differences from the controls are seen, but at 44 hr a slight inhibition of development can be detected, with less formation of the primary mesoderm. Incoordination of ciliary activity is also evident, most of the blastulae simply rotating rather

than swimming. At 64 hr, when the controls are beginning to gastrulate, the treated embryos are still spherical blastulae, and at 72 hr have not progressed beyond this point. In this species, malonate would appear to be a rather specific inhibitor of gastrulation without altering cleavage primarily. It may be noted that other cycle inhibitors, such as parapyruvate and fluoroacetate, also specifically block gastrulation in *Strongylocentrotus*. It is difficult to understand these marked differences between the behaviors of various sea urchin eggs, and especially the striking effects obtained by Rulon with such low malonate concentrations. The free malonate concentrations in his work would have been around 0.17 m*M* (he used Ca^{++}-free sea water) and the intracellular concentration presumably much less.

There is evidence that insect spermatogenesis is an aerobic process with the terminal electron transport through the cytochrome system, and that the cycle is the primary pathway for substrate oxidations. Yet no inhibition of meiosis or differentiation into spermatids and spermatozoa by 50 m*M* malonate is observed in hanging-drop cultures from the *Cecropia* silkworm (Schneiderman *et al.*, 1953). These experiments were carried out at pH 6.8–7.2 and it is possible that malonate failed to penetrate.

Amphibian gastrulation is inhibited by high concentrations of malonate. Frog embryos at the early dorsal lip stage were dissected to give explants which were exposed to 40 m*M* malonate at pH 8.0 for 18 hr. Development does not proceed beyond the next stage (Ornstein and Gregg, 1952; Gregg and Ornstein, 1953). There is no differential effect on the respiration of dorsal and ventral explants, both being inhibited about 60%. Unfortunately, there is some doubt that the block of development is due to any specific effect of the malonate since 45 m*M* NaCl apparently inhibits to the same degree. Thus the mechanism of the block could have been osmotic or a Na^+ effect. The chick embryo seems to be much more sensitive to malonate. Explants of chick embryo in the presence of glucose undergo morphogenesis and differentiation to the formation of the central nervous system and an actively beating heart. Malonate at 1–2 m*M* exerts striking differential effects (Spratt, 1950). Although no differences were noted during the first 20 hr, afterwards the central nervous system degenerates completely while the heart forms normally and beats. Malonate is the most specific inhibitor for the development of the nervous system. Some antagonism of this effect was seen with succinate but none with fumarate. No inhibition of mitoses in cultures of chick bone is observed with malonate from 65 to 138 m*M* (A. F. W. Hughes, 1950).

Mitoses in Mammalian Tissues

Epidermal mitotic activity in mouse ear fragments was determined over 4 hr periods at pH 7.4 and 38° by Bullough and Johnson (1951). From the effects of anaerobiosis and various substrates it was concluded that the

energy for mitosis is derived from cycle oxidations. Malonate at 20 m*M* prevents the cell from entering mitosis for 3 hr but evidence of recovery is seen after this time. However, at 4 hr, although some cells are progressing through mitosis, the number of mitoses is definitely less than in the controls. There is no evidence that malonate can stop mitosis once it has begun. Epidermal cells adjacent to the wound show a higher number of mitoses than normal epidermis and malonate depresses both strongly (see accompanying tabulation). (Bullough and Laurence, 1957). Malonate can thus

Malonate (m*M*)	Average number of mitoses	
	Epidermis adjacent to wound	Normal epidermis
0	8.20	0.96
10	1.23	0.22
20	0.05	0.18
30	0.05	0.01

inhibit healing without significant damage to the tissue, since no necrosis is seen following incubation with malonate. Similar results were obtained by Gelfant (1960) and concentrations as low as 0.5 m*M* are definitely inhibitory. After 4 hr 50 m *M* malonate produces some necrosis. The mitotic rate in the germinal epithelium of rat ovaries is also suppressed by malonate, 2 m*M* having variable effects but inhibiting 21% on the average and 10 m*M* inhibiting 59% (Weaver, 1959). The relatively high sensitivity of mammalian tissue mitosis to malonate would implicate the cycle as an important source of energy for this process.

Growth of Neoplastic Tissues

The respiration of various types of tumor cells is inhibited by malonate (Table 1-26) but comparisons with the appropriate normal tissues have seldom been made. Amino acid uptake is also inhibited (Kit and Greenberg, 1951) and high-energy phosphate compounds reduced (Creaser and Scholefield, 1960), but whether tumor tissue is more or less sensitive than normal tissue to malonate is not known. Fishgold (1957) obtained evidence that hepatoma succinate oxidase is inhibited more readily than the enzyme from normal mouse liver, but it is not certain if this is a true difference in the affinities of the dehydrogenase active center for malonate or the result of other factors. Other than this, there is no demonstrated metabolic difference between tumor and normal tissues with respect to inhibition by malonate. The time course of the accumulation of succinate in the Flexner-Jobling tumor is essentially the same as in other tissues (Fig. 1-11), but the relation-

ship of the accumulation to malonate concentration is not linear for the tumor (Fig. 1-12). As pointed out by Busch and Potter (1952 b), the accumulation of succinate in the tumor may be superficially indistinguishable from that in normal tissues, but there is reason to believe that the succinate in the tumor arises by somewhat different pathways, mainly from glutamate and related compounds. There is little reason to believe from the known metabolic characteristics of tumor tissues that malonate would selectively depress their growth; indeed, one might expect them to be less sensitive to malonate, except for the fact that tumor cells are often more rapidly proliferating and more active metabolically than normal tissues.

Neoplastic growth in general seems to be relatively resistant to malonate. Malonate at 30 m*M* is not toxic to cultures of various tumors but 50 m*M* is toxic to all types of cells in a few hours (Chambers *et al.*, 1943). It is interesting that no differences in susceptibility of lymphocytes and other wandering cells, whether from normal or neoplastic tissues, are seen. Eagle's KB strain of human carcinoma cells is more sensitive, the 50% inhibitory concentration of malonate being around 4 m*M* (Smith *et al.*, 1959). Malonate, like many metabolic inhibitors, is capable of producing acentric blebs on Sarcoma 37 ascites cells (Belkin and Hardy, 1961). Although this indicates some alteration of the membrane properties and the permeability to water, the relationship to growth inhibition is unknown.

Studies of the action of malonate on tumors growing in whole animals are more pertinent to the problem of the possible value of this inhibitor in therapy. The earliest work was done by Boyland (1940) following his investigations of the effects of malonate on tumor respiration. Definite suppression of growth was observed with malonate at a dose well below the lethal, the carcinoma being more sensitive than the sarcoma (see accompanying tabulation). Actual regression of the carcinomata was observed,

Inhibitor	Dose (mg/day)	% Inhibition of growth of		LD_{50} (mg)
		Grafted sarcomata	Spontaneous carcinomata	
Malonate	20	32	131	100
Malonamide	25	36	44	150
Ethylmalonate	40	27	25	160
Glutarate	25	8	48	150
Adipate	25	0	35	200

as indicated by the 131% inhibition. Whether ethylmalonate and malonamide are metabolized to malonate and are active for this reason is not known, but the lesser potencies compared to malonate do not suggest that these uncharged substances penetrate better. Malonate, fluoride, iodoacetate

and azide were administered to patients with advanced neoplasia and temporary suppressive effects were noted (Black and Kleiner, 1947; Black *et al.*, 1947). Sodium malonate was given orally at doses of 1–1.5 g/day. It is difficult to state clearly the effects of malonate, since the inhibitors were usually given sequentially or together, but it was stated that hematological remissions occurred in acute myeloblastic leukemia and that shrinkage of solid tumors, with relief of pain, was evident. The tumor cells usually become refractory to these inhibitors. After resistance to fluoride and iodoacetate has developed, a beneficial effect is seen with malonate. It would seem that these results are encouraging enough to warrant further study, particularly with combinations of the inhibitors to prevent or reduce the development of resistance. Several derivatives of malonate were tested against mammary Carcinoma 755 in mice and suppressive action was demonstrated (Freedlander *et al.*, 1956). Malonic acid at 1.2% in the diet did not affect either the tumor size or the growth of the mice. The most active ethyl ester was diethylethoxymethylenemalonate (the group =CH—O——CH_2CH_3 on C-2), which at 1.2% in the diet reduces the surface area of the tumors 83% while causing minimal loss of body weight. This substance is as effective as 8-azaguanine and is less depressant on the total body growth. Some diamides are also active, *N*-dimethylmalondiamide being the most active, reducing tumor area 68% with no effects on body growth. It was thought that these substances may be inhibitory to succinate dehydrogenase, probably after hydrolysis, but there is no evidence at present for this and it is quite possible that the mechanism is entirely different. Despite the lack of evidence for a high susceptibility of the metabolism or growth of isolated neoplastic cells to malonate, the *in vivo* work has brought out interesting effects that deserve more thorough investigation.

CELLULAR AND TISSUE FUNCTION

Many studies of the effects of malonate on physiological function with the object of relating the cellular activity to succinate oxidase or the cycle have been reported, but in only a few instances have the necessary data been obtained and a relationship adequately established. The general relations between enzyme inhibition and changes in cellular function were discussed in Chapter I-9, and several methods for demonstrating correlations were presented. The complexities of such studies were emphasized and the difficulties commonly encountered are well illustrated in the results of malonate inhibition. In addition to the various possible metabolic effects of malonate, one must bear in mind that malonate, or the cations added with it, can directly alter functional processes, actions which can be distinguished by the proper controls.

Single Cell Motility

A thorough study of the respiration and motility of the ciliate *Paramecium caudatum* was made by Holland and Humphrey (1953). Malonate at 20 m*M* inhibits the endogenous respiration 31% but has no effect on the ciliary activity over 1 hr. The oxidation of cycle substrates, such as citrate, α-ketoglutarate, fumarate, and malate, is well inhibited, and it is likely that the cycle is present. Furthermore, the succinate oxidase in homogenates is quite sensitive to malonate. One must conclude either that malonate does not penetrate sufficiently or that the motility is not entirely dependent on the cycle for an energy supply. The answer to this problem may lie in the observation that malonate does not inhibit the oxidation of pyruvate or acetate. Holland and Humphrey point out that there are many alternate pathways for metabolism in paramecia. Thus it is possible that normally energy is derived from the cycle but during a cycle block energy is provided by other alternate reactions. In the human parasitic ciliate *Balantidium coli*, malonate depresses both respiration, which is probably endogenous, and motility (Agosin and von Brand, 1953). The flagellar motility of *Bacillus brevis* is not affected by 10 m*M* malonate (De Robertis and Peluffo, 1951). The motility of bull sperm is reduced appreciably by 10 m*M* malonate and the endogenous respiration is simultaneously inhibited 55% (Lardy and Phillips, 1945). However, the results are quite different when various substrates are present and motility is depressed very little or not at all. Since motility is not depressed with glucose or pyruvate as substrate, whereas it is with acetate, it is likely that energy sources other than the cycle can be used. Ciliary and flagellar activities in different organisms are thus affected in various ways by malonate and no uniform picture emerges from the data at present available.

Chemotaxis and phagocytosis in guinea pig leucocytes are partially inhibited by malonate but the concentration used (140 m*M*) was so high that little significance can be attached to these results (Lebrun and Delaunay, 1951). Bacterial phagocytosis by human neutrophiles is depressed moderately by malonate from 33.5 to 100 m*M* but no effect is seen with 6.7 m*M* (Berry and Derbyshire, 1956). Malonate at 1 m*M* has no effect on the migration of amphibian chromatophores in cultures of the neural crest (Flickinger, 1949). At 10 m*M*, malonate is toxic to these preparations and not specifically depressant to the motility. These limited results point to a relative insusceptibility of ameboid-type movements to malonate, which is not surprising considering the known metabolic characteristics of such cells.

Renal Tubular Transport

Most of the studies of the effects of malonate on the kidney have involved the active transport of *p*-aminohippurate. The accumulation of this sub-

stance by kidney cortex slices, which is quite marked (slice/medium ratios around 10), is reduced by malonate: in slices from dogs, 5 mM inhibits 25%, 10 mM inhibits 35%, and 20 mM inhibits 55% (Shideman and Rene, 1951 b) and in slices from rabbits 20 mM inhibits 72% while reducing the respiration with acetate by 53% (Cross and Taggart, 1950). This action can be shown to occur in the whole animal also. Dominguez and Shideman (1953, 1955) removed one kidney from rats, administered solutions of sodium malonate, removed the other kidney, and determined the accumulation of *p*-aminohippurate. When approximately 10 millimoles/kg of malonate are injected subcutaneously, the uptake of *p*-aminohippurate is depressed 52%, the average slice/medium ratio falling from 9.28 to 4.47. The decrease in the slice/medium ratio is linear with intravenous doses of 4–7 millimoles/kg. Some effect occurs at 15 min after the injections, the maximal inhibition is around 60 min, and the transport mechanisms have returned to normal by 150 min, indicating the ready reversibility of the action. The transport inhibition can also be demonstrated by the renal clearance technique in dogs (Shideman and Rene, 1951 b). Malonate at a dose of 0.96 millimole/kg depresses the *p*-aminohippurate Tm* 73%. It was stated that 50% inhibition of renal succinate dehydrogenase is produced by 1.32 mM malonate and thus the dose given would be expected to inhibit *in vivo*, but the concentration of malonate in the tubular fluid is probably much higher than in the blood and permeability factors must also be important. Farah and Rennick (1954, 1956) studied the effects of many inhibitors on the *p*-aminohippurate uptake in guinea pig kidney slices and the results are summarized in Fig. 1-18. Malonate is one of the weakest inhibitors but yet exhibits a marked effect at 10 mM. Koishi (1959 a) confirmed the inhibition by malonate on *p*-aminohippurate accumulation in rat kidney slices, obtaining slight inhibition at 1 mM and 65% inhibition at 5 mM. The effects of malonate are expressed in the following equation:

$$\log (S/M) = 1.452 + 0.457 \log (I) \qquad (1\text{-}6)$$

where S/M is the slice/medium ratio and (I) is the molar concentration of malonate. The active transport of *p*-aminohippurate is thus definitely related in some manner to succinate dehydrogenase and the cycle, assuming a specific action of malonate, which is likely at the generally low concentrations used.

This conclusion is somewhat substantiated by the findings that the renal transport of other substances is frequently not inhibited potently by malonate. The accumulation of tetraethylammonium ion is scarcely affected by malonate up to 40 mM (Farah and Rennick, 1956; Farah, 1957), the metabolic requirements apparently being different than for *p*-aminohippu-

* Tm is the tubular transport maximal rate for a substance.

rate. Phenol red accumulation is also not depressed (Shideman and Rene, 1951 b), although in isolated flounder tubules it is suppressed by 10–20 m*M* malonate (Forster and Goldstein, 1961). It was claimed that there is a correlation between succinate oxidase activity and transport in different species. Clearance studies with glucose and phosphate show that there is little effect of malonate on their transport: e.g., when *p*-aminohippurate transport is inhibited 73%, glucose Tm is decreased only 10%. On the other hand, Malvin (1956) reported that malonate quite definitely depresses the phosphate Tm, although no data were given. Since malonate interferes with the uptake of inorganic phosphate in kidney homogenates, it was concluded that malonate in some manner suppresses the esterification of phosphate during its transport.

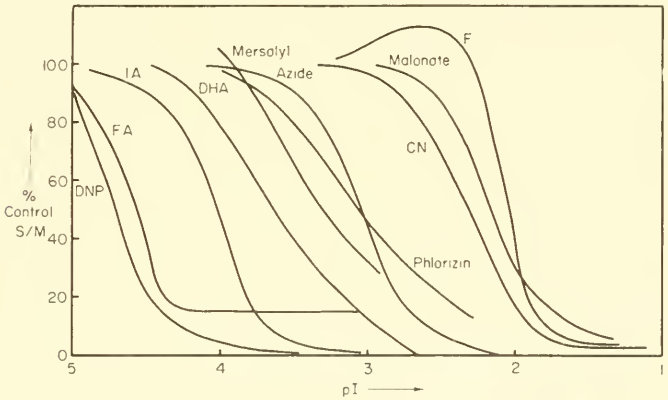


FIG. 1-18. Effects of inhibitors on the slice/medium (*S*/*M*) ratio for *p*-aminohippurate in kidney slices. DNP = 2,4-dinitrophenol, FA = fluoroacetate, IA = iodoacetate, DHA = dehydroacetate, CN = cyanide, and F = fluoride. (Modified from Farah and Rennick, 1956.)

Renal electrolyte transport is disturbed by malonate (Mudge, 1951). Rabbit renal cortex slices were leached for 2.5 hr in 0.15 *M* NaCl, this lowering the tissue K^+ concentration and reducing the endogenous respiration. The slices were then incubated for 30 min in medium containing 10 m*M* K^+, 10 m*M* acetate, NaCl to provide a constant osmotic pressure, and phosphate buffer, during which time K^+ enters the cells. Malonate at 50 m*M* almost completely blocks this return of K^+ into the cells and reverses the movement of water (see accompanying tabulation). The Na^+ changes are not so significant because the substitution of divalent anions for chloride increases the external, and presumably the internal, Na^+ concentration. If fresh slices are treated with malonate, a loss of cell K^+ and

	Initial	Control	Malonate 50 m*M*	% Change
Respiration (Q_{O_2})	—	0.69	0.37	− 46
Water content (%)	79.0	78.2	81.1	—
K^+ (meq/kg wet wt.)	24.7	58.0	28.1	− 90
Na^+ (meq/kg wet wt.)	112	93.8	139	—
$K^+ + Na^+$	136.7	151.8	167.1	+100

an equivalent gain of Na^+ would be expected due to the inhibition of the transport mechanism responsible for K^+ accumulation. The effect on water transport is really quite marked and greater than with some fifty other inhibitors used. Mudge suggested that malonate acts by depression of aerobic metabolism in general and not necessarily by a specific inhibition of succinate oxidase, which at the high concentration used is quite possible.

The results on malonate *in vivo* occasionally do not correspond to the *in vitro* experiments, nor do they always correspond to each other. The injection of 10 millimoles/kg malonate into rats leads to a considerable diuresis which lasts for several days (Angielski *et al.*, 1960 a). On the other hand, infusion of malonate into the renal artery of a dog at a concentration of 8.7 m*M* causes no significant change in creatine, *p*-aminohippurate, or Na^+ clearances, and produces a 15% suppression of urinary volume (Strickler and Kessler, 1963). The effects in intact animals are related to acid-base imbalance in addition to direct renal action, as shown by unpublished experiments by Goldberg (1963) in which rats were water loaded and received 17 millimoles/kg sodium malonate with 17 ml 10% mannitol/kg, this producing certain toxic symptoms (e.g., respiratory difficulty, sluggishness, and mild cyanosis). The results are summarized in the accompanying tabulation and it is clear that a systemic acidosis was produced. A rather

Determination	Control	Malonate
Urinary flow (ml/min)	0.054	0.058
Urinary pH	6.72	5.45
Titratable acidity (meq/liter)	17.0	46.7
Creatinine clearance (ml/min)	1.1	0.89
Na^+ excretion (meq/liter)	9.5	110
K^+ excretion (meq/liter)	10.3	57.5
NH_4^+ excretion (meq/liter)	25.7	16.3
Plasma pH	7.39	7.23
Plasma Na^+ (meq/liter)	150	198
Plasma K^+ (meq/liter)	5.4	9.5

clear increase in phosphate excretion with a fall in plasma phosphate was also observed.

The effects of malonate on the renal excretion of malate are interesting, but it is not known if this phenomenon is related to an action on transport mechanisms or to more general metabolic effects. Vishwakarma (1957, 1962) showed that malonate has no effect on the excretion df malate induced by malate infusion. However, when succinate is infused, the increased malate excretion is due to both an increased filtration and a marked tubular secretion. Malonate inhibits the tubular secretion of malate and converts the excretion to a purely filtration process or causes a net resorption. Vishwakarma and Lotspeich (1960) continued this study in chickens and found that when malonate is infused with succinate, instead of blocking the formation of malate, it further increases the malate excretion. Malonate was infused at a rate of about 6.8 μmoles/kg/min. This could mean that malonate (1) enhances the formation of malate from succinate, (2) facilitates the tubular secretion of malate, or (3) gives rise to malate by metabolic conversion. Since malonate infused alone did not significantly increase malate excretion, the last explanation is unlikely. In the dog, malonate inhibits the tubular secretion rather than stimulating it and does not inhibit the resorption of malate. The mechanism for this paradoxical effect is unknown. Reference may be made to studies of Lotspeich and Woronkow (1964), who unilaterally perfused chicken kidneys and found complex effects on the excretions of various organic acids, and concluded that the cycle must be involved in some manner.

Transintestinal Transport

Quastel has studied the effects of various inhibitors on the transfer of glucose across the guinea pig intestinal wall. This is an active transport and depends strongly on the aerobic metabolism (as shown by the marked inhibition by cyanide and azide) and the associated phosphorylations (as shown by the 2,4-dinitrophenol inhibition). When malonate at 20 mM is present in the lumen, there is 18.5% inhibition of the glucose transported, but if malonate is present both inside and outside, the inhibition is 44.3% (Darlington and Quastel, 1953). An increase of K^+ from 6 to 15.6 mM accelerates glucose transport about 50%. Malonate inhibits the K^+-stimulated transport completely at concentrations as low as 2 mM (Riklis and Quastel, 1958). This result may be related to the greater sensitivity of K^+-stimulated brain slices to malonate. It was claimed that 20 mM malonate depresses both the accumulation of L-monoiodotyrosine-I^{131} in the intestinal cells and its transport across the intestine, but no data were given (Nathans *et al.*, 1960). There is a marked difference between transintestinal transport and tissue accumulation of triiodothyroacetate, the former being inhibited much

more readily by a number of substances; malonate at 10 mM inhibits uptake 16% and transport 70% (Herz *et al.*, 1961).

Everted segments of the rat duodenum transport iron from the mucosal to the serosal surface against concentration gradients and this process is dependent on oxidative phosphorylations (Dowdle *et al.*, 1960). Malonate at 50 mM reduces the inside/outside ratio of Fe^{++} from 4.0 to 0.6. Ca^{++} is also transported actively and malonate at 20 mM reduces the inside/outside ratio of Ca^{45} from 5.0 to 2.5 (Schachter and Rosen, 1959). Ca^{++} is also accumulated by the intestinal cells, the tissue/medium ratio being 5.8, which is decreased by 20 mM malonate to 3.4 (Schachter *et al.*, 1960). The question of the chelation of Ca^{++} and Fe^{+++} by the malonate arises, since the high malonate concentrations would certainly reduce the free ions appreciably. This must play some role but in the case of Ca^{++} cannot explain the reduction in the transport, inasmuch as the inside/outside ratio is increased as the Ca^{++} concentration is lowered.

Gastric Acid Secretion

The secretion of hydrochloric acid by the parietal cells is dependent on oxidations and the formation of ATP, since it is strongly inhibited by cyanide, antimycin, and 2,4-dinitrophenol. However, the secretion in isolated rat stomachs is not affected by 10 mM malonate (Patterson and Stetten, 1949). Injection of malonate in mice subcutaneously inhibits the accumulation of *p*-aminohippurate in the kidney but does not inhibit acid secretion: 4.8 millimoles of malonate reduces the kidney/medium *p*-aminohippurate ratio from 6.1 to 2.9 but inhibits the secretion of hydrochloric acid only 6% (Davenport and Chavré, 1956). This is near the fatal dose of malonate and many of the mice did not live long enough to perform the test. Inasmuch as succinate oxidase activity is high in the stomach and is readily inhibited by malonate, and since fluoroacetate inhibits acid secretion, the most likely explanation for the lack of a malonate effect is a failure to penetrate into the parietal cells sufficiently. Some evidence for the participation of succinate oxidase in acid secretion was obtained by Vitale *et al.* (1956), who showed that stimulation of guinea pig or human gastric mucosa with histamine leads to significant increases in the succinate oxidase activity, although histamine has no such effect in liver or duodenum. Furthermore, succinate oxidase is concentrated in those regions of the stomach where the parietal cells are abundant.

Active Transport of Ions in Various Cells and Tissues

The effects of malonate on nerve and muscle, to be discussed in the following sections, depend in part on the modification of the active transport of ions in these tissues. Malonate depresses many types of active transport,

as we have seen for kidney and gastric mucosa, and the mechanism may be either a simple reduction in the energy supply or a more direct interference with electron transport associated with ionic movements across the membrane. One must also attempt to distinguish between effects on the active transport and increases in permeability. If the permeability to an ion is significantly increased, its intracellular accumulation may drop because the ion pump is no longer able to maintain the normal concentration; such an action would appear superficially to be an inhibition of active transport.

Malonate up to 10 m*M* has no effect on the transport of Na^+ and K^+ across the human erythrocyte membrane (Maizels, 1951), which is not surprising since the principal energy source in such cells is glycolytic. In ascites tumor cells, substrates (for example, glucose and succinate) increase the efflux of Na^+. Cyanide at a concentration inhibiting respiration 70% has no effect on either the influx or efflux of Na^+, presumably because the rate of aerobic glycolysis is simultaneously doubled, this compensating for the oxidative depression (Maizels *et al.*, 1958). Malonate at 12.5 m*M*, on the other hand, inhibits respiration 35% but produces only a 5% increase in the glycolysis, which may explain why the rate coefficient for Na^+ efflux drops from 6.5 to 5.1 hr^{-1}. The accumulation of intramitochondrial K^+ in preparations from rabbit heart is dependent on oxidative phosphorylation but is unaffected by 0.2 m*M* malonate (Ulrich, 1960). Inasmuch as α-ketoglutarate was the substrate used, even a complete block of succinate oxidation might not be expected to have much effect on ion movements because sufficient ATP may be generated in the single-step oxidation of α-ketoglutarate. Ca^{++} uptake and binding by kidney mitochondria depend on an oxidizable substrate and ATP; it is depressed 75% by 10 m*M* malonate, which suggests interference with the operation of the cycle, but could relate to the chelation of the Ca^{++} by the malonate (Vasington and Murphy, 1962). The uptake of iodide is inhibited by rather high concentrations of malonate in the brown alga *Ascophyllum nodosum* (79% inhibition at 25 m*M*) (Kelly, 1953), the rabbit ciliary body (50% inhibition at 50 m*M*) (Becker, 1961), and the rabbit choroid plexus (50% inhibition at 20 m*M*) (Welch, 1962), but 1 m*M* malonate has no effect on the uptake or incorporation of iodide in sheep thyroid particulate fractions (Tong *et al.*, 1957).

The accumulation of P_i^{32} in the roots of the loblolly pine *Pinus taeda* during a 3 hr incubation is inhibited 5% at pH 4.75 but stimulated 54% at pH 5.75 (Kramer, 1951). Similar results are obtained in mycorrhizal root tips but the inhibition is somewhat greater. It is possible that at the concentration (25 m*M*) of malonate, the stimulation is an ionic effect which is partially counteracted by a malonate inhibition at the lower pH. The uptake of K^+ and Br^- by barley roots is quite strongly inhibited by malonate at pH 4.5 (see accompanying tabulation) (Ordin and Jacobson, 1955). The inhibition is overcome to some extent by malate and fumarate; suc-

cinate, however, actually increases the inhibition. It is likely in this tissue that ion accumulation is obligatorily coupled to the operation of the cycle.

Malonate (m*M*)	% Inhibition of:		
	K^+ uptake	Br^- uptake	Respiration
5	39	42	30
10	92	70	55

Effect of Malonate on Mitochondrial Swelling

Rat liver mitochondria swell quite readily, as measured by changes in light scattering or optical density, when treated with various substances, and the effects of malonate on this phenomenon are interesting. Raaflaub (1953) established that succinate and phosphate promote swelling. This swelling is counteracted by ATP in both cases, but malonate prevents the swelling from succinate only. This was confirmed by Tapley (1956), who extended the list of substances causing swelling to fumarate, malate, glutamate, acetate, and α-ketoglutarate. Swelling is prevented by citrate, pyruvate, and oxalacetate, as well as malonate. Since malonate can prevent the swelling from substrates other than succinate, there is some question as to the specificity of the effect. It was claimed that the same results are obtained in the absence of oxygen and thus that the swelling is not related to the utilization of these substrates. Quite different conclusions were reached by Chappell and Greville (1958) inasmuch as they found a good correlation between swelling and utilizable substrates. Malonate prevents the swelling from succinate but not from α-hydroxybutyrate, and, in general, inhibitors blocking oxidations reduced swelling. Matters were further complicated by the results of Keller and Lotspeich (1959 b). They found that phlorizin caused swelling of kidney mitochondria and that this could be counteracted by Mg^{++}, ATP, 2,4-dinitrophenol, and malonate. Hunter *et al.*, (1959 a, b) considered the possibility that swelling is related to the fraction of NAD in the oxidized form, since amobarbital prevents oxidation of NADH and prevents swelling. However, succinate in the presence of amobarbital causes swelling and this is blocked by malonate. Glutamate-induced swelling is not prevented by malonate. It was concluded that swelling depends on electron flow between the substrate and oxygen, and whether or not an inhibitor will prevent swelling is determined by where in the electron transport chain the substrate and the inhibitor act. This does not very well explain the prevention of swelling by 2,4-dinitrophenol and it was suggested that there are at least two different types of mitochondrial swelling. Further confusion was introduced by Sabato and Fonnesu (1959), who found that swelling is

prevented by oxidizable substrates such as succinate, glutamate, and α-ketoglutarate, and that malonate counteracts this preventive action. These results were confirmed by Kaufman and Kaplan (1960), who again observed, in contrast to the earlier workers, that succinate inhibits swelling and malonate reverses this protection. They believe that swelling is correlated with the mitochondrial release of pyridine nucleotides (see tabulation).

	Pyridine nucleotide released (μg/20 min)	Optical density
No substrate	64	−0.710
Succinate (20 m*M*)	14	−0.095
Malonate (20 m*M*)	68	−0.740
Succinate + malonate	38	−0.520

Succinate reduces the loss of the pyridine nucleotides and malonate antagonizes this effect. One must conclude that there must be different mechanisms of swelling and that the mitochondrial behavior perhaps depends on the metabolic state and the nature of the suspension medium. The effects of malonate and other anions on the concentrations of free Ca^{++} and Mg^{++} should also not be ignored, inasmuch as EDTA has usually been shown to modify the swelling.

Conduction and Membrane Potentials of Nerve

Penetration of malonate into nerve axons in the physiological pH range must be very poor. This may account for the failures of Shanes and Brown (1942) to observe an effect of 20 m*M* malonate on the resting potential of frog nerve, and of Greengard and Straub (1962) to find an effect of 10 m*M* malonate on nonmyelinated nerve posttetanic hyperpolarization, despite the fact that this phenomenon is quite sensitive to other inhibitors. However, Jenerick (1957) reported some effect of 10 m*M* malonate on frog sciatic nerve although the action was presumably slow in developing. When the action potential spike amplitude is reduced by 80–90%, the threshold for stimulation begins to rise rapidly. Conduction block occurs when the resting potential has fallen by 30–40%. It is doubtful if the decrease in external Ca^{++} concentration, which was 1.3 m*M* initially, resulting from chelation by malonate could be held responsible for these effects, and it was felt that metabolic interference must occur. The preganglionic and postganglionic action potentials in preparations of cat sympathetic ganglia are depressed equally (75–80%) by 14 m*M* malonate and transmission through the ganglia is reduced (Larrabee and Bronk, 1952). The excitability of the

isolated cat carotid body is lowered by perfusion with malonate (Anichkov, 1953). These meager data are all we have to understand the actions of malonate on nerve function. Unfortunately, little has been done on junctional transmission, inasmuch as it might be predicted that the synapses would be more sensitive to malonate than are the axons, because of both a higher permeability of such regions to anions and a greater energy requirement for the synthesis of acetylcholine.

Skeletal and Smooth Muscle Function

Essentially nothing is known of the effects of malonate on skeletal muscle. Beckmann (1934) claimed that 6.7 m*M* malonate causes a swelling of muscle, indicating an alteration of permeability. This was termed a membrane-loosening effect. In the initial work of Ling and Gerard (1949) with intracellular microelectrodes, it was observed that 10 m*M* malonate drops the resting potential of frog sartorius muscle from 78 mv to 65.3 mv over a period of 3 hr. This may be correlated with the suppression of Na^+ extrusion observed by Kernan (1963) in the same muscle, 30% inhibition being produced by 1 m*M* malonate over 2 hr, an effect similar to that occurring in brain slices (Bilodeau and Elliott, 1963). No direct work on the contractile response to malonate has been done.

The contractions of isolated rabbit intestine are not inhibited by 10 m*M* malonate, whether in the absence of substrate or in the presence of either acetate or glucose (Weeks and Chenoweth, 1950; Weeks *et al.*, 1950). Indeed, there is a tendency for malonate to increase the contractile activity slightly, especially with glucose as the substrate. There is also no interference with the recovery of substrate-depleted strips produced by the addition of acetate or pyruvate. It was suggested that a lack of penetration of malonate into the smooth muscle cells might be responsible. Fluoroacetate is quite inhibitory under the same conditions so that some relationship of the contractility to the cycle is likely. The contractile properties of the vascular smooth muscle in the cat hind limb are not affected by 1 m*M* malonate (Hitchcock, 1946), and the behavior of electrically stimulated pig carotid artery is not altered by 10 m*M* malonate (Jacobs, 1950).

It would be important to know more about the possible effects of malonate on the formation and release of the neurohormones, such es acetylcholine and the catecholamines, but the data are not available. It is interesting to note, however, that malonate is reasonably effective in inhibiting the release of histamine from guinea pig lung slices during an anaphylactic reaction (Moussatché and Prouvost-Danon, 1958). The inhibition is 10% at 20 m*M*, 40% at 40 m*M*, and 50% at 60 m*M*. The inhibition was attributed to the effect on succinate dehydrogenase. Nevertheless, malonate at 40–60 m*M* has virtually no effect on the release of histamine brought about by the application of the histamine-releaser Compound 48/80 (Mous-

satché and Prouvost-Danon, 1957), although respiration of the lung slices is depressed fairly strongly. It would appear that malonate interferes with the anaphylactic release of histamine by a mechanism other than a direct effect on the formation or release of histamine. It may be noted that succinate accelerates the oxygen uptake but has no effect on the release of histamine.

Cardiac Membrane Potentials and Function

The physiological disturbances produced by malonate have been most thoroughly studied in the heart. Although the effects are often very slight, despite the evident importance of the cycle in the myocardium, under certain conditions the responses to malonate are very interesting. The earliest investigation was made by Forssman and Lindsten (1946) at Lund, who noted a marked discrepancy between the effects of malonate in the whole animal and on isolated hearts. Intravenous injections of malonate at doses around 3.7–7.5 millimoles/kg to cats and rabbits lead to an increase in the venous blood pressure and a fall in the arterial blood pressure, indicating cardiac depression. In rabbits the cardiac failure begins about 20 min after the injection whereas in cats the changes are immediate. In rabbits the heart may stop after 40 min but in cats recovery is the rule. At autopsy the heart is found to be dilated. The effects of malonate on the isolated perfused rabbit heart, however, are rather small and inconsistent (see accompanying tabulation). Moreover, succinate at the same concentrations

Malonate (m*M*)	% Change of		
	Amplitude	Coronary flow	Rate
10	−14	−23	− 5
20	−19	−16	− 8
40	−34	−29	0

acts similarly. These are the immediate effects of malonate and it is possible that the heart would recover from this depression after several minutes, as do rabbit atria (Webb, 1950). It is doubtful if these effects are related to inhibition of succinate oxidase; they are more likely ionic actions on the membrane. The reduction in the coronary flow may result from a vascular constriction, but is more probably the response to the decreased functional activity.

Isolated rabbit atria are depressed only by high concentrations of malonate, 30–40 m*M* producing a 30% decrease in contractile amplitude and rate at 2 min; the depression is temporary, complete recovery being observed after 8–10 min (Webb, 1950 b). Atria can continue to beat normally

for hours in 50 m*M* malonate. The temporary depression brought about by malonate is not counteracted by fumarate added either before, with, or after the malonate. In fact, fumarate, along with pyruvate, acetate, succinate, and malate, has an action very similar to that of malonate on the atria. It is not known if this inhibition and recovery are related to the somewhat slower but similar time course of ventricular respiration under the influence of malonate (page 181) (Webb *et al.*, 1949). The depression is not due to chelation of Ca^{++} or Mg^{++} since reduction in the concentrations of these ions produces a different response. Gardner and Farah (1954) confirmed the resistance of rabbit atria to malonate, finding that 10–20 m*M* has no significant effects on contractility, spontaneous rate, excitability threshold, refractory period, and conduction rate.

The effects of malonate were investigated more thoroughly on rat atria (Webb and Hollander, 1959). The contractility is depressed 21% immediately but slow recovery occurs: the inhibition is 13% during 5–25 min, 9% during 25–45 min, and 5% during 45–60 min. The malonate concentration used was 15 m*M*. The addition of 15 m*M* NaCl produces a rapid contractile depression about half as great as from malonate, so that at least part of the initial malonate effect is attributable to the Na^+ ion. A slight initial rise in the magnitude of the action potential is observed with both malonate and NaCl, but in the case of malonate this is soon replaced by a small depression. There is also some shortening of the action potential and a decrease in its area after the first 5 min, which could be responsible for the fall in tension. In summary, the addition of 15 m*M* malonate produces a rapid initial effect attributable mainly to the Na^+ and this is progressively replaced by changes due to the malonate, these latter changes being moderate depressions of the action potential and contractility. The importance of the cycle in the atrial function is indicated by the marked changes brought about by fluoroacetate, and thus the resistance to malonate is probably due to a low intracellular concentration of malonate. Greater effects on the contractility of rat atrium were observed by Venturi and Schoepke (1960), 5 m*M* depressing 22%, 10 m*M* 44%, and 20 m*M* 90%. It was stated that NaCl at these concentrations does not alter the contractility. Furthermore, succinate is as inhibitory as malonate. The greater depression observed here compared to my work is difficult to explain. Venturi and Schoepke used Locke solution at pH 7 whereas I used Krebs-Ringer-bicarbonate medium at pH 7.4. Part of the larger inhibition seen by Venturi and Schoepke thus might be due to the lower pH. In any event, these effects seem to be unrelated to the inhibition of succinate oxidase and again must be attributed to some action directly on the membrane. Venturi and Schoepke found that increasing Ca^{++} concentration can completely overcome the depressant actions of malonate, succinate, and the other organic anions used, leading them to suggest that the negative inotropic action is due to the chelation

of Ca^{++}. However, there are some reservations in accepting this explanation completely. In the first place, Ca^{++} stimulates atrial contractility and would be expected to counteract most depressants in a nonspecific manner. In the second place, reducing the Ca^{++} from 1.22 m*M* to 0.91 m*M* does not alter the contractility, although further reduction to 0.61 m*M* depresses 43%. Malonate at 15 m*M* would reduce the Ca^{++} from 1.22 m*M* to 0.82 m*M* and a small contractile depression may result from this. The total Ca^{++} in Locke solution is 2.16 m*M* and 20 m*M* malonate would reduce the free Ca^{++} to 1.31 m*M*, which alone could not produce the 90% depression seen by Venturi and Schoepke. In the third place, monocarboxylate ions, such as acetate, lactate, and pyruvate, at 20 m*M* depress the contractile amplitude 40–45% and these do not deplete the Ca^{++}.

The modifications in the electrocardiogram following intravenous injections of malonate into turtles were studied by Lenzi and Caniggia (1953). At a dose of 4.4 millimoles/kg malonate the following occurred: bradycardia, slowing of the a-v conduction, a tendency for the shortening of repolarization and electric systoles, with eventually a total a-v block and a prolongation of the depolarization time (see accompanying tabulation). Pacemaker and conduction depression are thus evident, and it is

	Control	Malonate at 30–35 min	Control	Malonate at 57–65 min
Rate	50	23	78	32
pq interval	0.30	0.465	0.24	block
qrs interval	0.15	0.18	0.10	0.18
st-t interval	0.57	0.895	0.44	1.06
qt interval	0.72	1.075	0.54	1.24

quite possible that similar changes would be observed in mammals, considering the general behavior of the heart in cats and rabbits treated with malonate (Forssman and Lindsten, 1946). The electrocardiogram from the embryonic chick heart is not altered by 4 m*M* malonate (Boucek and Paff, 1961).

In contrast to the depressant effects of malonate on the whole heart and isolated atria, the rat ventricle strip is usually strongly stimulated, as first noticed by Masuoka *et al.* (1952). Substrate-depleted and hypodynamic strips recover to almost the initial contractile amplitude upon addition of 10 m*M* malonate at pH 6.2 (which was used to facilitate penetration of the malonate), and simultaneously the stimulatory action of succinate is blocked. The ability of glucose to induce recovery is augmented by malonate, and that of pyruvate is slightly reduced (Berman and Saunders, 1955). This interesting positive inotropic action was analyzed in detail because it was

felt that such an action might have bearing on the mechanisms whereby the cardioactive glycosides stimulate the failing heart. Only the major results will be summarized here. The positive inotropic action occurs most strongly when glucose is present, less in substrate-free medium, and not at all with pyruvate or α-hydroxybutyrate as substrate (Covin and Berman, 1956). These results suggested that malonate might stimulate the Embden-Meyerhof glycolytic pathway, resulting in an accelerated conversion of glucose and glycogen to pyruvate. If this were so, pyruvate should produce a comparable positive inotropic effect and it does in both substrate-depleted and glucose-supplemented strips. Furthermore, iodoacetate at 0.2 m*M* blocks the stimulation by malonate, whereas it does not affect the response to pyruvate. The chelation of Ca^{++} was shown to contribute to the depression produced by malonate at high concentrations (20–50 m*M*), and it probably reduces the amount of stimulation seen at the lower concentrations since lowering the Ca^{++} to the degree calculated to occur in 10 m*M* malonate depresses the contractile activity 23%. The effects of malonate on the oxidation of C^{14}-labeled substrates by ventricle strips were then studied in chambers in which the respiration and contractile activity could be determined simultaneously (Rice and Berman, 1961). Malonate at 5.6 m*M* under conditions in which a positive inotropic effect is observed has very little effect on the utilization of glucose-1-C^{14}, glucose-6-C^{14}, and pyruvate-2-C^{14}, slight inhibition of glucose oxidation being noted although this is possibly not significant. These results indicate that the stimulatory action is not related to (1) acceleration of glucose metabolism, (2) inhibition of the cycle, or (3) stimulation of the pentose-phosphate pathway. It was found that $C^{14}O_2$ is produced from malonate-2-C^{14} in ventricle strips, and possibly part of the positive inotropic action in substrate-depleted strips is related to the oxidation of malonate via formation of acetyl-CoA and its incorporation into the cycle. However, the explanation for the greater effect of malonate in the presence of glucose and the inhibition of its action by iodoacetate is not immediately evident. It may be noted that other metabolic inhibitors, such as fluoride, arsenate, fluoroacetate, and dehydroacetate, can exert positive inotropic actions under the appropriate conditions, so this paradoxical effect of malonate is not unique.

Wenzel and Siegel (1962) determined the dose-response curves for the positive inotropic effects of malonate and ouabain on the rat ventricle strip, and then constructed an isobologram, plotting the malonate concentration against the ouabain concentration for a chosen contractile stimulation. Since the isobol sags, i.e., is concave upwards, they claimed it is clear that potentiation occurs and that this indicates the sites of action of malonate and ouabain are different. There is some doubt that a moderately sagging isobol can be interpreted as potentiation, inasmuch as pure summation often elicits such a curve (see Figs. I-10-7, 8).

The response of the heart to neurohormones is not altered by malonate. The positive chronotropic effect of epinephrine on the frog heart is not changed by 0.1 m*M* malonate (Nickerson and Nomaguchi, 1950), which is not surprising considering the concentration. A brief report by Ellis and Anderson (1951 a) stated that malonate does not affect the stimulation by epinephrine except after prolonged treatment when the frog heart is depressed. The malonate concentration was not given. It would seem likely that any severe metabolic disturbance producing marked cardiac depression would prevent, or at least reduce, any type of stimulation, since the additional functional activity would demand more energy, so that an antagonism of epinephrine by malonate is not of much significance unless it occurs when the heart is not too much depressed. Malonate has no demonstrable effect on the response of the heart to acetylcholine, with respect to reduction of either rate or contractility (Webb, 1950 b), whereas fluoroacetate alters the response markedly, indicating again that the cycle is of importance in these myocardial functions but that malonate does not reach sufficiently high intracellular concentrations.

EFFECTS OF MALONATE IN THE WHOLE ANIMAL

A summary of the miscellaneous results relating to toxic and lethal effects of malonate is given in Table 1-28. One may conclude that in mammals injected doses of 1.5–2.5 g/kg (10–17 millimoles/kg) of sodium malonate are generally lethal. Such doses, especially when given intravenously, probably produce plasma levels in excess of 10 m*M* malonate at peak concentrations. A dose of 12 millimoles/kg subcutaneously in rats leads to a plasma concentration of 4.5 m*M* at 30 min, and another similar dose raises the plasma level to around 8 m*M* (Busch and Potter, 1952 a). Intravenous injection would give higher peak levels. When compared in the same experiments, the acid is more toxic than the sodium salt. It is difficult to know if this is due to a nonspecific acid effect or to better penetration into the tissues. It would appear that malonate is more toxic to mice at 38° than at 30° environmental temperature (Gruber *et al.*, 1949).

The sequence of symptoms resulting from the injection of malonate into rats and mice was described by Gruber *et al.* (1949) as: champing, air hunger, maintenance of the head in a dorsally flexed position, and clonic convulsions. Busch and Potter (1952 a) found dyspnea and convulsions in rats following injection of toxic doses. The cause of death has been attributed to various actions. Forssman (1941) and Forsmann and Lindsten (1946) believed that death is due to cardiac failure (the cardiovascular effects observed were discussed in the previous section). Handler (1945) also favored a cardiac mechanism for death and found the succinate oxidase to be inhibited 50–75% in homogenates prepared from poisoned animals. He also noted that

TABLE 1-28

TOXIC AND LETHAL DOSES OF MALONATE

Species	Route	Form	Dose (g/kg)	Dose (millimoles/kg)	Effect	Reference
Frog	Lymph sac	Acid	0.9 [a]	8.6	Lethal dose	Heymans (1889)
		Na salt	8.4 [b]	57	Not lethal	Heymans (1889)
	SC	Na salt	0.71 [b]	4.8	Toxic but not lethal	Davenport and Chavré (1956)
Mouse	Oral	Na salt	3.3 [c]	22	LD_{50}	Boyland (1940)
	Oral	Acid	1.2 [d]	—	No effect on growth	Freedlander *et al.* (1956)
	SC	Na salt	17.5 [c]	119	Many died within 2 hr	Davenport and Chavré (1956)
	IP	Acid	1.55	14.9	LD_{50}	Gruber *et al.* (1949)
	IV	Na salt	2.10	14.2	LD_{50}	Gruber *et al.* (1949)
Rat	IP	Na salt	2.50	16.9	LD_{50}	Gruber *et al.* (1949)
		Acid	1.54	10.4	LD_{50}	Gruber *et al.* (1949)
	IP	Na salt	0.80 [e]	5.4	Toxic but not lethal	Mookerjea and Sadhu (1955)
	SC	Na salt	1.78	12.0	Toxic and often lethal	Busch and Potter (1952 a)
	SC	Na salt	0.80	5.4	Toxic	Tinacci (1953)
Rabbit	SC	Na salt	1.60	10.8	Lethal in 3–5 hr	Handler (1945)
	IV	Na salt	0.50	3.4	Nontoxic	Forssman (1941)
			1.22	8.2	Lethal in 35 min	Forssman (1941)
	IV	Na salt	0.55	3.7	Smallest lethal dose	Forssman and Lindsten (1946)
	IV	Acid	0.66	6.3	LD_{50}	Gruber *et al.* (1949)
	IV	Na salt	0.685	4.6	Toxic	Wick *et al.* (1956)

[a] Dose calculated on the basis of 25-g frog weight as given by Heymans. [b] Dose per frog inasmuch as weights are not given. [c] Dose calculated on the basis of 30-g mouse weight. [d] Administered at 1.2% in the diet. [e] Daily dose for 3–4 weeks.

the heart is dilated at death and that there is an accumulation of ascitic and pleural fluid. Forssman and Lindsten, from their failure to obtain appreciable direct action on the isolated heart, suggested an indirect mechanism, possibly mediated through the effect of malonate on liver metabolism. The dyspnea and various central nervous system effects may result from a direct action of malonate but could also arise from the ionic and acid-base imbalances produced. Handler (1945) showed that malonate causes a marked fall in CO_2 capacity in the blood from 53 to 6 vol%. Although no study of the alterations in the plasma electrolytes undoubtedly produced by malonate has been made, Wick *et al.* (1956) noted that the ability of the blood to coagulate is reduced, and attributed many of the actions of malonate to the chelation of divalent cations. Other changes in the blood composition may also be responsible for some of the toxic effects. Handler (1945) reported that 1.6 g/kg malonate given subcutaneously to rabbits increases the blood glucose 368%, blood lactate 545%, blood pyruvate 163%, serum inorganic phosphate 262%, and serum organic phosphates 155%. (The ketonemia produced by malonate was discussed previously). There is thus much opportunity for secondary mechanisms to play a role in the toxicity of malonate. At the present state of our knowledge it is even difficult to evaluate the importance of succinate oxidase inhibition in these effects.

The kidneys have the highest concentration of malonate after administration and therefore the renal effects and nephrotoxicity have been investigated. Early arguments about the renal toxicity of glutarate were published between 1907 and 1912. Rose (1924) reinvestigated this and found that glutarate is a nephrotoxic substance in rabbits, as indicated by the increases in nonprotein nitrogen, urea, and creatinine, and the almost complete disappearance of renal function as measured by the phenolsulfonphthalein test. A single experiment with malonate was reported. No renal damage was observed after 2 g given on successive days and 3 g 2 days later, nor was there a change in the rate of dye excretion. Corley and Rose (1929) reported that methylmalonate and ethylmalonate are slightly toxic to the kidneys at doses of about 1 g/kg in rabbits, there being a definite increase in the nonprotein nitrogen and some reduction in dye secretion, although both effects are transitory. Extensive renal damage was observed by Becker and Rieken (1954) following the intraarterial injection of 20 mg potassium malonate (Fig. 1-19 a). The vessel walls become edematous, potocytosis is evident, and many perinuclear vacuoles appear in the loops of Henle. However, it requires much higher doses to depress the respiration of kidney slices prepared from injected animals, 80 mg potassium malonate giving no effect and 150 mg inhibiting 30.5%. Similar histological changes occur after incubating kidney tissue *in vitro* with 110 m*M* potassium malonate for 20 min (Fig. 1-19 b), vacuolization being intense. Tinacci (1953) found not only kidney damage but widespread degenerative changes 2–8 days after sub-

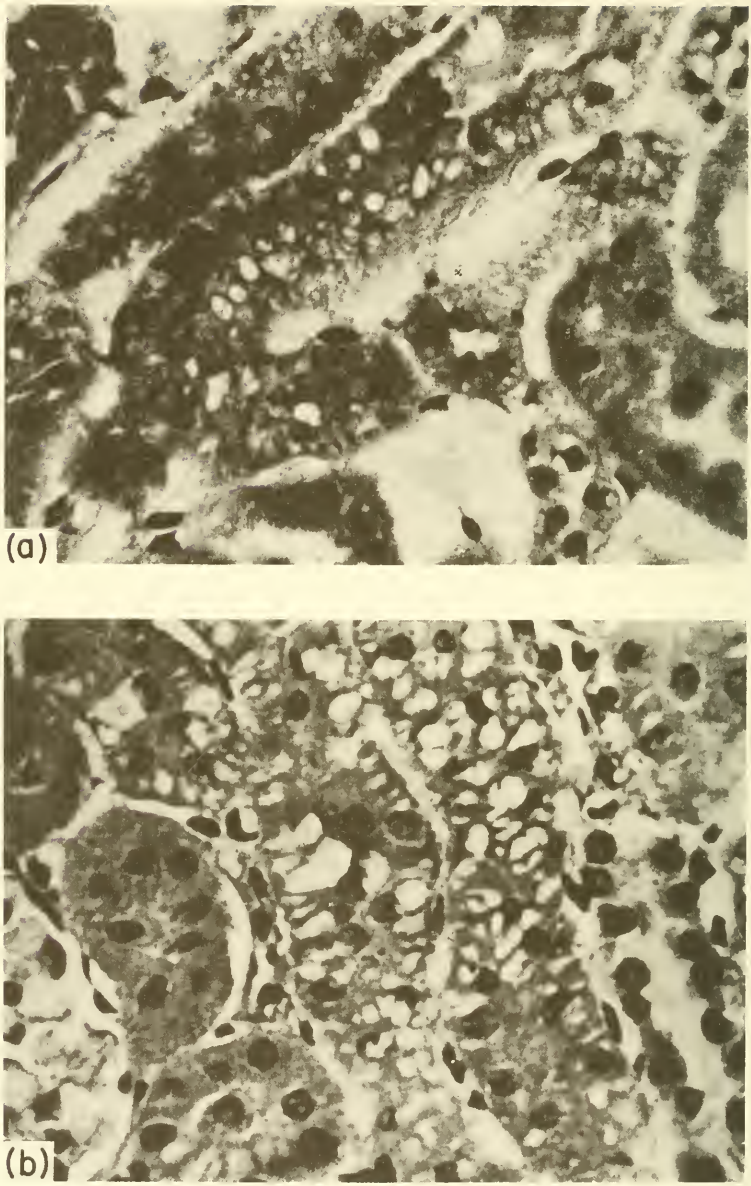


FIG. 1-19. Renal damage resulting from (a) intraarterial injection of 20 mg potassium malonate into rabbits, and (b) incubation of slices with 110 m*M* malonate for 20 min. (From Becker and Rieken, 1954.)

cutaneous injections of 0.4–0.8 g/kg of sodium malonate, almost all organs being involved. Malonate is diuretic when subcutaneously injected into hydrated rats at a dose of 11.5 millimoles/kg, this dose being sufficient to inhibit kidney succinate oxidase (Fawaz and Fawaz, 1954). However, the effect is probably osmotic rather than due to enzyme inhibition by the malonate, since KNO_3 at the same dosage induces the diuresis, and also because diethylmalonate, which inhibits kidney succinate oxidase, has no diuretic activity. Summarizing these results, one may conclude that renal damage may occur from high doses of malonate, but that minimal changes in the kidney occur after the usual toxic doses. It seems unlikely that the renal action is of major importance in poisoning or death from malonate.

EFFECTS OF MALONATE ON BACTERIAL INFECTIONS

The influence of enzyme inhibitors on the course of bacterial infections is of great interest because the results have bearing on the fundamental question of the metabolic basis of the resistance to infection. The effects of malonate on *Salmonella typhimurium* infection in mice have been studied by Berry and co-workers at Bryn Mawr in a series of excellent investigations. Mice injected intraperitoneally with a *Salmonella* suspension show evidence of the infection on the third day and most succumb by the sixth day. If mice are given intraperitoneal injections of 20 mg sodium malonate in saline every hour for 8 hr, they die much more rapidly from the infection than animals given only saline injections (Berry and Mitchell, 1953 a). The striking effects is illustrated in Fig. 1-20. Noninfected mice show no effects of the malonate. Thus sublethal doses of malonate are able either to accelerate bacterial proliferation or to decrease the resistance of the host markedly. These are the basic observations and the later work attempts to elucidate the mechanisms by which these effects are brought about.

The reduction of the survival time in mice infected with *Salmonella* is not unique. Similar effects of malonate on infections with *Proteus morganii*, *Staphylococcus aureus*, *Streptococcus pyogenes* (Berry and Mitchell, 1954), *Diplococcus pneumoniae*, and *Klebsiella pneumoniae* (Berry *et al.*, 1954 a) have been observed. Furthermore, reduced survival times have been found in *Salmonella* infected rats, guinea pigs, and chickens (Berry and Beuzeville, 1960). Finally, the phenomenon has been seen with other inhibitors, such as fluoroacetate and arsenite (Berry *et al.*, 1954 a, b). This, then, is a general effect of certain types of inhibitor, especially those affecting the cycle in some manner, and the problem is thus more important because it must relate to some fundamental metabolic relationship between host and bacteria.

We shall now examine in greater detail some of the characteristics of the malonate effect. Malonate not only can reduce survival time, but in some instances can change a nonlethal infection into a lethal one, this being

observed with *Diplococcus*, *Staphylococcus*, and *Proteus*. That is, an infection which does not kill plus a dose of malonate which is non-toxic may cause the death of all the animals within several hours (Berry *et al.*, 1954 a). This is a true case of synergism. It has been found that *Salmonella* bacteremia in mice is much greater in malonate-treated animals than in the controls (Berry and Mitchell, 1953 b, 1954). The bacteremia in the controls reaches a low peak value soon after inoculation and then falls off, whereas in the presence of malonate it continues to progress. At 9 hr, the blood of

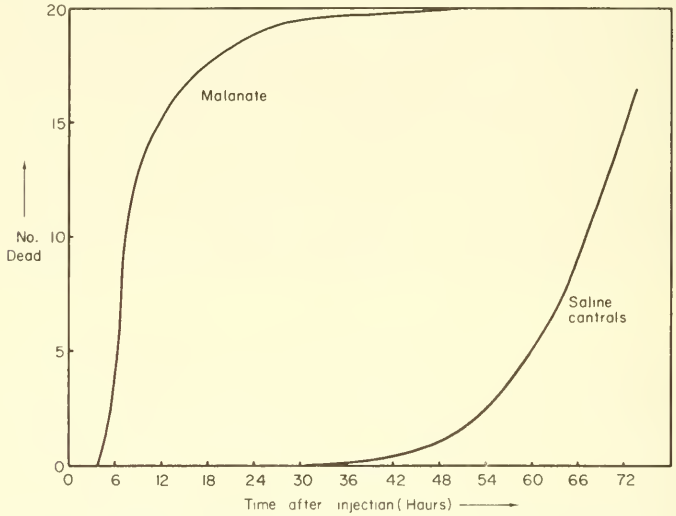


FIG. 1-20 Effect of malonate given in 8 hourly injections of 20 mg sodium malonate on the mortality of mice intraperitoneally injected with suspensions of *Salmonella typhimurium*. (From Berry and Mitchell, 1953 a.)

the controls contains 10,000 to 20,000 bacteria/ml whereas the blood of malonate-treated animals has a count of around 3,000,000 bacteria/ml. Thus the bacteremia is over 100-fold as severe in the treated animals as in the controls. This bacteremia is a reflection of the situation throughout the body. The total number of bacteria in the body 6.5 hr after the injections is 1.65×10^6 in the controls and 32.1×10^6 in the malonate-treated mice (Berry, 1955). The ratio of counts in the treated and control series is 20 for the whole body and 19 for the blood at this time. Now, the interesting thing is that, at the time of death, the number of bacteria in the body is the same in both the controls and malonate-treated mice. This clearly shows that malonate does not alter the susceptibility of the mice to the bacteria, but reduces the time required for the bacteria to multiply to that number necessary to kill.

The rapid proliferation of bacteria could be due to a weakening of the body defenses for disposing of the bacteria. This does not seem to be the case, inasmuch as malonate has no effect on the uptake of thorotrast by the reticuloendothelial system (Berry, 1955) nor does it depress phagocytosis except at very high concentrations (Berry and Derbyshire, 1956). Instead it would appear that malonate disturbs metabolism in such a way that it creates a more favorable environment in the host for bacterial growth. Malonate itself may be metabolized slightly by *Salmonella*, but not to the extent required to explain the explosive proliferation (Berry and Beuzeville, 1960). Growth medium was prepared from the eviscerated carcasses of control and malonate-treated animals and it was found that the bacteria grow more rapidly in the latter (Berry, 1955). It has also been shown that *Salmonella* grows more rapidly in the peritoneal fluid of malonate-treated mice than in the controls (Berry and Beuzeville, 1960). Citrate is known to accumulate following the administration of malonate. This was confirmed in mice given the doses of malonate capable of reducing survival times of infected animals (Berry *et al.*, 1954 b). Both malonate and endotoxin from *Salmonella* increase citrate levels in most tissues, and together the increases are often greater than with either alone (see accompanying tabulation). It

Treatment	Citrate (μg/g) in					
	Blood	Spleen	Kidney	Heart	Duodenum	Liver
None	42	94	76	111	132	109
Malonate	43	245	225	120	115	170
Endotoxin	50	273	190	187	170	443
Malonate + endotoxin	70	173	173	407	543	240

is thus possible that a summation of effects on the cycle could be partially responsible for the increased mortality. However, *Salmonella* infection does not increase citrate levels (Berry and Beuzeville, 1960). Could the increased citrate be favorable to the growth of the bacteria? It is unlikely that this is a major factor because malonate is the most potent inhibitor for reducing survival times and yet both arsenite and fluoroacetate cause greater accumulations of citrate. The primary cause of the augmented bacterial proliferation has not been found but the range of possible mechanisms has been narrowed. Since there are many other possible substrates for *Salmonella* that accumulate during malonate inhibition, it will be necessary to examine these in mice and their effects on the growth of *Salmonella*.

Some work on this problem in other laboratories may be briefly mentioned. Malonate reduces the antibacterial activity of guinea pig blood toward

Salmonella enteritidis but this is not due to a decrease in the number of leucocytes (Yamauchi, 1956). The survival times of chicks infected with *Salmonella pullorum* are reduced by 500–800 mg/kg of malonate injected simultaneously or shortly after the bacterial inoculation (Gilfillan *et al.*, 1956). On the other hand, the diethyl ester of malonate increases the survival time of mice infected with *Mycobacterium tuberculosis*, when administered daily for 2 weeks at oral doses of 250–500 mg/kg (Davies *et al.*, 1956). Compounds of this type are thus considered worthy of study as chemotherapeutic agents in tuberculous infections.

METABOLISM OF MALONATE

Malonate occurs normally in many types of organism and occasionally at concentrations possibly inhibitory to succinate dehydrogenase. Many organisms are capable of metabolizing malonate by various pathways and some are able to utilize it for growth or cell functions. In some cases, indeed, it is difficult to demonstrate the inhibitory action of malonate in the presence of its own oxidation. The metabolism of malonate must always be considered in studies of the effects of malonate on any type of cellular activity. It is often impossible to detect and correct for the metabolism of malonate without using labeled malonate. It is possible that many studies of the inhibition of respiration or cycle activity by malonate have been complicated by the oxidation of the malonate.

Occurrence of Malonate

Malonate has been isolated or demonstrated chromatographically from a number of microorganisms, plants, and animals, and it is likely, considering the recent demonstration of its role and the role of malonate derivatives in fatty acid metabolism that its occurrence is widespread. The accompanying incomplete tabulation will serve to illustrate this. Malonate has also been found in winter wheat, barley, oats, alfalfa, kidney bean leaves, clover, pea leaves, vetch leaves (Soldatenkov and Mazurova, 1957), sake (Kawano and Kawabata, 1953), and several products prepared from plants. Although no thorough studies of animal tissues have been made, it is evident that malonate must occur in rat, dog, and human tissues to some extent if it is found in the urine. Although the name of malonate comes ultimately from the Latin *malus*, it has never been identified in apples or other fruit.

Methylmalonate has been found in *Propionibacterium* (Stjernholm and Wood, 1961; Wood and Stjernholm, 1961), pigeon liver (Bressler and Wakil, 1961), pig heart (Flavin *et al.*, 1955), mouse adipose tissue (Feller and Feist, 1957), and rat and human urine (Boyland and Levi, 1936; Barness *et al.*,

Source of malonate	Reference
Achromobacter guttatum	Sguros and Hartsell (1952 a)
Nocardia corallina	Lara (1952)
Penicillium funiculosum	Igarasi (1939)
Aspergillus niger	Challenger *et al.* (1927) Walker *et al.* (1927), Subramanian *et al.* (1929)
Phaseolus vulgaris (bush bean)	Young and Shannon (1959), Rhoads and Wallace (1957), Huffaker and Wallace (1961)
Phaseolus coccineus (runner bean)	Bentley (1952)
Wheat plants	Nelson and Hasselbring (1931)
Bunias orientalis (Cruciferae)	Jermstad and Jensen (1951)
Tobacco leaves	Wada and Kobashi (1953), Bellin and Smeby (1958), Vickery and Palmer (1956 b, 1957), Vickery (1959)
Lucerne (green alfalfa)	Turner and Hartman (1925)
Hevea brasiliensis	Fournier *et al.* (1961)
Helianthus anuus	Bentley (1952)
Umbelliferae (*Anthriscus and Apium*)	Bentley (1952)
Leguminosae (18 species)	Bentley (1952)
Rat urine	Stalder (1958), Thomas and Stalder (1958)
Dog urine	Thomas and Kalbe (1953)
Human urine	Stalder (1958)

1957; Stalder, 1958; Thomas and Stalder, 1958). Ethylmalonate has been found in rat and human urine (Stalder, 1959). Hydroxymalonate (tartronate) occurs in *Acetobacter* (Stafford, 1956) and malonic semialdehyde in *Pseudomonas* (Nakamura and Bernheim, 1961).

The concentrations of malonate in plant tissues are often surprisingly high. The legumes and umbellifers analyzed by Bentley (1952) contain 0.5–2 mg/g fresh tissue. These values correspond to 7–30 mM malonate if distributed uniformly throughout the tissue water. The stems of the runner bean (*Phaseolus coccineus*) contain 2.1 mg/g and the expressed juice of the stem is 30 mM in malonate. Since 20 mM malonate at pH 4.5 inhibits the respiration of these stems 50% and causes accumulation of succinate, one would expect the metabolism in these plants to be constantly suppressed by the malonate, unless the malonate is in some manner segregated from the metabolic systems. Soldatenkov and Mazurova (1957) reported similar

values in legumes (2–3% of the plant dry weight) and that in kidney-bean and clover leaves malonate represents 45% of the total di- and tricarboxylates present. Bush-bean (*Phaseolus vulgaris*) leaves often contain as much as 10 mg/g dried tissue and malonate is more concentrated than fumarate or succinate, although less than malate and citrate (Young and Shannon, 1959). In man malonate is excreted in the urine at an average rate of 0.0047 mg/kg/day and in the rat at 10 times this rate (Stalder, 1958). This amounts to only 0.32 mg/day in man (only two individuals were tested so these averages are not accurate). Since malonate is apparently metabolized in mammals, the tissue concentration or rate of excretion will reflect a balance between formation and destruction. In other words, these excretion values do not necessarily represent the rates of malonate formation.

Relatively little is known about the pathways for the formation of malonate, but the miscellaneous observations make it likely that different reactions are involved in various organisms. Malonate can arise from many different substrates but in most cases the pathways are complex and the immediate precursors are not known. Malonate can be formed from pyrimidines and barbiturates in the mycobacteria (Hayaishi and Kornberg, 1952), from pyrimidines in *Nocardia* (Lara, 1952), from acetate in *Hevea brasiliensis* (Fournier *et al.*, 1961) and avocado (Mudd and Stumpf, 1961), from citrate in *Aspergillus niger* (Challenger *et al.*, 1927), from succinate in *Aspergillus niger* (Subramanian *et al.*, 1929), from asparagine in rats (Thomas and Stalder, 1959), from oxalacetate in pig heart extracts catalyzed by metmyoglobin and Mn^{++} (Vennesland and Evans, 1944; Vennesland *et al.*, 1946), and from malonyl-CoA in *Penicillium cyclopium* (Bentley and Keil, 1961). The high concentrations of malonate in bush-bean plants led Huffaker and Wallace (1961) to study the mechanism of the accumulation. They found that the malonate synthesis is related to the dark CO_2 fixation in the roots, phosphoenolpyruvate being carboxylated to oxalacetate and this going to malonate with the help of one or more enzymes. The addition of phosphoenolpyruvate and Mg^{++} to root homogenate leads to the formation of labeled malonate from $C^{14}O_2$. It was also found that any other reactions utilizing oxalacetate decrease the yield of malonate. It is very interesting that frogs accumulate malonate-C^{14} from $C^{14}O_2$, along with other dicarboxylates (Cohen, 1963). In normal strains the malonate accounts for only 0.3–0.5% of the total incorporation but in hybrids (*R. pipiens* × *R. sylvatica*) the value is 6–23%. This increased accumulation in the hybrids was attributed to some defect in the metabolism of malonate.

Methylmalonate can be formed from propionate (Flavin *et al.*, 1955) in a variety of tissues, and in rat liver the pathway has been shown to go through succinate (Katz and Chaikoff, 1955). The feeding of isobutyrate and valine to rats leads to the formation of methylmalonate (Thomas and Stalder, 1958) and the feeding of isoleucine leads to ethylmalonate (Stalder, 1959).

The first reaction appears to be an oxidative deamination. Since methylmalonyl-CoA is a common intermediate in many tissues, methylmalonate probably arises from any substance forming the coenzyme A derivative. (This will be discussed in greater detail in the following sections).

General Occurrence and Nature of Malonate Metabolism

The metabolism of malonate by many organisms and tissues has been conclusively demonstrated by a variety of techniques. The ideal method is the determination of $C^{14}O_2$ or other labeled products formed from labeled malonate, but in some instances good evidence is provided by studies of oxygen uptake, especially when the endogenous respiration is very small, or by growth in media containing only malonate as a utilizable substrate. In other cases, the evidence is more indirect. For example, a stimulation of growth rate or a rise in respiration in the presence of other substrates may suggest the utilization of malonate but does not prove it. In the tabulation on page 228 of organisms in which malonate metabolism has been claimed to occur, those that are probable and based on indirect evidence are designated by (P). It may be noted in addition that Shannon *et al.* (1959) found malonate to be metabolized by the excised leaves of 15 different common plants (such as fig, peach, eucalyptus, azalea, and lantana) and 30 other plants representing 27 families, from which it must be concluded that plants are generally capable of utilizing malonate.

The bacterial oxidation of malonate occasionally shows a lag period, first observed by Lineweaver (1933). The rate of oxidation by *Azotobacter* is very low for 2–3 hr, rises to a maximum around 6–8 hr, and falls off by 10 hr. The malonate is eventually 99% metabolized with an R.Q. of 1.6. The theoretical R.Q. for complete oxidation:

$$CH_2(COOH)_2 + 2\,O_2 \rightarrow 3\,CO_2 + 2\,H_2O$$

is 1.5. Lineweaver postulated that two separate reactions are involved: the decarboxylation of malonate to acetate, and the oxidation of the acetate. He attributed the lag phase to the slow decarboxylation, which was supported by the progressive decrease in the R.Q. with time. This interpretation was criticized by Karlsson (1950) because Lineweaver had not used cells adapted to malonate. Malonate-grown *Azotobacter* does not decarboxylate malonate appreciably under anaerobic conditions, so Karlsson concluded that oxygen is required for the initial attack on malonate, either because malonate must be oxidized before decarboxylation or because oxygen may be required for some activation of malonate (for example, by a phosphorylative mechanism). A lag phase was also demonstrated for *Aerobacter* by Barron and Ghiretti (1953), the maximal oxidative rate occurring around 2–3 hr after malonate addition. Only 40% of the malonate

Organisms and tissues metabolizing malonate	Reference
Pseudomonas aeruginosa	Gray (1952)
Pseudomonas fluorescens	Hayaishi (1953, 1954, 1955 a), Wolfe and Rittenberg (1954), Wolfe *et al.* (1954 a, b, 1955)
Escherichia coli (P)	Grey (1924), Quastel and Whetham (1925), Cook (1930)
Aerobacter aerogenes	Barron and Ghiretti (1953)
Azotobacter agilis	Lineweaver (1933), Karlsson (1950)
Salmonella typhimurium (P)	Berry and Beuzeville (1960)
Mycobacterium tuberculosis	Hayaishi and Kornberg (1952), Bernheim *et al.* (1953), Horio and Okunuki (1954), Kusunose *et al.* (1960)
Mycobacterium phlei	Müller *et al.* (1960)
Aspergillus niger	Walker *et al.* (1927), Challenger *et al.* (1927)
Aspergillus (6 species) (P)	Berk *et al.* (1957)
Penicillium cyclopium (P)	Bentley and Keil (1961)
Penicillium (3 species) (P)	Berk *et al.*, (1957)
Streptomyces olivaceus	Maitra and Roy (1961)
Pullularia pullulans (P)	Clark and Wallace (1958)
Avena coleoptile (P)	Albaum and Eichel (1943)
Chlorella pyrenoidosa (P)	Eny (1951)
Pollen of *Camellia*, *Thea*, and *Lilium*	Okunuki (1939)
Tobacco leaves	Vickery (1959), Vickery and Palmer (1957)
Bush bean leaves	Young and Shannon (1959)
Peanut mitochondria	Giovanelli and Stumpf (1957)
Euglena gracilis	Danforth (1953)
Hymenolepis diminuta (cestode) (P)	Read (1956)
Locusta migratoria fat body	Tietz (1961)
Chickens (P)	Clementi (1929), Pupilli (1930)
Pigeon liver	Menon *et al.* (1960)
Mice	Lifson and Stolen (1950)
Rats	Lee and Lifson (1951), Busch and Potter (1952 a), Nakada *et al.* (1957), Thomas and Stalder (1959)
Rat liver	Menon *el at.* (1960)
Rat ventricle	Rice and Berman (1961)
Rabbits	Wick *et al.* (1956)
Dogs	Pohl (1896)
Dog heart and muscle	Menon *et al.* (1960)
Human placenta	Hosoya and Kawada (1958), Hosoya *et al.* (1960)
Human prostate	Andrews and Taylor (1955)

is oxidized but the R.Q. is 1.47, close to that for complete oxidation to CO_2 and water. Again, no decarboxylation is observed in nitrogen. Horio and Okunuki (1954) reported a 30–60 min lag period for *Mycobacterium* and also found that decarboxylation presumably precedes oxidation, because CO_2 formation always is ahead of O_2 uptake, and acetate can be demonstrated in the culture after 2 hr. The explanation of this behavior is found in the work of Gray (1952) on *Pseudomonas*. Unadapted cells show a lag period of 2 hr whereas cells cultured for 70 hr in 22 m*M* malonate are able to oxidize malonate immediately (Fig. 1-21). It was postulated that mal-

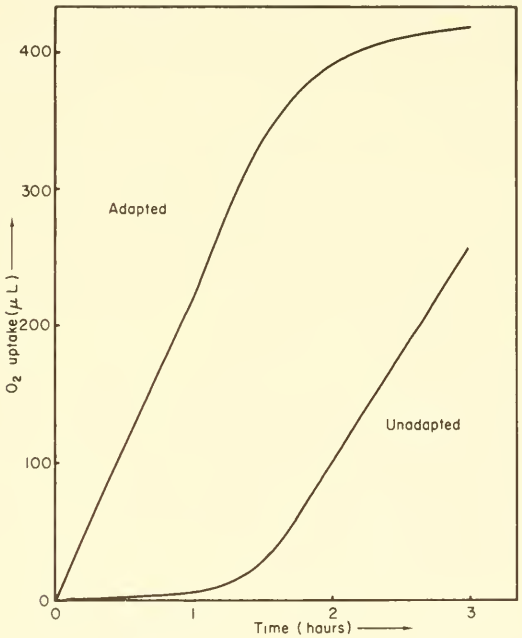
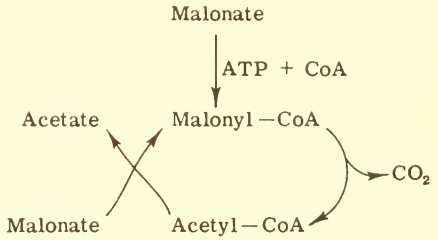


Fig. 1-21. Oxidation of malonate by normal and adapted *Pseudomonas aeruginosa*. (From Gray, 1952.)

onate decarboxylase is an adaptive enzyme, and it was pointed out that the resistance of certain microorganisms to malonate may be due to such an enzyme as well as to permeability barriers. Horio and Okunuki showed that streptomycin does not directly inhibit the decarboxylation of malonate or the oxidation of acetate, but inhibits malonate oxidation in unadapted cells, probably by preventing the synthesis of the decarboxylase. The oxygen requirement may also relate to the adaptive enzyme synthesis.

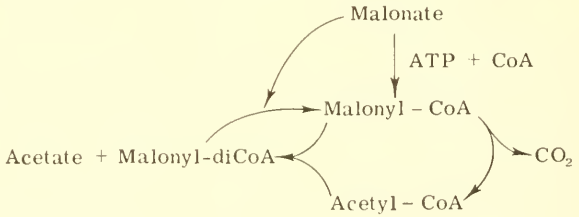
Pathways of Malonate Metabolism in Microorganisms

An analysis of the pathways of malonate oxidation was made simultaneously in the laboratories of Hayaishi and Rittenberg between 1953 and 1955. The work was done on *Pseudomonas fluorescens*, a soil isolate capable of utilizing malonate as the sole carbon source, and adapted to malonate by culture in 27–33 m*M* malonate media. Hayaishi (1953) observed that the decarboxylation of malonate requires ATP and CoA and postulated that malonate must first be activated, probably to malonyl-CoA. Using partially purified extracts from *Pseudomonas*, it was shown that malonate is quantitatively converted to CO_2 and acetate, no other products being detectable chromatographically. The proposed scheme (1-7) may be represented as follows (Hayaishi, 1954, 1955 a):



(1-7)

The cyclic process thus involves the transfer of CoA back and forth between the acetyl and malonyl groups. Wolfe *et al.* (1954) also ruled out the direct decarboxylation to acetate by showing a dependence on ATP and CoA, and in later work (Wolfe *et al.*, 1954 b, 1955; Wolfe and Rittenberg, 1954) proposed the following scheme (1-8) based mainly on chromatographic analyses of intermediates and products:



(1-8)

The principal difference between the two schemes is the participation of malonyl-diCoA. Hayaishi's results do not exlude it but provide no evidence for it, while Wolfe *et al.* claim to have detected it chromatographically. Although the decarboxylation of malonate is characterized by $\Delta F = -7$ kcal/mole, the activation energy is presumably so high that the more complex mechanisms above are necessary. The malonate decarboxylase and

CoA-transferase have been partially purified. The acetyl-CoA formed from malonate may enter the cycle directly or participate in other reactions, such as the formation of acetoacetate if the cycle is blocked by malonate, or transfer its CoA to malonate, or simply be hydrolyzed to acetate. It is very interesting that a lag period was noticed in the oxidation of malonate by cell-free extracts (Wolfe *et al.*, 1955). Little oxygen uptake occurs with malonate until it is all decarboxylated; during the period of the most rapid CO_2 evolution, the respiration is low. Yet acetate is activated and oxidized rapidly. One possible explanation is a block of the cycle by malonate so that acetyl-CoA cannot enter until most of the malonate has been metabolized. Another explanation involves a distribution of CoA in favor of the malonyl derivatives with little acetyl-CoA present during the active decarboxylation reaction.

Cryptococcus terricolus can grow on malonate as the sole source of carbon and malonate stimulates the endogenous respiration and CO_2 formation after a lag phase (Pedersen, 1963). It would appear that malonate is completely oxidized to CO_2 and water, since the R.Q. in the presence of malonate is 1.54. In other microorganisms where the oxidation is not complete, malonate may be incorporated into a variety of substances, especially lipids (Bu'Lock *et al.*, 1962), although the rate of incorporation is seldom very rapid.

Pathways of Malonate Metabolism in Plants and Animals

Despite the widespread occurrence of malonate metabolism in plant tissues, little is known of the reactions involved, although perhaps they are not significantly different from those described for microorganisms. Peanut mitochondria supplemented with ATP, CoA, and other factors are able to oxidize malonate (Giovanelli and Stumpf, 1957). Incubation with malonate-2-C^{14} for 2 hr leads to the appearance of labeled citrate, malate, and succinate, indicating the sequence

$$\text{Malonate} \rightarrow \text{malonyl-CoA} \rightarrow \text{acetyl-CoA} \rightarrow CO_2 + H_2O$$

the last step occurring in the cycle. The participation of malonyl-CoA in the oxidation of propionate by peanut mitochondria is suggested by the tracing of the label from propionate-1-C^{14} (Giovanelli and Stumpf, 1958). The following sequence involving malonic semialdehyde may be formulated:

$$\text{Propionate} \rightarrow \text{propionyl-CoA} \rightarrow \text{acrylyl-CoA} \rightarrow \beta\text{-hydroxypropionyl-CoA} \rightarrow \beta\text{-hydroxypropionate} \rightarrow \text{malonic semialdehyde} \rightarrow \text{malonyl-CoA} \rightarrow \text{acetyl-CoA} \rightarrow CO_2 + H_2O$$

It is also possible that CoA derivatives are retained throughout, since a malonic semialdehyde-CoA dehydrogenase which catalyzes the formation

of malonyl-CoA has been found in *Clostridium kluyveri* (Vagelos, 1960). In bush bean (*Phaseolus vulgaris*) leaves, incubation with malonate-2-C^{14} leads to labeled citrate, isocitrate, and malate, indicating a pathway through acetyl-CoA and the cycle (Young and Shannon, 1959). Malonate is incorporated in isolated spinach chloroplasts at about half the rate for acetate, much of the label appearing in lipids (Mudd and McManus, 1964).

Metabolism of malonate was first described in the dog by Pohl (1896), who found that only a fraction of the malonate administered to the animals can be recovered in the urine. This subject was not taken up again until 1950 and since that time much has been learned of how the body deals with malonate, and of the role of malonate and its derivatives in normal metabolism. It would appear from the limited data that about 30% of the administered malonate is metabolized (Lifson and Stolen, 1950; Busch and Potter, 1952 a). However, the rate of oxidation is relatively slow and in rabbits represents less than 2% of the total respiration (Wick *et al.*, 1956). The rate of oxidation may be in part limited by the transfer from the extracellular space into the tissues, since this is slow.

Various mammalian tissues can decarboxylate malonate and utilize the acetate formed. This has been investigated most completely in rat tissue slices by Nakada *et al.* (1957), who determined the $C^{14}O_2$ arising from malonate-1-C^{14} during 1 hr incubation at pH 7.4, the total concentration of malonate being 5 m*M* (see accompanying tabulation). Kidney, liver, and

Tissue	Added C^{14} as $C^{14}O_2$ (%)
Kidney	27.0
Liver	18.0
Heart	15.2
Diaphragm	6.8
Spleen	1.7
Brain	1.5
Lung	1.0
Testis	0.6

heart are particularly active, and the tissues show a wide range of decarboxylative ability, part of which may be due to different rates of penetration. The variation of malonate oxidation with concentration is shown in Fig. 1-22 for rat kidney slices, and an inhibition of its own metabolism is seen at concentrations above 5 m*M*. The inhibition of acetate-1-C^{14} oxidation is shown for comparison. The relative rates of activation of malonate, succinate, and glutarate by several tissues were determined by measuring the rates of formation of hydroxamic acid from hydroxylamine during the

incubations (see accompanying tabulation) (Menon *et al.*, 1960). The results show not only differences between the tissues, but also that the activating system for malonate is different from that for succinate and glutarate.

Tissue	Hydroxamic acid formed (mμmoles/mg/30 min)		
	Malonate	Succinate	Glutarate
Dog heart	10.3	7.4	28.4
Dog muscle	2.6	8.2	16.0
Pigeon liver	4.7	20.5	31.7
Rat liver	11.5	62.9	86.7

The metabolic pathways for malonate in mammalian tissues appear to be very similar to those in bacteria. The oxidation of malonate requires ATP, coenzyme A, and Mg^{++} in extracts or homogenates of rat kidney (Nakada *et al.*, 1957), human placenta (Hosoya and Kawada, 1958), and, incidentally, locust fat body (Tietz, 1961). However, the kinases, decarbox-

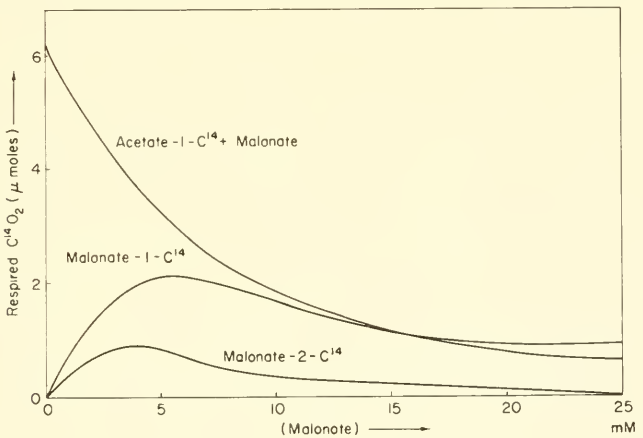


FIG. 1-22. Effects of malonate concentration on the oxidation of malonate and acetate by rat kidney slices at pH 7.4 and 37° during 2-hr incubation. (From Nakada *et al.*, 1957.)

ylases, and CoA-transferases for these reactions have not been studied. An important transfer of coenzyme A between acetoacetyl-CoA and malonate has been shown to occur in pig heart extracts (Beinert and Stansly, 1953). Acetoacetyl-CoA is formed by the condensation of two acetyl-CoA

molecules, and various carboxylate anions can accept the coenzyme A so that acetoacetate is formed:

$$2 \text{ Acetyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{CoA}$$
$$\text{Acetoacetyl-CoA} + \text{malonate} \rightleftharpoons \text{malonyl-CoA} + \text{acetoacetate}$$

succinate and butyrate being normally the most active. In this way malonate can give rise to acetoacetate as well as by its block of the cycle. Labeled malonate forms labeled acetoacetate in rat liver (Nakada *et al.*, 1957). This type of reaction has also been shown to occur in yeast, and dog heart and skeletal muscle (Menon and Stern, 1960). The enzyme, succinyl-β-ketoacyl-CoA transferase, was purified from pig heart and catalyzes the transfer to both malonate and glutarate. The following reaction:

$$\text{Succinyl-CoA} + \text{malonate} \rightleftharpoons \text{malonyl-CoA} + \text{succinate}$$

also occurs. It is interesting to speculate that some of the succinyl-CoA formed in the oxidation of α-ketoglutarate transfers its coenzyme A to malonate when it is present; if the malonyl-CoA is not readily metabolized, this could deplete the α-ketoglutarate oxidase of coenzyme A and slow down the reaction. Condensation of malonyl-CoA with coenzyme A derivatives may be important in fatty acid synthesis. In pigeon liver and carrot roots there is an enzyme catalyzing the condensation of malonyl-CoA with either acetyl-CoA or butyryl-CoA; although the product is unknown, it was isolated chromatographically (Steberl *et al.*, 1960). This product can form palmitate with other enzyme fractions. When malonyl-2-C^{14}–CoA is incubated with extracts from various rat tissues, various C_{13}–C_{18} acids are formed, depending on the acyl-CoA acceptor used (Horning *et al.*, 1960). One acyl-CoA unit is incorporated into long-chain fatty acids and the rest of the C-chain in supplied from malonyl-CoA. Labeled fatty acids are also formed from malonate-1-C^{14} in particle suspensions of the locust fat body (Tietz, 1961). A highly purified preparation from pigeon liver, which converts malonyl-CoA and acetyl-CoA to palmitate in the presence of NADPH, has been reported (Bressler and Wakil, 1961). In the absence of NADPH, malonyl-CoA and acetyl-CoA condense to form an unknown product (which is not acetoacetate, butyrate, or β-hydroxybutyrate). The conversion of malonyl-CoA to fatty acids is perhaps mediated through such condensations to C_5 acids, forming butyryl-CoA, which would again condense with malonyl-CoA, and so lengthen the chain.

A few words should be said about the pathways of methylmalonate metabolism since it has been recently found to be an important intermediate in fatty acid metabolism, and malonate can interfere markedly with at least one of these reactions. Methylmalonate was shown to be an intermediate in the metabolism of propionate in various tissues (Flavin *et al.*, 1955; Katz and Chaikoff, 1955; Feller and Feist, 1957). In the course of this work

a novel reaction was discovered, namely, the interconversion of methylmalonate and succinate. The enzyme, which has been called a methylmalonyl-CoA isomerase, catalyzes the reaction:

$$\text{Methylmalonyl-CoA} \rightleftharpoons \text{succinyl-CoA}$$

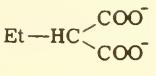
and has been purified from ox liver (Stern and Freidman, 1960) and *Propionibacterium shermanii* (Wood and Stjernholm, 1961; Stjernholm and Wood, 1961). At equilibrium the ratio (succinyl-CoA)/(methylmalonyl-CoA) is 10.5 (pH 7 and 25°). This reaction functions in both the oxidation and formation of propionate. Another reaction of importance in propionate metabolism is

$$\text{Methylmalonyl-CoA} + \text{pyruvate} \rightleftharpoons \text{propionyl-CoA} + \text{oxalacetate}$$

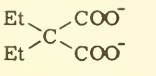
It is catalyzed by methylmalonyl-oxalacetate transcarboxylase, whereby a carboxylation may be effected without the intervention of CO_2 or the expenditure of energy to activate CO_2. It may finally be noted that the feeding of malonate to dogs leads to a marked excretion of methylmalonate in the urine (Thomas and Stalder, 1958). This does not necessarily mean that the methylmalonate is formed from malonate, since malonate inhibits the interconversion of methylmalonyl-CoA and succinyl-CoA very strongly (Flavin *et al.*, 1955).

INHIBITORS STRUCTURALLY RELATED TO MALONATE

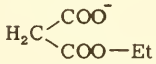
The inhibition of succinate dehydrogenase by various dicarboxylate ions was treated earlier (page 34). Maleate will be dealt with in Chapter III-2 and oxalate will be discussed in Volume IV. Inhibitors related to malonate will also be found in the following chapter on analogs. It then remains to take up the esters of malonate, hydroxymalonate, and those compounds in which the carboxylate groups have been replaced by other anionic groups. As mentioned previously, there is often confusion in the nomenclature; we shall adopt the following (using the ethyl derivatives as examples):



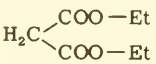
Ethylmalonate



Diethylmalonate



Malonic monoethyl ester



Malonic diethyl ester

Malonic Esters

Malonic monoethyl and diethyl esters have been found to have some interesting actions. They can increase the survival period of mice infected with mycobacteria (Davies *et al.*, 1956), are occasionally carcinostatic (Freedlander *et al.*, 1956), can inhibit the breakdown of hexobarbital in liver homogenates and prolong the narcotic action (Kramer and Arrigoni-Martelli, 1960), and inhibit sporulation of *Bacillus cereus* (Nakata and Halvorson, 1960). However, the relation of these effects to succinate dehydrogenase inhibition is obscure. In most cases, malonic esters have been used to circumvent the permeability barriers to malonate, inasmuch as the esters should penetrate into cells readily. It has often been assumed that hydrolysis to malonate occurs within the cells. This hydrolysis must be enzymatic because the esters are quite stable. A lipase from pig liver hydrolyzes one ethyl group from malonic diethyl ester but does not remove the other ethyl group, the product being malonic monoethyl ester (Christman and Lewis, 1921). I have been able to find no direct evidence for the hydrolysis to malonate. Malonic esters are neutral at physiological pH's, since they are very weak acids with pK_a values around 15.75 (Rumpf *et al.*, 1955) and ionize very slowly with a rate constant of 1.8×10^{-3} min^{-1} (Pearson and Mills, 1950).

The effects of the malonic esters on metabolism will now be discussed in order to determine if there is any indirect evidence for the intracellular hydrolysis to malonate and the inhibition of succinate dehydrogenase. When injected into fluoroacetate-poisoned rats, malonate and malonic diethyl ester have approximately the same effects on the accumulation of citrate in the heart and kidneys (Fawaz and Fawaz, 1954). Furthermore, in kidney slices, the diethyl ester inhibits succinate oxidation 92% at 20 m*M*, and malonate at the same concentrazion inhibits 75%. Less inhibition by the ester compared to malonate is observed in heart slices. Evidence for hydrolysis by both tissues was adduced from the decreases in the pH observed. The respiration of the fungus *Zygorrhynchus moelleri* is not inhibited readily by malonate although the succinate dehydrogenase from this organisms is quite sensitive, indicating a failure to penetrate (Moses, 1955). Malonic diethyl ester was tested and found to inhibit the oxidation of both glucose and acetate, but at high concentrations (see tabulation). It was felt

Malonic diethyl ester (m*M*)	% Inhibition of:	
	Glucose oxidation	Acetate oxidation
10	Stim 49	10
30	30	76
100	96	98

that the greater inhibition of acetate oxidation is evidence for the hydrolysis of the ester and that the inhibition is exerted by malonate. Malonic diethyl ester was also used to facilitate penetration into *Penicillium chrysogenum*, since even 100 m*M* malonate does not effect acetate metabolism (Goldschmidt *et al.*, 1956). The ester at 20 m*M* inibits the production of $C^{14}O_2$ from labeled acetate 75–85% and simultaneously decreases the incorporation of C^{14} into cellular materials, the labeling of glutamate being particularly depressed. The utilization of acetate by *Bacillus cereus* is also interfered with by malonic diethyl ester, so that acetate and pyruvate accumulate (Nakata and Halvorson, 1960). Malonate and the diethyl ester have been compared with respect to their effects on the respiration of *Mycobacterium phlei* (Müller *et al.*, 1960). Malonate stimulates the endogenous respiration, presumably through its oxidation, and the ester stimulates even more potently at 1–10 m*M*, although at 100 m*M* the ester inhibits and malonate still stimulates. The respiration with glycerol as the substrate behaves similarly. Finally, malonic diethyl ester markedly stimulates the endogenous respiration of *Chlorella vulgaris* at 4–10 m*M*, but inhibits the oxidation of glucose and acetate. the latter more strongly (Merrett and Syrett, 1960). All of these results show that the diethyl ester is inhibitory but certainly do not constitute conclusive evidence for a hydrolysis to malonate. This is a subject that should be pursued further and more extensive tests should be made for enzymes hydrolyzing the esters. Until the intracellular hydrolysis can be established, the results obtained with the malonic esters cannot be interpreted.

Hydroxymalonate (Tartronate)

The substitution of a methylene hydrogen of malonate by any group seems to reduce rather strongly the ability to inhibit succinate dehydrogenase. Even the small hydroxyl group almost abolishes the inhibitory activity and this is evidence that the binding of malonate to the active center of succinate dehydrogenase must involve severe steric restrictions. Although tartronate does not inhibit succinate dehydrogenase, it has other actions of some interest. Quastel and Wooldridge (1928) found that 71.4 m*M* tartronate does not inhibit succinate oxidation at all whereas it inhibits the oxidation of lactate 90%, as measured by methylene blue reduction in toluene-treated *E. coli*. In fact, the marked differences in the effects of malonate and tartronate led Quastel and Wooldridge to postulate the specific structure of the active centers of enzymes. Tartronate inhibits the respiration of rat liver slices and is strongly ketogenic, acetoacetate being formed to a greater extent that with equivalent concentrations of malonate (Edson, 1936). Pig heart malate dehydrogenase is inhibited 24% by 60 m*M* tartronate (Green, 1936) but in pigeon liver extracts it is about 1000 times as effective, inhibiting malate oxidation competitively with a K_i of 0.09 m*M*

(Scholefield, 1955). Tartronate is also a competitive inhibitor of the decarboxylating malate dehydrogenase (malic enzyme) of pigeon liver with a K_i of 0.1 mM, the inhibition being stronger than with malonate (Stickland, 1959 b). The carboxylation of pyruvate, catalyzed by the same enzyme, is also strongly inhibited, but there is a large noncompetitive element (Stickland, 1959 a). The inhibition of lactate and malate oxidations and decarboxylations is not surprising since tartronate is structurally similar. Although tartronate occurs in plant and animal tissues to the extent of 8–15 μg/g wet weight of tissue, which would correspond to about 0.1 mM in the tissue water (Veitch and Brierley, 1962), it is doubtful if it could exert a regulatory action on the metabolism. Tartronate is not metabolized in the rat and consequently is near 8 mM in the urine.

The respiration of guinea pig brain slices is depressed by tartronate at concentrations of 67–75 mM (Jowett and Quastel, 1937). The degree of inhibition depends on the substrate provided and is maximal with lactate and minimal with pyruvate. Since lactate is probably metabolized through pyruvate, the inhibition here may be mainly on lactate dehydrogenase. However, the anaerobic breakdown of pyruvate and anaerobic glycolysis are also well inhibited. The respiration of *Mycobacterium phlei* with lactate as substrate is inhibited 65% with 66 mM tartronate, although the endogenous respiration is stimulated and the oxidation of glucose unaffected (Edson and Hunter, 1947). This relatively specific effect on lactate dehydrogenase was used by Fiume (1960) to inhibit aerobic glycolysis in tumor cells, inasmuch as lactate dehydrogenase is involved. It was postulated that aerobic glycolysis might be inhibited more strongly in the tumor than in normal tissues, depleting the ATP supply more severely. It was found that tartronate inhibits aerobic glycolysis of the Yoshida ascites hepatoma — 26% at 10 mM, 34% at 20 mM, and 58% at 50 mM — but comparisons with normal tissue were not made.

The inhibition of phosphatases should perhaps also be considered in work with tartronate, since prostatic acid phosphatase is inhibited competitively with a K_i of around 50 mM. The inhibition by tartronate is much greater than by malonate and about twice as potent as by ketomalonate (Kilsheimer and Axelrod, 1957).

Aminomalonate

This substance was considered to be a possible substrate for the synthesis of δ-aminolevulinate but was found upon examination to be a potent inhibitor of δ-aminolevulinate synthetase (Matthew and Neuberger, 1963). The inhibition of the enzyme from *Rhodopseudomonas spheroides* and chicken erythrocytes is competitive; the K_i for the bacterial enzyme is 0.0225 mM. Pyridoxal-P is a coenzyme in these systems and the inhibition depends on its concentration. Aminomalonate may be considered to be an analog of

glycine (it is carboxyglycine) and presumably inhibits δ-aminolevulinate synthetase by binding to the glycine site and complexing with pyridoxal-P as well. Aminomalonate condenses with aldehydes nonenzymatically in the presence of pyridoxal-P. Furthermore, other pyridoxal-P-dependent enzymes are inhibited, e.g. serine hydroxymethyltransferase, whereas enzymes involved in glycine metabolism but not requiring pyridoxal-P are not inhibited. Aminomalonate can be formed in the tissues by transamination between ketomalonate and glutamate, and can be decarboxylated by an enzyme found in silkworm larvae and rat heart and liver to glycine. This derivative of malonate is a good illustration of how a simple change in the structure can create an inhibitor with quite different properties and inhibitory spectrum.

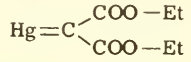
Substituted Malonates

Although the alkylmalonates are not particularly interesting as inhibitors, there are two malonate derivatives that may warrant further investigation. Fluoromalonate was studied by Chari-Bitron (1961) on the principle that if malonate is metabolized through acetyl-CoA, fluoromalonate might follow the same pathway and enter the cycle as fluoroacetyl-CoA, producing the same effects as fluoroacetate, namely, a block of the cycle at the aconitase step. The toxicity of fluoromalonate is a good deal less than fluoroacetate but the ester is as toxic in mice (see accompanying tabulation).

Animal	LD_{50} (mg/kg)		
	Fluoromalonate	Fluoromalonic diethyl ester	Fluoracetate
Mouse	80	15	15
Rat	60	70	5
Guinea pig	2	—	0.25

Death is associated with a marked accumulation of citrate in the tissues and it differs strongly from malonate in this respect. Accumulation of citrate also occurs in kidney mitochondria with fluoromalonate at concentrations around 1 m*M*. It was further established that decarboxylation of fluoromalonate occurs in kidney preparations. Finally, fluoromalonate is only about one tenth as effective as malonate in the inhibition of succinate dehydrogenase. The results thus conform quite well to the predicted mechanism. Difluoromalonate and its amide inhibit quite readily the oxidations of succinate and fumarate by *Pseudomonas* (around 70% inhibition at 0.7 m*M*), but there is no inhibition of succinate dehydrogenase in sonicates; the mechanism is unknown (Bernheim, 1963).

Inasmuch as the active center of succinate dehydrogenase possesses a sulfhydryl group close to the cationic binding sites, this being the basis for the inhibition by mercurials and other sulfhydryl agents, the possibility of combining a sulfhydryl inhibition with malonate presents itself. Mercurimalonamide and mercurimalonic diethyl ester have been prepared (Naik and Patel, 1932) and these compounds, or particularly the hydrolyzed



forms if they are stable, might be interesting to examine as inhibitors of succinate dehydrogenase.

Acetylene-dicarboxylate and Propane-tricarboxylate

Succinate dehydrogenase is inhibited competitively by acetylene-dicarboxylate (Dietrich *et al.*, 1952). The order of addition of the succinate and acetylene-dicarboxylate is important; for example, if acetylene-dicarboxylate is added 20 min after the succinate, the rate of oxygen uptake decreases slowly and does not become equal to that observed when the succinate is added after the inhibitor until 5 hr (Thomson, 1959). Acetylene-dicarboxylate is about as effective as malonate on long incubation with the enzyme. The constants obtained on rat kidney succinate dehydrogenase are $K_m = 4.12$ mM and $K_i = 0.81$ mM when substrate and inhibitor are added together; after 18 hr incubation with the inhibitor, $K_i = 0.171$ mM. It is difficult to understand why the rate of inhibition is so slow. Succinate dehydrogenase from pig heart is less readily inhibited, K_i being 1.4 mM with *N*-methylphenazine as electron acceptor and 16.5 when ferricyanide is the acceptor (Hellerman *et al.*, 1960). Possibly insufficient time for equilibrium was allowed. The inhibitions of succinate and pyruvate oxidations by acetylene-dicarboxylate in suspensions of rate heart mitochondria are shown in Fig. 1-23 (Montgomery and Webb, 1956 b). Acetylene-dicarboxylate is less potent than malonate against succinate oxidation and more potent against pyruvate oxidation, indicating that an inhibition is exerted at some other point in the cycle.

Propane-tricarboxylate is a rather weak inhibitor of succinate dehydrogenase from rat heart, 5 mM inhibiting around 30% when the succinate is also 5 mM. The oxidation of α-ketoglutarate is inhibited 60% under the same conditions, suggesting some effect on the α-ketoglutarate oxidase. The oxidation of pyruvate is inhibited even more strongly (Fig. 1-24). It might be thought that propane-tricarboxylate would inhibit aconitase or isocitrate dehydrogenase, but very little inhibition is noted, either of citrate oxidation or the ability of citrate to function as a source of oxalacetate for the oxidation of pyruvate.

Trans-1,2-Cyclopentane-dicarboxylate inhibits the succinate dehydrogenase of *Tetrahymena geleii* homogenates, around 50% inhibition being observed when the inhibitor/substrate ratio is 0.5 (Seaman and Houlihan, 1950). On the other hand, in intact cells this substance increases the utilization of pyruvate, acetate, and succinate. The uptake of acetate is in-

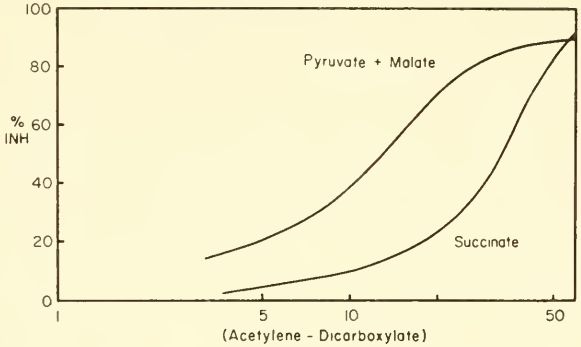


FIG. 1-23. Effects of acetylene-dicarboxylate (in m*M*) on the oxidations of pyruvate + malate and succinate by rat heart mitochondria. (From Montgomery and Webb, 1956 b.)

creased as much as 50% by *trans*-1,2-cyclopentane-dicarboxylate and in its presence succinate is taken up and oxidized whereas succinate does not enter the cells normally. It was postulated that *trans*-1,2 cyclopentane-dicarboxylate increases the permeability of the membrane to these substrates, possibly by an action on the metabolic systems involved in the

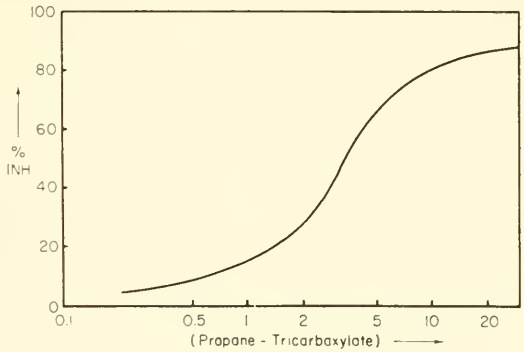


FIG. 1-24. Effect of propane-tricarboxylate (in m*M*) on the oxidation of pyruvate + malate by rat heart mitochondria. (From Montgomery and Webb, 1956 b.)

inward transport. It is not known if such an effect is observed with other dicarboxylate anions.

Sulfonate, Phosphonate, and Arsonate Analogs of Succinate

Succinate and malonate are bound to the active center of succinate dehydrogenase in part through electrostatic forces involving the negatively charged carboxylate groups. It might be expected that substances in which the carboxylate groups are replaced by other anionic groups would also be inhibitory. The structures for some of the compounds and the intercharge distances are given in Table 1-1. Klotz and Tietze (1947) first demonstrated the inhibition of succinate dehydrogenase (rat liver) by 1,2-ethanedisulfonate and β-sulfopropionate, 50% inhibition being observed in both cases when the inhibitor is 13 mM and succinate is 20 mM, these inhibitions being approximately equivalent to those of malonate. Intact *E. coli* cells are not affected by 1,2-ethanedisulfonate but the oxidation of succinate by cell-free preparations is well inhibited, corresponding to the results with malonate (Ajl and Werkman, 1948). Some inhibitor constants for these substances are shown in Table 1-29.

There appears to be definite differences in the susceptibility of the succinate dehydrogenases from different sources. Seaman (1952) noted that β-phosphonopropionate inhibits the *Tetrahymena* enzyme more strongly than does malonate, and yet no inhibition is observed on the enzymes from rat heart, liver, brain, or muscle. The inhibitions are competitive wherever they have been tested and there is no evidence that the mechanism is in any way different from that of malonate. Since these groups are larger than the carboxylate group, the interchange distances are greater than in malonate or succinate, and this must play some role in determining their binding to the enzyme. However, the distances for the malonate analogs, methanedisulfonate and arsonoacetate, are between those for malonate and succinate, so that binding should be as tight as for these latter substances if this were the only factor. Another factor of importance is the total net charge on these inhibitors, inasmuch as each of the sulfonate, phosphonate, and arsonate groups can ionize more than once. In other words, a disulfonate can exist in five different forms with charges 0, −1, −2, −3, and −4. Furthermore, the third and fourth ionization constants are in the physiological range of pH (see accompanying tabulation). The

	pK_{a_3}	pK_{a_4}
Arsonoacetate	7.7	—
1,2-Ethanediphosphonate	6.84	8.17
1,4-Butanediphosphonate	7.28	9.05
Pyrophosphate	5.69	7.76

TABLE 1-29

INHIBITOR CONSTANTS FOR SULFONATE, PHOSPHONATE, AND ARSONATE ANALOGS OF SUBSTRATES [a]

Inhibitor	Preparation	K_i	Reference
Malonate	*Tetrahymena* succinate	6.67	Seaman (1952)
β-Phosphonopropionate	dehydrogenase	1.51	
Arsonoacetate		9.25	
1,2-Ethanedisulfonate	Rat liver succinate	2.73	Klotz and Tietze
β-Sulfopropionate	dehydrogenase	3.69	(1947)
Malonate	Mouse liver succinate	0.19	Tietze and Klotz
1,2-Ethanedisulfonate	dehydrogenase	26.5	(1952)
o-Sulfobenzoate		11.7	
β-Phosphonopropionate		No inh	
Arsonoacetate		No inh	
Methionate		20.6	
Malonate	Beef heart succinate	0.014	Rosen and Klotz
Methanediphosphonate	dehydrogenase	0.5	(1957)
Phosphonoacetate		0.15	
Pyrophosphate		0.0011	
Hypophosphate		0.14	
1,2-Ethanediphosphonate		9.6	
1,4-Butanediphosphonate		8.9	
Arsonoacetate		15.3	
Malonate	Fumarase	40	Massey (1953 b)
α-Hydroxy-β-sulfo-propionate		16.5	

[a] The K_i values for rat and mouse liver succinate dehydrogenases were calculated assuming $K_m = 6 \times 10^{-3}$ M, and for beef heart succinate dehydrogenase $K_m = 4 \times 10^{-4}$ M. Although these values are undoubtedly inaccurate, the K_i values so calculated are useful for comparisons since within each experiment the relative values are reliable.

rather poor inhibition produced by the arsono and phosphono derivatives was postulated by Tietze and Klotz (1952) as possibly due to the extra negative charge carried by these substances. In the more recent work of Rosen and Klotz (1957), the ionization was taken into account and the inhibitor constants calculated on the basis of the concentration of doubly charged anion present. Evidence that too high a charge reduces the inhibitory potency is provided by the falling off of the inhibition as the pH is raised. On the other hand, it is difficult to understand why there should

be less affinity between cationic groups on the enzyme and doubly charged anions, compared to singly charged anions, and one cannot help but wonder if the alterations in pH in the experiments of Rosen and Klotz were not affecting the ionizable groups on the enzyme. These workers suggested that the binding of these substances involves an iron ion on the enzyme and correlated affinities with the ability to chelate iron. This is certainly a type of binding that should be kept in mind, but it is difficult to reconcile with the fact that iron-chelating agents such as 1,10-phenanthroline and 2,2′-bipyridine do not interfere with the binding of succinate to the dehydrogenase even though their attachment to the enzyme can be demonstrated spectroscopically.

An interesting succinate dehydrogenase inhibitor, not fundamentally related to the compounds previously discussed, is 3-nitropropionate (hiptagenate), found to be the toxic principle of *Indigofera endecaphylla* (Morris *et al.*, 1954). It inhibits succinate dehydrogenase competitively with $K_i =$ 0.19 m*M* (Hylin and Matsumoto, 1964,) although no inhibition of the enzyme is found after administration of the substance to animals, so that it is not possible to correlate the toxicity with an effect on this enzyme. It is rather surprising that 3-nitropropionate binds so well to succinate dehydrogenase, lacking two anionic groups, and it would be interesting to know more of the nature of the interaction of the nitro group with the enzyme.

With respect to inhibitors related to malonate in one way or another, it must be concluded that none possesses particular advantages over malonate for the specific inhibition of succinate dehydrogenase, although certain derivatives have interesting properties and metabolic actions. Progress could be made by the finding of forms of malonate, or related inhibitors, that are uncharged, reasonably stable outside the cell, and easily split to the active inhibitor intracellularly. It would also be valuable to have an inhibitor which would initially bind to the succinate dehydrogenase at the substrate site, because of its complementary configuration, and then react chemically with an adjacent group, so that the inhibition of this enzyme would be not only specific but slowly reversible.

CHAPTER 2

ANALOGS OF ENZYME REACTION COMPONENTS

An enzyme-catalyzed reaction involves the combination of the components with specific sites on the apoenzyme protein surface, these areas possessing the particular molecular configuration and the electrical field distribution required for the attachment and the electronic displacements characterizing the activated complex. If one of these components is modified in any way, its behavior in the system will usually be altered, due primarily to the new pattern of interaction between the modified component and the enzyme. The development and study of such analogs of substrates and coenzymes have been very active fields during the past few years for several reasons. First, the determination of the relative affinities of analogs that are substrates or inhibitors for enzymes is one of the most effective means for analyzing the topography of active centers and establishing the types of interaction involved in the catalysis. Second, it is hoped that inhibitors more specific for blocking certain enzymes than the inhibitors previously available will be found and this has been justified to a certain extent. Third, it has been realized that analog inhibition has direct bearing on the important phenomenon of feedback control of metabolic sequences and on the general regulation of cellular metabolism. Last, it is anticipated that the use of proper analogs may be useful in the specific correction of certain abnormal metabolic patterns and growth processes, such as occur in hereditary enzyme defects or neoplastic changes.

The previous chapter is concerned with malonate, a classic analog inhibitor, and in the present chapter it is proposed to extend this principle to a variety of enzymes in order to establish some basic concepts. There are a number of inhibitors which act, at least occasionally, because they are structurally related to some enzyme reaction component, but which for various reasons will be discussed in separate chapters. Such are carbon monoxide, fluoroacetate and fluorocitrate, parapyruvate, arsenate, pyrophosphate, monoamine oxidase inhibitors, certain inhibitors of cholinesterase, and various drugs. Furthermore, it is necessary to point out that no attempt will be made to review the vast literature on the depression of

growth and proliferation exerted by many analogs, inasmuch as my purpose here is to restrict the discussion to enzymic and metabolic levels.

TERMINOLOGY

The term *analog* is defined very broadly as any substance that is in some way structurally related to a substrate, coenzyme, or cofactor.* It may either participate in the enzyme reaction to a greater or lesser extent than the normal components, or inhibit the reaction by interfering with the functioning of these normal components. The commonly used term *antimetabolite* generally implies that the substance is a biologically abnormal compound synthesized in the laboratory and capable of interfering with the reactions of some cellular metabolite. We shall in this chapter frequently be concerned with inhibitions produced by substances such as carbohydrates, amino acids, purines, and nucleotides, which naturally occur in most cells, and thus the more general term analog is preferred. The use of the term *isostere* has generally been restricted to a substance produced by the substitution of an atom or group in the normal compound by another atom or group with similar electronic or steric properties. A *homolog* is a member of a series in which some part or property of a basic chemical type is progressively varied, as is the case when an aliphatic chain is lengthened by adding successive methylene groups. Most of the analogs to be discussed therefore fall in to one or more of these latter categories, but it is felt that there is little benefit to be derived from using these more specific terms.

POSSIBLE SITES AND MECHANISMS OF INHIBITION

The most common mechanism of inhibition is a competition between the analog and the normal reactant for a specific site on the enzyme surface. However, the frequently made assumption that this is the only mechanism involved is often unjustified. Other mechanisms which should be borne in mind will be mentioned here; they will be illustrated and discussed in greater detail later in the chapter. Let us first consider the mechanisms which may apply particularly to the inhibition of pure enzymes.

(*A*) Binding of the analog to the enzyme sites for substrate, coenzyme, or activator by interactions which are at least in part those involved in the binding of the normal reactant and which allow reversibility.

(*B*) An irreversible, or practically irreversible, reaction with the enzyme

* The definition of *analog* must be imprecise because it is impossible to limit accurately how much structural deviation can occur before the derivative can no longer be thought of as related to the parent compound.

site subsequent to binding. Inasmuch as substrates occasionally form a temporary chemical bond with the enzyme, an analog may do likewise but fail to complete the reaction, remaining chemically attached to the site.

(*C*) Binding of the analog to an enzyme site other than that with which the normal reactant interacts. Such binding may be simply fortuitous or the site may be specifically for the purpose of allowing feedback inhibition by a product formed in the sequence in which the enzyme participates. Regions outside the catalytic areas with which inhibitors can react are often called allosteric sites.

(*D*) The analog may be a substrate of the enzyme and will inhibit the reaction of the normal substrate to a degree dependent on the relative binding affinities and reaction rates.

(*E*) Binding of the analog to a complex of the enzyme with the normal substrate, coenzyme, or activator.

(*F*) The formation of a molecular complex of the analog with the normal reactant as a result of their structural complementarity. Although such complexes are probably uncommon and have seldom been considered in work with analogs, we shall see that examples of this mechanism are known.

(*G*) Inhibition by a mechanism only indirectly related, or completely unrelated, to the structural similarity of the analog to the normal substrate. An analog may, for example, possess chelating properties not exhibited by the substrate, or it may react with SH or carbonyl groups.

When one is investigating more complex systems, particularly cellular preparations, a number of other mechanisms for analog inhibition may be proposed, and these should be added to the above list.

(*H*) The analog may interfere with the transport mechanism by which the normal substance is taken through the cell membrane, since the two substances may both combine with some membrane carrier or enzyme system required for efficient transport or accumulation.

(*I*) The analog may not be the actual inhibitor, but may be transformed through a metabolic sequence into a substance which blocks a later reaction, a process frequently termed lethal synthesis. In certain instances the analog may complete a long and complex metabolic journey to terminate as a component in some important cellular product. The incorporation of pyrimidine and purine analogs (e.g., the 5-halouracils, 2-thiouracil, 8-azaguanine, and 8-azathymine into RNA and DNA) and amino acid analogs (e.g., tryptazan, 7-azatryptophan, ethionine, *p*-fluorophenylalanine, and *β*-2-thienylalanine into proteins) has been frequently demonstrated. The products containing the analogs may be so abnormal as to fail to function properly in the cells, thereby producing far-reaching and complex disturbances.

(*J*) The analog may act not on the enzyme attacking the normal substrate but on an enzyme involved in the formation of this substrate, since the precursors of the substrate will usually be structurally similar to it. In the linear sequence:

$$X \xrightarrow{E_1} S \xrightarrow{E_2} P$$

an analog of S may have been designed to inhibit E_2 but actually in cellular metabolism acts primarily on E_1 to reduce the rate of formation of P.

KINETICS OF ANALOG INHIBITION

The kinetics of competitive inhibition have been presented in Chapter I-3, and the graphical analyses for the proof of competition and the determination of the constants discussed in Chapter I-5. Type A plots of $1/v$ against $1/(S)$ have almost invariably been used to demonstrate the types of inhibition produced by analogs, but other types of plotting may be more satisfactory in certain situations, especially when the inhibition is not clearly and completely competitive. It is my opinion that many kinetic analyses of inhibition would be improved if several types of plotting procedure were used, allowing comparison of the results and more accurate calculations of the constants.

It is quite often the case that the inhibition by an analog is not, by the usual methods of analysis, competitive with the substrate or coenzyme to which the analog is structurally related, and such results have puzzled many workers. The plotting may indicate noncompetitive, coupling, mixed, or indeterminable inhibition mechanisms. It has been pointed out (Chapter I-3) that true noncompetitive inhibition must be rather rare and this is particularly true for inhibition by analogs. Coupling or uncompetitive inhibition is perhaps more common among analogs than with other inhibitors, especially with regard to coenzymes or cofactors, inasmuch as the substrate combines with enzyme-coenzyme or enzyme-cofactor complexes in many reactions. It is important to determine in any case if the inhibition is really competitive and the kinetics modified to obscure this, or whether the mechanism is actually other than competitive, in those instances in which the graphical analysis does not demonstrate the typical and expected competitive picture.

There are several reasons why an analog inhibition may not turn out to be competitive by the usual plotting procedures, and it may be useful to list them at this point.

(*A*) The analog may be acting by some mechanism other than specific attachment to an active site on the enzyme, that is, by one of the mechanisms listed in the previous section.

(*B*) The analog may be bound very tightly to the enzyme, in which case the order of addition of the substrate and the analog may be of great importance. If the analog is added to the enzyme and the mixture is incubated before the substrate is added, the inhibition may be very marked and the substrate may be unable to displace the inhibitor from the enzyme in a reasonable time. On the other hand, if both substrate and analog are added together, the inhibition may be very low initially but progress slowly, due to the relatively small fraction of the active centers available for reaction with the inhibitor. In either case secondary changes in the enzyme may occur and complicate the kinetics (Chapter I-12). The basic problem in the interpretation of such inhibitions is the inability experimentally to achieve satisfactory equilibrium conditions, and it must be stressed that the usual inhibition equations and plotting procedures apply only to equilibrium conditions. There has been confusion between noncompetitive and irreversible inhibitions. The arsenicals, the mercurials, and iodoacetate, for example, are often thought to inhibit succinate dehydrogenase noncompetitively, and yet succinate and these inhibitors react at the same site on the enzyme, as shown by the protection afforded by the presence of succinate when it is added with the inhibitors. These inhibitions are indeed competitive under certain conditions and during specific time intervals, but once the inhibition has been established it is difficult to demonstrate a competitive effect. Several examples of this situation using analog inhibitors will be encountered in this chapter.

(*C*) The concentration of free inhibitor may be depleted due to its combination with the enzyme or other materials and one is then dealing with a mutual depletion system (Chapter I-3). The quantitative aspects of competition may be modified quite markedly in such cases. This behavior must be looked for particularly when one is working with very potent analog inhibitors.

(*D*) An irreversible or semi-irreversible change in the configuration or properties of the active center may occur following reaction with the inhibitor, so that even after dissociation of the inhibitor from the enzyme the affinity of the enzyme for the substrate is altered. The active centers of certain enzymes appear to be flexible and adapt in some way to the interacting molecules, and it is possible that such a change would not be readily reversible. The active center, or at least the immediately adjacent region, of the penicillinase of *Bacillus cereus* is altered by combination with the competitive analog of benzylpenicillin, in that there is a marked increase in the sensitivity to iodination, and this is prevented by the presence of substrate (Citri and Garber, 1961). Although this alteration is presumably reversible, since the ability to hydrolyze benzylpenicillin after removal of the inhibitor is unimpaired, it is easy to imagine changes only slowly reversible.

(*E*) In cellular systems, or possibly in subcellular preparations of some structural complexity, the failure of the analog to penetrate to the site of inhibition as readily as the substrate would obviously distort the kinetics, although the inhibition itself is truly competitive.

(*F*) If the substrate possesses several groups through which it is bound to the enzyme, an analog which has only one of these groups (perhaps an analog comprising only a part of the normal substrate molecule) may block off one enzyme attachment point but allow the substrate to bind through the remaining groups. In many cases this will prevent catalysis, but in others it could only reduce the rate at which the substrate is reacted. Simultaneously there will be a reduction in the affinity of the enzyme for the substrate. The plots will give evidence of a mixed inhibition, which is the actual situation, but nevertheless competition of a type is occurring.

Analogs of coenzymes often present a special problem in this connection since the coenzyme may be bound quite tightly to the apoenzyme. The addition of analog to the complete enzyme may not result in significant displacement of the coenzyme and little inhibition will be observed. However, if the enzyme is resolved into its components by dissociating the coenzyme in some manner, competition between the coenzyme and analog may be demonstrated in recombination experiments in which the analog reduces the ability of added coenzyme to reactivate the enzyme.

The type of inhibition may depend on the concentration of the analog. It has been noted several times that an inhibition may be competitive at low analog concentrations and partially or completely noncompetitive at higher concentrations. The noncompetitive elements of the inhibition probably reflect the increasing unselectivity of action that is a common property of all inhibitors when the concentration is raised beyond a certain level.

An analog of a substrate will occasionally undergo reaction in the presence of the enzyme and, since both substrate and analog bind at the same site on the enzyme, competition will occur. The behavior in such a situation was discussed in Chapter I-3 (page 96). The inhibition observed will depend on what is determined. If we designate the substrate by S and its analog by S′:

$$\mathrm{E} + \mathrm{S} \rightleftarrows \mathrm{ES} \xrightarrow{k_s} \mathrm{E} + \mathrm{P} \tag{2-1}$$

$$\mathrm{E} + \mathrm{S}' \rightleftarrows \mathrm{ES}' \xrightarrow{k_s'} \mathrm{E} + \mathrm{P}' \tag{2-2}$$

and the individual rate expressions may be written as:

$$v_1 = \frac{V_m(\mathrm{S})}{(\mathrm{S}) + K_s\left[1 + \dfrac{(\mathrm{S}')}{K_s'}\right]} \tag{2-3}$$

$$v_2 = \frac{V_m'(S')}{(S') + K_{s'}\left[1 + \frac{(S)}{K_s}\right]} \tag{2-4}$$

If the disappearance of S or the formation of only P is measured, the inhibition by S′ will be typically competitive, but the inhibitor constant determined by the plotting procedures, $K_{s'}$, will not necessarily be a true dissociation constant. The result will depend on the ratio $k_{s'}/k_s$, which in most cases will be considerably less than unity. If P and P′ are identical and determined together, the inhibition will be less than in the previous case. It is important in work with analogs to establish whether they are catalytically reacted and, if so, to plan the inhibition experiments accordingly. It is also necessary in many instances to determine initial rates, since the concentration of the analog may be reduced significantly because of its conversion to the product.

The product, or one of the products, of the enzyme-catalyzed reaction may generally be considered as an analog of the substrate, or of part of the substrate molecule. Inhibition by products was taken up in Chapter I-4 (page 140) and it was pointed out there that the inhibition is not necessarily competitive, since the product can react with the enzyme or other components of the reaction in a variety of ways. Several instances of product inhibition will be encountered in this chapter and in none of these is the inhibition due to a simple reversal of the forward reaction, but to actual combination with the enzyme at or near the active center.

A somewhat more complex situation, in which the analog is transformed into a product that inhibits a subsequent reaction of the substrate, is fairly common and warrants some discussion although a complete kinetic analysis is difficult. The simplest system may be represented by:

$$A \xrightarrow{E_1} B \xrightarrow{E_2} C \tag{2-5}$$

$$A' \xrightarrow{E_1} B' \tag{2-6}$$

The substrate, A, and its analog, A′, both are reacted by E_1 and the analog product, B′, inhibits E_2. There are several ways in which the rate can vary with time and the behavior of the system will depend on many factors. In the first place, the presence of A′ may slow down reaction 1 whereby A is transformed to B, this being a case of competing substrates. The formation of C may thus be initially slowed for this reason. The concentration of B′ will progressively rise and the inhibition on E_2 increase. However, the concentration of A′ will decrease and the competition with A be reduced, leading to a relative acceleration of reaction 1. The change in the rate of formation of C will depend on the balance between these two types of inhibition. For example, if A′ is reacted fairly rapidly but B′ is not a very

potent inhibitor of E_2, the rate may first rise as the inhibition on E_1 is released, and then fall later when the concentration of B′ rises sufficiently. On the other hand, if A′ is not readily depleted and B′ inhibits well, the inhibition will steadily increase. It is clear that the kinetics may not indicate competitive inhibition when the rate of formation of C is measured. The concentration of the intermediate B may rise and fall in a complex manner, as discussed in Chapter I-9 (page 438). This is actually the simplest case of lethal synthesis complicated by competition in the initial reaction.

MEANS OF EXPRESSING RESULTS

The results in the study of analogs have usually been given in terms of inhibitions at different concentrations of substrate and analog.* Such results are often valuable but rather difficult to interpret, especially when different analogs at different concentrations must be compared. It would be more valuable if inhibitor constants were calculated and presented, along with Michaelis or substrate constants, and fortunately this practice is becoming more common. With values of K_m and K_i it is possible to determine the inhibition expected at any combination of substrate and analog concentrations. Furthermore, it is possible to calculate relative interaction energies from a series of K_i's obtained for a group of analogs, and thereby put the inhibition on a more molecularly interpretable basis.

There are fundamentally two types of K_i. The actual dissociation constant for the EI complex may be called the *true inhibitor constant* and refers to a particular free and active form of the inhibitor. The experimentally determined K_i, on the other hand, often differs from the true K_i and may be termed the *apparent inhibitor constant*. This apparent K_i may depend on a number of factors, the most important of which is usually the pH since many inhibitors are weak acids or bases. This problem has been discussed in some detail in Chapter I-14. If the inhibitor is a weak acid only one form may be the inhibitor, say the ionized I^- form, in which case the apparent K_i will vary with pH in the range around the pK_a of the inhibitor. The true K_i, which refers to the equilibrium

$$E + I^- \rightleftarrows EI^-$$

will not vary with the pH because of changes in the ionization of the inhibitor, although it may for other reasons. The apparent K_i, in fact, will depend on any type of equilibrium between active and inactive forms of the inhibitor, for example an enol-keto isomerism, or on the binding of the inhibitor to nonenzyme components of the preparation. The K_i's given

* The substrate concentration has been omitted in some reports and this vitiates the results and makes them quantitatively meaningless.

in this chapter will in almost all cases be apparent inhibitor constants because the true K_i's have generally not been calculated in the reports, although in many instances they must be very close to the true K_i's because of the nature of the analog or the conditions of the experiments. I have taken the liberty in certain cases where the data are adequate of calculating the K_i's from the inhibitions reported. In every instance where plotting procedures have been used to determine the type of inhibition, it would have been possible to determine the appropriate constants, but these constants have been seldom reported.

An especially unsatisfactory means of expressing the results is to give the inhibition for a certain ratio of inhibitor to substrate concentrations, (I)/(S), which has often been called the inhibition index. If the actual concentrations are not given, it is not possible to visualize the inhibition quantitatively, because (I)/(S) is not constant for a certain degree of inhibition even when the inhibition is competitive. This has been clearly pointed out in Chapter I-3 (page 106) but it is important to re-emphasize it here. The ratio, (I)/(S), is constant and meaningful only at high substrate concentrations which saturate the enzyme. This may be seen from the following expression, obtained by rearranging the equation for competitive inhibition:

$$\frac{(\mathrm{I})}{(\mathrm{S})} = K_i \left[\frac{1}{K_s} + \frac{1}{(\mathrm{S})} \right] \frac{i}{1 - i} \tag{2-7}$$

It has sometimes been assumed that for 50% inhibition, $(\mathrm{I})/(\mathrm{S}) = K_i/K_s$, but this is not necessarily true, which can be easily seen by rewriting Eq. 2-7 for $i = 0.5$ and $(\mathrm{S}) = nK_s$:

$$\frac{(\mathrm{I})}{(\mathrm{S})} = \frac{K_i}{K_s} \left[\frac{\mathrm{I}}{n} + 1 \right] \tag{2-8}$$

$(\mathrm{I})/(\mathrm{S}) = K_i/K_s$ only when the substrate concentration is high relative to K_s (i.e., when n is much greater than unity). This can also be seen in another way: If $K_s = 1$, $K_i = 0.3$, and (I)/(S) is kept constant at 0.3, the inhibition will vary with the absolute magnitudes of the concentrations as shown in the following tabulation:

(S)	(I)	i
0.1	0.03	0.083
0.3	0.09	0.19
0.5	0.15	0.25
1	0.3	0.33
2	0.6	0.40
5	1.5	0.45
10	3	0.48
20	6	0.49

It is obvious that a statement that the inhibition was a certain value at (I)/(S) = 0.3 would be of little significance.

When a series of analogs is tested, quantitative expression of the relative affinities of the enzyme for the various analogs is desirable when possible. The term *affinity* implies no units and has a vague meaning, having been used in a variety of ways. A common method of expressing the affinity is to equate it to $1/K_i$ in the case of an inhibitor, and to $1/K_s$ for a substrate. The ratio of the affinities of an inhibitor and a substrate is thus often expressed as K_s/K_i. If $K_s = 1$ mM, and $K_i = 0.001$ mM, it would be stated that the affinity of the enzyme for the inhibitor is 1000 times that for the substrate. This may sound dramatic but is misleading in a way. It would seem that affinity might be better expressed in terms of binding energies. The ratio of the free energies of binding of inhibitor and substrate is given by:

$$\frac{\Delta F_i}{\Delta F_s} = \frac{\mathrm{p}K_i}{\mathrm{p}K_s} \tag{2-9}$$

In the hypothetical case above, $\Delta F_i/\Delta F_s = 2$, and the inhibitor is bound twice as tightly as the substrate, which is a more reasonable way of designating the relative affinities.

One might consider three types of ΔF for the binding of an inhibitor to an enzyme. There is the *true* ΔF corresponding to the true K_i and an *apparent* ΔF corresponding to the apparent experimental K_i. In addition there is the *theoretical* ΔF corresponding to the interaction energy of the enzyme and inhibitor in a vacuum, uncomplicated by solvent and ions. In order to compare different analogs with respect to their affinities for the enzyme, it is actually this last ΔF one would wish in most cases, but it is impossible to obtain. Lacking this, one must use the true ΔF values, but, as pointed out above, true K_i's are not often available. It may be quite misleading to compare calculated ΔF values for a series of analogs if these analogs have different pK_a's and the experimental pH is in the region of these pK_a's. The differences in the ΔF's may reflect mainly the different degrees of ionization rather than the differences in binding energies of the inhibitory forms. In several instances in this chapter, I have calculated the ΔF values for such series of analogs and it must be remembered that the validity of comparing these values is sometimes questionable. It is sometimes impossible to calculate even an apparent ΔF and one must be satisfied with values that are relative to some chosen compound; these will be called *relative* ΔF values. Although the ΔF itself may not be meaningful, occasionally the difference of the ΔF values for two inhibitors will be significant in attributing interaction energies to certain groups, as discussed in Chapter I-6 (page 268), since all of the other factors involving the solvent and ionic atmosphere may remain relatively constant for the inhibitors. The relative binding energies may be calculated in some cases even though the K_i's

are not known, since for two inhibitors:

$$\Delta F_1 - \Delta F_2 = 1.422 \log \frac{i_2 (1 - i_1)}{i_1 (1 - i_2)} \qquad (2\text{-}10)$$

if $(I_1) = (I_2)$.

It is easy to derive an expression for the relationship between the true ΔF difference and the apparent ΔF difference for two inhibitors when the sole factor involved is the ionization of the inhibitors. If we designate the true free energy of binding as ΔF and the apparent free energy of binding as $\Delta F'$, the difference in the true binding energies for the two inhibitors will be $\Delta F_1 - \Delta F_2$, and the difference in the apparent binding energies will be $\Delta F_1' - \Delta F_2'$. The relationship between these for analogs that are singly ionizing weak acids is:

$$(\Delta F_1 - \Delta F_2) = (\Delta F_1' - \Delta F_2') - 1.422 \log \frac{1 + [(H^+)/K_{a_1}]}{1 + [(H^+)/K_{a_2}]} \qquad (2\text{-}11)$$

It is therefore possible to correct for the pH effect if the pK_a's of the inhibitors are known and the true interaction energy difference may be obtained.

IMPORTANT TYPES OF MOLECULAR ALTERATION PRODUCING INHIBITING ANALOGS

A substrate or coenzyme might generally be considered to have three different types of molecular region: (1) groups involved primarily in the binding to the enzyme, (2) groups involved in the catalytic reaction, and (3) groups or regions not directly involved in either binding or reaction. There is overlap between these in some cases, of course, because the groups undergoing chemical change usually participate to a certain extent in the binding. Furthermore, some substrates, such as succinate, do not possess the third type of group, all of the molecule being directly involved in binding and reaction. The properties of a substance produced by altering a single group or region of a substrate will depend on the type of group or region that is modified, that is, its function in the reaction of the substrate with the enzyme. A change in a binding group will usually alter the interaction energy in the combination of the substance with the enzyme and may or may not affect the susceptibility to chemical reaction, whereas a change in a group directly involved in the catalysis will generally reduce the reactivity without necessarily modifying the binding to the enzyme. It is also evident that a change in the third type of neutral group will not be so likely to alter the behavior of the substrate, unless such a change in some way secondarily modifies the interactions of the other groups. The aim in the design of analogs for enzyme inhibition is to produce a compound which will bind reasonably tightly to the enzyme (preferably more tightly than

the substrate), but which is resistant to chemical reaction in its complex with the enzyme. It would appear that the most effective inhibitors would result from modifications of the reactive groups, or of groups adjacent to the reactive region, rather than changes in binding groups. Malonate illustrates this in a simple way because here the $—CH_2CH_2—$ group of succinate has been altered to the nonoxidizable $—CH_2—$ group, while the binding $—COO^-$ groups remain. Amidation of the $—COO^-$ groups of either succinate or malonate, thereby eliminating the negative charges, produces a substance that is neither a substrate nor an inhibitor because the affinity for the enzyme has been lost.

The total interaction energy between a substrate or an inhibitor and the enzyme active center is the result of all the forces of attraction and repulsion summed over all the participating groups. Every atom or group of a substrate or its analog contributes to some degree to the interaction energy but, practically, the binding may be attributed usually to two or three groups that serve to orient the molecules on the enzyme surface. The subtraction, addition, or alteration of substrate groups may change the binding energy in various ways. Modifying a region vicinal to a binding group may sterically interfere with the normal approach of this group to the enzyme group with which it interacts, or it may by inductive or resonance effects alter the properties of the binding group, as discussed in Chapter I-6 (page 304). It is worthwhile emphasizing again that a change in a particular region of a molecule may produce variations in the electronic configurations throughout the entire molecule, and that a change in the interaction energy cannot generally be attributed solely to this altered region. Furthermore, the volume and configuration of a substrate and its analog may involve water of hydration, so that a group change can secondarily affect the binding by modifying the disposition of the bound water molecules. The introduction of so-called neutral groups, such as hydrocarbon chains, can bring about an increase in the binding energy through nonspecific van der Waals' interactions, providing these groups do not interfere sterically with the approach of the important binding groups to the enzyme surface.

Most analogs of substrates have less affinity for the enzymes than do the natural substrates, which is reasonable in view of the enzyme active center conformation to the substrate configuration. However, occasionally an analog will exhibit a much tighter binding than the substrate, the extra binding energy being more than could be attributable simply to a new group introduced into the molecule. In such cases it is likely that a qualitative change in the binding is involved. A substrate frequently forms a covalent bond with the enzyme during the catalytic reaction, and normally this constitutes only an intermediate state in the sequence of changes. Certain analogs may be able to form this type of bond but are unable to complete the sequence, so that the analogs remain firmly attached to the enzyme.

This is known to occur in the case of diisopropylfluorophosphate and related cholinesterase inhibitors, as well as with monoamine oxidase inhibitors such as iproniazid.

An analog may be related to the corresponding substrate in one of two general ways: it may be either an isomer of the substrate, or a substance obtained by the replacement of one or more groups on the substrate. An *isomeric analog* may be a geometric isomer (e.g., one of a *cis* and *trans* pair), optical isomer, or any stereoisomer of the substrate. It might be thought that such analogs would often be specific and useful inhibitors but actually, except for certain optical isomers (see page 268), this is seldom the case, the reason being that the configuration of the analog is more important than a simple equivalence of all the atoms and groups. A *substitution analog* can result from a variety of molecular changes in the substrate. What is often called *addition of groups* is usually only a substitution of the new group for a H atom (e.g., the replacement of a H atom with a F atom or a CH_3 group), and what is called *deletion of groups* is usually a substitution of a H atom for the group that is removed. On the other hand, an important type of analog is derived by the substitution of one functional group with another group (e.g., the replacement of an OH group with a SH group, or of a CH_3 group with a Cl atom). Some commonly interchangeable groups might be put in the following families:

(*a*) —NH_2 —OH —SH —CH_3 —Cl —F —H

(*b*) —COO^- —SO_3^- —AsO_3H^- —PO_3^-

(*c*) —$CONH_2$ —SO_2NH_2

(*d*) —S— —O— —NH— —CH=CH— —CH_2—

(*e*) —Phenyl —benzyl —pyridyl —pyrimidyl —cyclohexyl

A good deal has been written about isosteric and isomorphic groups in the production of analogs (for an excellent review see Schatz, 1960), especially with respect to the development of new drugs, but this has limited bearing on the elaboration of enzyme inhibitors. The replacement of substrate groups with isosteric and approximately isomorphic groups usually leads to substances that are also substrates. It is generally necessary to alter the proper region of the substrate molecule significantly in order to produce an effective inhibitor. There are actually at the present time no general rules for the most efficient procedures to be used for the modification of substrates to produce inhibitors. Various enzymes exhibit quite different reaction mechanisms and an effective transformation of the substrate in one case will not work for other enzymes. For this reason the most important thing to establish initially is the nature of the particular enzyme mechanism, if an attempt is to be made to design analogs rationally. The binding groups, reactive groups, and relatively neutral groups in the substrate must be

determined so that modifications in the structure may be made in the proper regions. The examples discussed in this chapter will clearly demonstrate that many analog inhibitors are not isosteric or isomorphic with the substrate and that, indeed, many of the most useful inhibitors appear to differ quite markedly from the substrate. In this connection it is necessary to call attention to the danger of visualizing molecules on the basis of their classic two-dimensional formulas. One must also realize that usually not all of a substrate molecule is involved in the binding and reaction with the enzyme; the side of the molecule more distant from the enzyme may be relatively less important than the rest of the molecule and modifications on this side may give the appearance of producing radically different substances, whereas from the standpoint of the enzyme surface these substances may be very similar to the substrate. Conversely, just because two substances look alike when written in the usual structural formulas is not enough to ensure that they will exhibit comparable interactions with the active center of an enzyme. Ideally, one should learn to conceive enzyme reactions on an electronic and molecular level and to visualize analogs with three-dimensional molecular imagination, in other words, to approach the problem of analog design from the point of view of a rational active center.

Some analogs are not directly inhibitory but are metabolically transformed into inhibitory substances that block a sequence at a later step. Such analogs are often very interesting and useful, being in many cases specific and potent. The design of an analog to be an inhibitor precursor presents a slightly different problem than in the general case. If the analog is to enter into the metabolic sequence it must be a substrate of the initial enzymes and, hence, structurally similar to the natural substrate in the region of the reactive groups. Isosteric and isomorphic substitution is often useful in this situation. This probably accounts for the popularity of fluorine as a replacement for a H atom in the design of this type of analog. Many F-substituted analogs enter into metabolic sequences and interfere at a more distal region, for example, fluoroacetate, 5-fluorouracil, *p*-fluorophenylalanine, and 6-deoxy-6-fluoro-D-glucose. The F atom is the smallest of the common atoms that may be substituted for a H atom, and approaches the H atom in size (van der Waals' radii for H and F atoms being 1.2 and 1.35 Å, respectively) (see Table I-6-8). The F atom is also relatively unreactive and forms a stable bond to carbon. However, it is strongly electronegative and alters the electronic configuration in comparison to the parent compound. The dipole moment of the C—F bond will be quite different from the C—H bond, this altering neighboring bonds as well as inducing an ability to form hydrogen bonds with the enzyme. Thus the F analog not only may be much like the substrate in over all size and configuration, allowing it to be metabolically reactive, but eventually may be transformed

into a substance in which the F atom, because of its electronegative character, interferes in some way with the catalytic reaction. Another consideration arises when phosphorylation of the substrate is an initial step in the metabolic sequence. Here one must be careful not to alter the groups involved in the phosphorylation, but to modify groups that are reactive in a later enzymic step.

DEVELOPMENT OF THE CONCEPT OF INHIBITION BY ANALOGS

This important concept, which today plays a major role in the fields of enzymology and biochemistry and is becoming more and more important in pharmacology, chemotherapeutics, and pathology, has an interesting history illustrating a typical growth pattern of a scientific idea. The development of the general concept has been traced by Martin (1951), Woolley (1952), and Albert (1960), so that here it is necessary only to present a cursory exposition related particularly to enzyme inhibition. However, it must be realized that many fields of study — including immunology, drug antagonism, and microbial growth inhibition among others — contributed in one way or another to this concept.

Despite the fact that numerous examples of competitive inhibition by analogs had been demonstrated since 1910, and that the analog concept had been quite clearly stated around 1930, general recognition of the basic principles did not occur until after 1940. Wohl and Glimm (1910) reported the inhibition of amylase by glucose and galactose, as well as by the reaction product, maltose, and shortly Michaelis described the inhibition of β-fructofuranosidase by fructose and α-methylglucoside (Michaelis and Pechstein, 1914), and the inhibition of α-glucosidase by glucose (Michaelis and Rona, 1914). Isolated, unpremeditated, and unrecognized discoveries, such as the inhibition of arginase by ornithine (Gross, 1921) and the inhibition of β-fructosidase by β-glucose but not by α-glucose (Kuhn, 1923) unfortunately gave no impetus to the formulation of a general concept. Even the classic work of Quastel on malonate inhibition, described in the previous chapter, wherein competitive inhibition by structural analogs was considered in a modern fashion, apparently did not activate anyone outside Cambridge, where Bernheim (1928) studied the aconitate inhibition of citrate oxidation, Murray (1929) and Murray and King (1930) applied the principles of analogs (although in a somewhat naive way) to the inhibition of lipase by various ketones and alcohols, Richter (1934) proved the inhibition of catechol oxidase by resorcinol to be competitive, Keilin and Hartree (1936) reported potent competitive inhibition of uricase by the methylurates, and Green (1936) extended the malonate inhibition of succinate dehydrogenase to the inhibition of malate dehydrogenase by several dicarboxylates.

Several accidental observations were made of the toxicity of metabolite analogs while they were being tested for activity in animals, for example, the discovery that ethionine is toxic to rats (Dyer, 1938) and that pyridine-3-sulfonate is lethal to dogs suffering from nicotinic acid deficiency (Woolley *et al.*, 1938), but the importance was not immediately recognized. Apparently the stimulus for the formulation of the general theory had to come from a discovery of clinical importance, and this was provided by the finding of the antagonism by *p*-aminobenzoate of the action of the sulfonamides by Woods (1940). Between 1940 and 1943 many examples of analog antagonism were reported, and from 1943 to 1946 it was shown that vitamin deficiency symptoms could be produced in animals by the administration of the appropriate analogs of all the known vitamins. It was then possible to return to the enzyme level and apply the principles formulated long before to the new results. Since 1946 there has been a steadily increasing interest in analog inhibitors, which is reflected in the large numbers of publications on this subject each year (Fig. 2-1). At the present time a paper on analog enzyme inhibition as covered in this chapter appears every other day; this does not include all of the investigations on analog inhibitors that are to be treated separately (such as fluoroacetate, arsenate, carbon monoxide, parapyruvate, and others), or analogs commonly used clinically

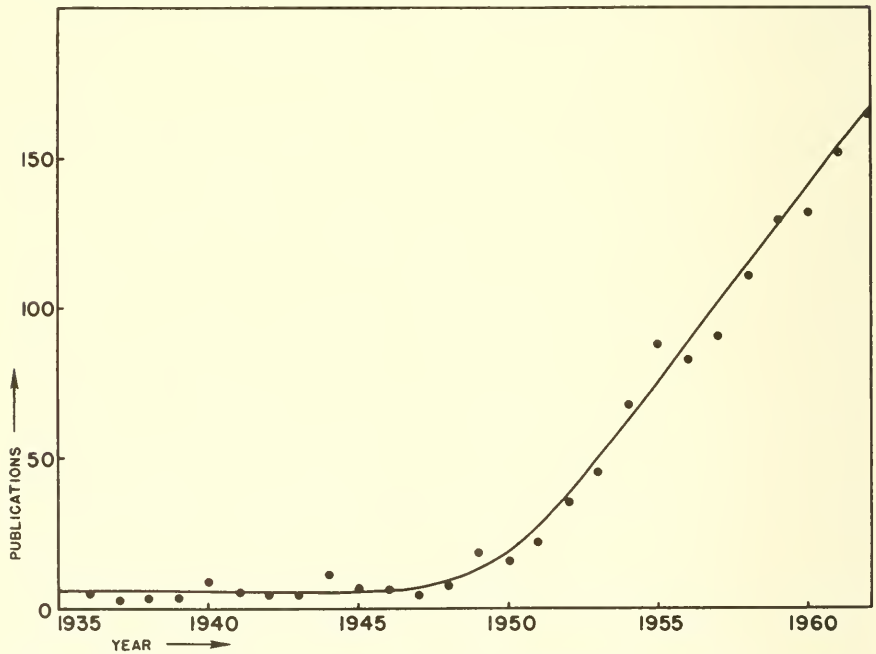


FIG. 2-1. Curve showing the annual number of publications on analog inhibition of enzymes.

(such as the cholinesterase and monoamine oxidase inhibitors), or antimetabolites used in microbial growth suppression or tumoristasis.

A few general references treating aspects of the subject not included in the present work are suggested for additional information: Welch (1945), Work and Work (1948), Woolley (1950 b, 1952), Martin (1951), Rhoads (1955), Matthews (1958), Albert (1960), Schueler (1960), Schatz (1960), Kaiser (1960), and the *Symposium on Antimetabolites* sponsored by the National Vitamin Foundation (1955). A great deal of information on the biological actions of many types of analog will be found in Volume I of "Metabolic Inhibitors" edited by Hochster and Quastel (1963), and this aspect of the subject will be mainly omitted in the present work so that the enzymic effects may be discussed in sufficient detail.

ANALOG INHIBITION OF MEMBRANE TRANSPORT

Before discussing specific enzyme inhibitions by analogs, we must turn our attention to the possibility that the depression of the utilization of some substrate or metabolite by an analog is not due to an action on any enzyme involved in the metabolism, but is the result of a specific interference with the transport of the substrate or metabolite into the cell. It is quite probable that some of the actions of analogs on metabolism, attributed to competition at the enzyme level, are actually exerted at the cell membrane; indeed, certain instances will be discussed. It is becoming more and more evident that many substrates and metabolic precursors are taken up by cells by processes other than simple diffusion, and this applies particularly to certain carbohydrates, amino acids, coenzymes, or coenzyme precursors. It is not necessary that the transport be active to be influenced by analogs; any movement of a substance through a membrane which involves a carrier, a special size or configuration of pores, or a specific type of mechanism, active or passive, can be slowed in the presence of an analog of this substance. It is possible, of course, that the membrane trasport is mediated through an enzyme reaction, such as a phosphorylation, and the competition by the analog is then truly an enzymatic one.

It is sometimes difficult to determine if there is inhibition of a transport process. The demonstration of a reduced uptake of a substrate from the medium is generally not sufficient evidence, inasmuch as a decreased intracellular utilization might also be responsible. The best procedure is to determine directly the concentration of the substrate within the cells in the absence and presence of the analog, but sometimes the concentration is too low to measure accurately, especially when the substrate is rapidly metabolized. The demonstration of typical competitive inhibition with respect to the action of an analog on some metabolic process is also not sufficient evidence for an enzymic site of action, because the inhibition of

membrane transport may exhibit competitive kinetics. The kinetics will often depend on whether the transport is limiting the metabolic utilization of the substrate or not. Membrane transport involving a carrier (C) molecule can often be represented by:

$$S + C \rightleftarrows SC_o \rightarrow SC_i \rightarrow C + S \tag{2-12}$$

where the subscripts refer to the outside and inside of the membrane. The later reactions may be written in one direction only because of the utilization of the substrate as it enters the cell. If an analog also combines with the carrier:

$$S' + C \rightleftarrows S'C \tag{2-13}$$

whether it is transported into the cell or not, typical competitive behavior will be observed since the forms of Eqs. 2-12 and 2-13 are the same as those for competitive enzyme inhibition.

Plots of transport rates against the external concentrations of the transported substance usually yield hyperbolic curves, and double reciprocal plots are often linear, allowing the calculation of a constant which corresponds to the Michaelis-Menten constant in enzyme kinetics. It is frequently assumed that this is the dissociation constant for the complex of the substance with the carrier, but it is not necessarily true for the same reasons that K_m is not always K_s. The kinetics of transport inhibition are likewise commonly similar to those observed with enzymes and values of K_i may be determined by appropriate plotting, this constant representing the dissociation constant of the carrier-analog complex. The kinetics of carrier transport and its inhibition have been elaborated by Wilbrandt and Rosenberg (1961) and Rosenberg and Wilbrandt (1962) for facilitated diffusion and certain restricted types of active transport. It is interesting in connection with certain types of inhibition work to note that the accumulation ratio, $(X)_i/(X)_o = V_m/kK_m$, where V_m is the maximal transport rate, K_m is the Michaelis-Menten constant for transport, and k is the passive diffusion constant for the membrane. Thus the cell/medium ratio may be altered by the inhibitor as a result of changes in any of these three parameters.

Carbohydrate Transport

Competition between sugars for entrance into cells has been observed in many tissues but has seldom been studied quantitatively, so that in most cases it is impossible to know if true competition kinetics are followed. Occasionally a reduction in the inhibition with increase in the concentration of the transported substrate has been noted; for example, the active accumulation of D-galactose by rabbit kidney cortex slices is inhibited 61% by 5.6 mM glucose when D-galactose is 0.1 mM and only 28% when D-galactose is 0.2 mM (Krane and Crane, 1959), but these results do not fit

a simple competitive formulation. Indeed, the exact site of inhibition has not been established in any case. The entrance of L-arabinose into rat heart cells is inhibited 92% by glucose at equimolar concentration and, since L-arabinose is not metabolized, the inhibition is presumably on some phase of membrane transport (Morgan and Park, 1958). In the same tissue the competition between glucose and 3-methylglucose at the outer but not the inner surface of the cell membrane leads to a net outward transport of 3-methylglucose against a concentration gradient, and it was concluded that there are stereospecific combining sites at both surfaces. A comparison between cellular and subcellular preparations can occasionally indicate a membrane site for an inhibition. Galactose markedly inhibits the utilization of fructose by intact ascites tumor cells but not in homogenates, pointing to a competition before the hexokinase step and probably in transport (Nirenberg and Hogg, 1957). The effects of 2-deoxy-D-glucose on the fermentation of glucose, fructose, and mannose by yeast (to be discussed in more detail on page 391) led Scharff (1961) to assume a transport system which is stable, since it still functions in acetone-dried cells, and perhaps bound to a complex or "bundle" of fermentation enzymes somewhere in the outer regions of the yeast cells.

Although the nature of the transport mechanisms and the site of inhibition are generally not known, it is clear that the interference is quite specific and dependent on the molecular configurations of the sugars. The transport of D-galactose (5 m*M*) by hamster jejunum is inhibited to varying degrees by other sugars and derivatives at 25 m*M* (see tabulation) (Wilson

Sugar	% Inhibition
D-Mannose	Stim 4
D-Xylose	7
3-*O*-Methyl-D-glucose	59
α-Methyl-D-glucoside	95

et al., 1960), and the uptake of glucose by lymph node cells is likewise inhibited differently by several sugars at 9 m*M* (see tabulation) (Helmreich and Eisen, 1959), both observations pointing to stereospecific trans-

Sugar	% Inhibition
D-Arabinose	7
D-Galactose	9
D-Fructose	40
D-Mannose	61

port systems presumably involving carriers in the membranes. The patterns of competition between various sugars have often led to the assumption of two or more different transport mechanisms for carbohydrates and that these systems can operate simultaneously and independently. Competition for transport can be demonstrated in rat diaphragm muscle for members of the group including D-glucose, D-mannose, D-xylose, D-arabinose, L-arabinose, D-lyxose, and 3-*O*-methyl-D-glucose, but not between these and D-galactose, D-fructose, maltose, α-methyl-D-glucoside, and β-methyl-D-glucoside (Battaglia and Randle, 1960), so that different sites for entry were postulated. A number of sugars are transported into erythrocytes: all the aldoses penetrate by a transport system characterized for glucose, while the ketoses penetrate according to a pattern of passive diffusion (LeFevre and Davies, 1951). The aldoses compete with each other, e.g., the uptake of glucose is strongly inhibited by mannose, but any aldose delays the entrance of a ketose, e.g., the uptake of fructose is prevented by glucose and galactose, while the ketoses do not perceptibly alter the transport of the aldoses. One carrier system in the erythrocyte has been characterized as reacting only with those monosaccharides in which the pyranose ring tends to assume the "chair" configuration, which illustrates a unique type of stereospecificity (LeFevre and Marshall, 1958). On the other hand, in hamster intestine only a single transport mechanism or carrier seems to be present, mutual inhibition occurring between D-glucose and D-galactose, D-glucose and 1,5-anhydro-D-glucitol, D-galactose and 1,5-anhydro-D-glucitol, D-glucose and 6-deoxy-D-glucose, and 6-deoxy-D-glucose and 1,5-anhydro-D-glucitol, whereas sugars that are not transported do not interfere with those that are (R. K. Crane, 1960).

Amino Acid Transport

The situation here is very much the same as in sugar transport and there is good evidence for stereospecific systems and multiple pathways. There is competition between L-leucine and DL-isoleucine for renal tubular resorption in the dog, these being well resorbed amino acids, and there is also competition between the poorly resorbed L-arginine and L-lysine (Beyer *et al.*, 1947). However, no interference is observed between L-leucine and L-arginine. It is possible to classify the amino acids into groups with respect to their mutual interference in resorption. In the rat kidney, dibasic amino acids (arginine, lysine, and cystine) are actively accumulated and there is mutual inhibition of the transport (Rosenberg *et al.*, 1962). Monobasic amino acids (alanine, phenylalanine, and histidine) do not interfere with the uptake of the dibasic amino acids, nor does arginine depress the uptake of the monobasic amino acids. It seems likely that separate transport systems are present. The transport system for basic amino acids in the hamster intestine is distinct from that for other amino acids and similar to the renal

system (see accompanying tabulation) (Hagihira *et al.*, 1961). Arginine and cystine interfere more with lysine transport than with glycine transport, whereas methionine behaves in the reverse manner, and there is

Inhibitor	Concentration (m*M*)	% Inhibition of transport of:	
		Glycine	L-Lysine
L-Arginine	2	16	89
L-Cystine	0.8	0	45
L-Methionine	1	73	32
L-Lysine	1	14	—
Glycine	1	—	0

little or no interference between glycine and lysine. Proline, histidine, and glycine are actively transported across the intestinal wall and methionine at equimolar concentration completely inhibits this (Wiseman, 1954). This intestinal transport carrier is limited to monoamine-monocarboxylates and they compete with each other. In addition to a common carrier, there may be an additional carrier for glycine and proline (Newey and Smyth, 1964), and it was pointed out that although each carrier system would conform to Michaelis-Menten kinetics, the total transport with two or more carriers involved would not necessarily.

Intestinal transport systems may react with only the L- or the D-form of an optically isomeric pair. D-Methionine is accumulated and transported against a concentration gradient by the rat intestine and this is blocked completely by equimolar concentrations of L-methionine (Jervis and Smyth, 1960). Similarly, the transport of L-I^{131}-monoiodotyrosine, which involves active accumulation of the amino acid in the gut wall, is inhibited by many L-amino acids (the most effective being L-tryptophan, L-methionine, L-leucine, and L-isoleucine) but scarcely at all by any of the four D-amino acids tested (Nathans *et al.*, 1960). L-Tryptophan, for example, at 10 m*M* reduces the tissue/medium ratio from 8.85 to 1.55 and the inhibition is apparently competitive.

The active cumulative uptake of amino acids by ascites carcinoma cells comprises several transport systems, each with a specific range of substrates. The uptake of glycine-1-C^{14} (K_m = 6.4 m*M*) is most strongly inhibited by 1-aminocyclopentanecarboxylate (K_i = 1.47 m*M*), and this is competitive, while the uptake of DL-methionine-S^{35} (K_m = 1.7 m*M*) is inhibited best by allylglycine (K_i = 0.86 m*M*). Glycine transport is moderately inhibited by allylglycine and less readily by furylglycine and thienylglycine (Scholefield, 1961). On the other hand, the uptake of DL-leucine-1-C^{14} is stimulated by most of these inhibitors. Transport of L-tryptophan in

ascites cells occurs by both diffusion and active transport (Jacquez, 1961). Certain amino acids accelerate this at lower concentrations (1 m*M*) and competitively inhibit at higher (5 m*M*), while other amino acids (such as L-alanine, L-lysine, and L-arginine) only inhibit. Oxender and Christensen (1963) thoroughly studied the effects of many amino acids on the uptake of neutral amino acids by ascites cells and found they fall into two overlapping clusters, the transport systems apparently not being very specific.

The penetration of amino acids into the brain is probably important for the metabolism and function of that tissue, and there appear to be several transport systems available. Tyrosine enters the brain readily *in vivo* and a specific transport is probably involved, since L-tyrosine penetrates more rapidly than D-tyrosine and the entry is potently inhibited by certain other amino acids, particularly L-tryptophan, L-leucine, L-valine, β-fluorophenylalanine, and L-histidine (Chirigos *et al.*, 1960). In phenylketonuria the blood levels of phenylalanine are high due to the inability of the tissues to metabolize it to tyrosine. It is possible that these high concentrations can interfere with the entry of other amino acids into the brain and partially account for the central nervous system disturbances. The uptake of five amino acids by rat brain slices is indeed inhibited by L-phenylalanine (see accompanying tabulation) and it was felt that such

Amino acid (2 m*M*)	% Decrease of concentration gradient
L-Proline	9
L-Histidine	42
L-Arginine	46
L-Ornithine	47
L-Tyrosine	70

could occur *in vivo* (Neame, 1961). The transport of L-histidine is inhibited by neutral aliphatic amino acids and short-chain diamino acids to a degree dependent on the length of the carbon chain (Neame, 1964). Inhibition by the dicarboxylic amino acids is not dependent on the chain length. In general, the L-isomers inhibit more potently than the D-isomers. It was suggested that histidine is transported by a system which transports most other amino acids, but with different affinities, since the inhibitions are all competitive. The synthetic amino acid, 1-aminocyclopentanecarboxylate, is not metabolized but is actively transported in brain slices and ascites cells, and the system involved must be the same as for methionine since it is affected similarly by the same competitive amino acids (Ahmed and Scholefield, 1962). Such transported but nonmetabolized amino acids may well be of use in studying transport inhibition, since effects can be clearly

distinguished from possible inhibitions of amino acid incorporation in the cell. Reference should be made to the very complete investigation of amino acid transport in brain slices by Abadom and Scholefield (1962), in which the many competitive inhibitions established point to several separate amino acid transport systems.

The entrance of valine, proline, and hydroxyproline into the human erythrocyte is not mutually competitive, but is inhibited markedly by certain sugars, such as glucose, galactose, and xylose, although not by fructose (Rieser, 1961). These results would indicate that some amino acids and sugars follow the same transport pathway. If this is a general phenomenon, one must consider in the use of analogs of these substances the possibility that an amino acid analog might depress glucose uptake, and thereby secondarily interfere with the transport by suppressing energy generation.

The accumulation of L-histidine by the parasitic fungus *Botrytis fabae* is inhibited by most other amino acids, and one transport system seems to be available to all the amino acids (Jones, 1963). Substitution at the NH_2 or COOH groups lessens or abolishes the inhibitory activity, indicating that the binding to the carrier is at least partly electrostatic.

Miscellaneous Transport Systems

The oxidation of protocatechuate by a *Flavobacterium* is competitively inhibited by *p*-aminosalicylate and one might conclude that mutual interaction with some enzyme is responsible. However, *p*-aminosalicylate does not affect the rate or extent of the oxidation in extracts, measured in different ways (Hubbard and Durham, 1961). These results thus point to competition for a transport system in the membrane, rather than the more usual explanation. The active transport of biotin across the hamster intestine is inhibited by various analogs (e.g., biocytin, desthiobiotin, diaminobiotin, and biotin methyl ester), but the nature of the inhibition was not investigated so it may not be competitive (it is not with lipoate) (Spencer and Brody, 1964). The active influx of urate into erythrocytes is competitively inhibited by hypoxanthine with $K_i = 0.1$ mM, whereas the efflux consists of two components, one sensitive to hypoxanthine (Lassen and Overgaard-Hansen, 1962).

There are several instances in which the transport of inorganic ions is inhibited by other related ions. The transfer and exchange of phosphate across the membrane of *S. aureus* are inhibited by chlorate, borate, and arsenate (Mitchell, 1954), although only arsenate is able to substitute completely for phosphate in the exchange. The uptake of sulfate by yeast is competitively inhibited by thiosulfate (Kleinzeller *et al.*, 1959). Nitrate inhibits quite well the accumulation of iodide in the rabbit ciliary body, 50% reduction of the tissue/medium ratio occurring at 3 mM, but it is not known if this is truly competitive (Becker, 1961).

The examples chosen to illustrate transport inhibition do not always involve analogs, except in the most general sense, but clearly demonstrate the importance of considering such a type of interference whenever analogs are used in cellular preparations.

ANALOGS WHICH ARE ISOMERS OF SUBSTRATES

The behavior of the isomers of normal metabolites, particularly optical isomers, constitutes, in a way, a special field and therefore some of the more interesting results will be discussed in this section rather than under the specific enzymes that are involved. The concept that a proper fit of a substrate to the enzyme surface is necessary for reaction implies that enzymes will usually be stereospecific, and this has been demonstrated many times. Since an enzyme commonly attacks only one form of an isomeric pair, the unreactive form may be either an inhibitor or completely inert. In most cases the unreactive form does not bind to the enzyme at all, as might be expected from the different spatial configurations of most isomeric pairs, and is not inhibitory.

Enantiomeric Analogs

These analogs are related to the corresponding substrates on the basis of molecular asymmetry and the most common examples are optically active due to an asymmetric carbon atom. Unnatural enantiomers often exhibit no affinity for the enzymes. Indeed, it has been generally found that D-amino acids do not interfere with the microbial growth-promoting activity of L-amino acids, although there are exceptions. Some examples of a lack of inhibition by optical isomers may be mentioned. The oxidation of L-phenylalanine by the L-amino acid oxidase of *Neurospora* is not inhibited by D-phenylalanine, even when the latter is present at 500 times the concentration of the substrate (Burton, 1951 b), and D-tryptophan does not inhibit *E. coli* L-tryptophanase even though it interferes with growth (Gooder and Happold, 1954). D-Malate is not oxidized by the malate dehydrogenase of *Mycobacterium tuberculosis* nor does it inhibit the oxidation of L-malate (Goldman, 1956 b). A rather unusual case is presented by potato tyrosinase in that both L- and D-tyrosine are attacked at the same rate, but there is no evidence of mutual inhibition, perhaps because of the limited range of concentrations used (Spencer *et al.*, 1956). Occasionally a slight inhibition is noted but one which would not be of any practical significance, as in the just detectable inhibition by D-leucine of the oxidation of L-leucine by the L-amino acid oxidase of the hepatopancreas of *Cardium tuberculatum*, an inhibition actually much less than exerted by other amino acids and hence probably not specific (Roche *et al.*, 1959).

However, the primary purpose of this section is to discuss instances in which significant inhibition by optical isomers is observed.

The asparaginase of *Mycobacterium phlei* attacks only L-asparagine and this deamidation is quite well inhibited by D-asparagine (Grossowicz and Halpern, 1956 a). It is a particularly clear and straightforward instance of stereomeric inhibition and a $1/v - 1/(S)$ plot shows it to be completely competitive. Around 75% inhibition is produced by 40 mM D-asparagine when L-asparagine is 10 mM. On the other hand, the asparaginases of *Bacillus coagulans* and *B. stearothermophilus* are inhibited by D-asparagine but not competitively (Manning and Campbell, 1957). The type of inhibition is difficult to designate since the double reciprocal plots intersect to the right of the ordinate, that is, the inhibition does not tend toward noncompetitive kinetics. A yet more complex situation is presented in the depression of the formation of α-amylase by D-aspartate in *Pseudomonas saccharophila*, 0.2 mM blocking the protein synthesis completely (Eisenstadt *et al.*, 1959). The inhibition is readily reversed by L-aspartate and is characterized by a fairly long lag period before inhibition is observed. The inhibition was assumed to be on the reaction:

$$\text{L-Aspartate} + \text{IMP} + \text{GTP} \rightarrow \text{fumarate} + \text{AMP} + \text{GDP} + \text{P}$$

thus producing an impairment in AMP synthesis, this secondarily disturbing the formation of ATP and the activation of amino acids for protein synthesis. Examination of this reaction in cell-free extracts showed that D-aspartate does indeed inhibit competitively. Another type of inhibition is exhibited by pea glutamine synthetase with L-glutamate, ammonia, and ATP as substrates (Varner, 1960). D-Glutamate inhibits the formation of L-glutamine. However, D-glutamate is also a substrate and actually has a lower K_m although the reaction rate is slower than with L-glutamate (K_m for L-glutamate is 10 mM and for D-glutamate is 2 mM). This is then an example of a competitive inhibition between isomeric substrates. It may also be mentioned that D-glutamate inhibits, although quite weakly, the decarboxylation of L-glutamate by bacterial glutamate decarboxylase (Roberts, 1953).

The splitting of L-histidine by rat liver histidase is inhibited by D-histidine, 11% inhibition being given by 2 mM, 36% by 12 mM, 55% by 24 mM, and 85% by 48 mM when the L-histidine is 12 mM (Edlbacher *et al.*, 1940). The formation of L-histidine from L-histidinol occurs in two steps:

$$\text{L-Histidinol} \rightarrow \text{L-histidinal} \rightarrow \text{L-histidine}$$

The enzymes catalyzing both these reactions are inhibited by D-histidinol and D-histidinal competitively (Adams, 1955). The K_i for D-histidinol is 0.05 mM for both oxidations by a yeast preparation and it is possible that only a single enzyme is involved.

Germination of *Bacillus cereus* spores is induced by certain amino acids, such as L-alanine, and it would appear in this case that L-alanine dehydrogenase is essential for the activation process (O'Connor and Halvorson, 1961 b). D-Alanine and some other analogs, such as D-α-amino-n-butyrate, inhibit the germination when it is induced by L-alanine, and also inhibit the oxidative deamination of L-alanine and to a lesser extent other amino acids, there being a good correlation between these two inhibitory actions in the series of analogs used (see accompanying tabulation). The inhibition

Substrate	% Inhibition of deamination by D-alanine (100 mM)
L-Alanine	61
L-α-Amino-n-butyrate	59
L-Norvaline	48
L-Serine	28
L-Valine	24
L-Cysteine	15
L-Isoleucine	12
L-Leucine	0
L-Phenylalanine	0

by D-alanine is, however, by no means specific for L-alanine. It is possible that several enzymes, with different susceptibilities to D-alanine, are involved in the deamination of the various amino acids.

An interesting illustration of optical specificity is provided by the *O*-phosphoserine phosphatase from chicken liver (Neuhaus and Byrne, 1960). Both L-phosphoserine and D-phosphoserine are substrates and both L-serine and D-serine inhibit. The L-serine is a much more potent inhibitor (see accompanying tabulation). L-Alanine also inhibits, being between

Substrate	K_i (mM)	
	L-Serine	D-Serine
L-Phosphoserine	0.68	27
D-Phosphoserine	0.70	29

L- and D-serine in potency, but D-alanine does not inhibit. The inhibition by L-serine seems to be uncompetitive from a double reciprocal plot, but

this is apparent only and the type of inhibition does not fit into the usual classical categories. The following scheme was proposed:

$$E + PS \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EPS \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EP + S$$
$$\downarrow k_5$$
$$E + P$$

where PS is phosphoserine. It was shown to fit the data kinetically if k_{-1}/k_1 is small. This example shows well the danger of uncritically accepting the usual interpretation of a plotting procedure since, as emphasized previously, there are types of inhibition different from those included in the classic formulations.

α-Chymotrypsin hydrolyzes the L-isomers of various tryptophanamides and tyrosinamides, and these reactions are usually inhibited by the D-isomers (Huang and Niemann, 1952; Manning and Niemann, 1958). When the substrate is nicotinyl-L-tryptophanamide ($K_s = 2.7$ mM), the reaction is inhibited by nicotinyl-D-tryptophanamide ($K_i = 1.4$ mM) and a number of other derivatives of D-tryptophanamide. The hydrolysis of several derivatives of L-tyrosinamide is similarly inhibited by the D-isomers (see accompanying tabulation). It is to be noted that in every case the D-isomer is bound more tightly than the L-isomer, assuming that K_s does indeed represent a dissociation constant. The possible forces binding these substances to the enzyme will be discussed in a later section (pages 370-375).

Tyrosinamide	K_s for L-isomer	K_i for D-isomer
Nicotinyl-	12	9
Chloroacetyl-	27	6.5
Trifluoroacetyl-	26	20
Acetyl-	32	12

Anomeric Analogs

Michaelis and Pechstein (1914), in their early work on β-fructofuranosidase, and Michaelis and Rona (1914), studying yeast maltase inhibition, concluded that the configuration around carbon 1 of carbohydrates (i.e., α- and β-anomers) is of importance in determining the affinity of these substances for the enzymes, since α-methylglucoside inhibits both enzymes quite potently whereas β-methylglucoside inhibits very little or not at all. The splitting of phenol-β-glucosides by taka-β-glucosidase is also inhibited by phenol-α-glucoside (Ezaki, 1940). The synthesis of polysaccharide from α-D-glucose-1-phosphate by muscle phosphorylase is not inhibited by β-D-glucose-1-phosphate; however, it is interesting that α-methyl-

glucoside inhibits while β-methylglucoside does not, indicating the importance of the carbon 1 configuration (Campbell *et al.*, 1952). In general, the β-anomers cannot act as either substrates or inhibitors of phosphorylase. The β-glucuronidase of mouse liver is also stereospecific, since menthyl-β-glucoronide is a substrate but menthyl-α-glucuronide only a very weak inhibitor (Levvy and Marsh, 1952).

Positional Analogs

Isomers in which a ring group is moved from one position on the ring to another are generally not inhibitory due to the fairly marked structural changes involved. There are exceptions, however, and one of the most striking is the inhibition of the oxidation of *p*-hydroxyphenylpyruvate to homogentisate by *m*-hydroxyphenylpyruvate in preparations from dog liver (La Du and Zannoni, 1955). A depression of 50% is seen with 0.2 m*M* and over 90% with 0.5 m*M* *m*-hydroxyphenylpyruvate when the substrate concentration is presumably 1.2 m*M*, indicating a tighter binding to the enzyme of the *m*-isomer. Other examples of positional isomers will be encountered in later sections.

Geometric Isomeric Analogs

One would not expect that potent inhibitors would be found in *cis* and *trans* pairs because of the different molecular configurations. We have already seen that fumarate and maleate differ markedly in their reactions with succinate dehydrogenase (page 34). The outstanding exception to this rule is the well-known inhibition of aconitase by *trans*-aconitate. This enzyme catalyzes the interconversion of the tricarboxylates:

$$\text{Citrate} \rightleftarrows \textit{cis}\text{-aconitate} \rightleftarrows \text{isocitrate}$$

although perhaps *cis*-aconitate is not an obligatory intermediate between citrate and isocitrate. Bernheim (1928), impressed by the results obtained by Quastel with malonate, tested the effect of *trans*-aconitate on liver "citric dehydrogenase" (the reduction of the methylene blue used in this system was actually due to the oxidation of isocitrate formed from citrate via aconitase) and found definite inhibition. He believed the inhibition to be related to the structural similarity between citrate and *trans*-aconitate, stating, "The curve obtained seems to indicate that the aconitic acid is adsorbed on the enzyme so that part of the surface is unavailable for citric acid." Twenty years later a thorough study of this inhibition was made by Saffran and Prado (1949), using aconitase from pigeon breast muscle. Both the conversion of *cis*-aconitate to citrate and the disappearance of citrate are inhibited by *trans*-aconitate. However, *trans*-aconitate is

not bound as tightly to the enzyme as are the substrates. When citrate is 3.3 m*M*, 50% inhibition is found with 16 m*M* *trans*-aconitate; since K_m for citrate is roughly 1 m*M*, K_i is approximately 4 m*M*. The inhibition is competitive although the $1/v - 1/(S)$ plots are not ideal, perhaps because of some enzyme inactivation or failure to achieve equilibrium. The inhibition of rat mammary gland aconitase seems to be somewhat more potent, since equimolar concentrations of citrate and *trans*-aconitate lead to around 50% inhibition (Abraham *et al.*, 1960). Studies on the stereospecificity and deuterium transfer during reactions catalyzed by aconitase (Speyer and Dickman, 1956; Englard and Colowick, 1957) point to a three-point attachment of the tricarboxylates (and perhaps an intermediate carbonium ion) to the apoenzyme and Fe^{++}. The carboxyl groups in *trans*-aconitate would not appear to be in such a favorable position as in *cis*-aconitate for the formation of this complex and this might explain the relatively weaker binding. The effects of *trans*-aconitate on other enzymes have been little investigated but it has been found to be a fairly potent inhibitor of fumarase, K_i being 0.63 m*M* at pH 6.35 (Massey, 1953 b). The K_i increases with rise in the pH (Fig. I-14-11) and, as with malonate, the formation of the EI complex is exothermic at low temperatures and endothermic at high temperatures.

It is somewhat surprising that *trans*-aconitate is a reasonably effective inhibitor of the respiration of intact cells, inasmuch as penetration into the cells should be difficult. The following inhibitions have been observed: 22–36% of endogenous respiration of various tumor slices and 28% of endogenous respiration of liver slices at unspecified concentration (Weinhouse *et al.*, 1951), 25% of *Paramecium* respiration at 10 m*M* (Holland and Humphrey, 1953), and 40% of the ion-linked respiration of barley roots at 20 m*M* (Ordin and Jacobson, 1955). However, no inhibition of the respiration of *Australorbis* mince at 10 m*M* was reported (Weinbach, 1953). The oxygen uptake resulting from the addition of citrate or *cis*-aconitate to rat liver slices is strongly inhibited by 30 m*M* *trans*-aconitate (Sherman and Corley, 1952). The most complete study of respiratory inhibition is by Saffran and Prado (1949) with rat liver and kidney slices. In the latter the inhibition is 27% at 2 m*M* and 73% at 20 m*M*, which is quite comparable to malonate. The inhibition by 2 m*M* *trans*-aconitate is not altered by adding malate or fumarate, but is reversed with 5 m*M* citrate or *cis*-aconitate. In other experiments the sensitivity to *trans*-aconitate is unexplainably less. The inhibition of aconitase in liver and mammary gland homogenates leads to a fairly marked depression of the conversion of citrate to CO_2 and of acetate to fatty acids by *trans*-aconitate (Abraham *et al.*, 1960). The synthesis of mammary fatty acids is inhibited 45% by 7.1 m*M* and 75% by 21.4 m*M*. Accumulation of citrate accompanies the inhibition in kidney, liver, and tumor slices (Weinhouse *et al.*,

1951; Saffran and Prado, 1949), and this is augmented by the addition of cycle intermediates such as pyruvate or malate. These results indicate clearly that aconitase is being inhibited intracellularly.

Essentially nothing is known of the possible effects of *trans*-aconitate on cellular functions. No depression of *Paramecium* motility is seen at 10 m*M* (Holland and Humphrey, 1953). However, the active transport of ions by barley roots is markedly reduced (Ordin and Jacobson, 1955). K^+ and Br^- uptakes are inhibited 32% and 33%, respectively, by 10 m*M* *trans*-aconitate, and 63% and 47%, respectively, by 20 m*M*. These inhibitions are probably not specific but the result of the depression of respiration.

trans-Aconitate is known to occur naturally in many plant tissues and is abundant in sugar cane juice. It is formed from acetate-C^{14} in corn tissues and 95% of the aconitate which accumulates is in the *trans* form, it being out of equilibrium with the cycle acids; further evidence for its compartmentalization during endogenous formation is provided by the fact that it is metabolized quite readily when it is added to corn roots (MacLennan and Beevers, 1964). It was suggested by Rao and Altekar (1961) that it may arise from *cis*-aconitate through the mediation of an aconitate isomerase, which they isolated from soil organisms. Some pseudomonads are capable of metabolizing *trans*-aconitate without previous exposure to it and other strains can adapt to utilizing it (Altekar and Rao, 1963).

FUMARASE

Fumarase has been studied more intensively than most enzymes with regard to interactions with competitive inhibitors, the effects of pH on these interactions, and the nature of the active center. A generalized representation of the bindings of fumarate and L-malate to the enzyme is shown in Fig. I-6-2, the pH effects are discussed in Chapter I-14 (page 691), the apparent pK_i's for various competitive inhibitors are given in Table I-14-2, and the pK_a's of the two catalytically active sites for fumarase and its substrate complexes are given in Table I-14-3.

Emphasis in this section will be directed to a more accurate delineation of the active center configuration and to a more quantitative expression of the ways in which competitive inhibitors interact with the active center. Fumarase possesses four important groups: two cationic groups for binding the COO^- groups of the substrate in the *trans* position, and two ionizable groups interacting with the groups on the α- and β-carbon atoms and involved in the addition or removal of water. The latter enzyme groups will be designated as R_L and R_D in conformity with Wigler and Alberty (1960); each may exist in the protonated form, R_LH or R_DH. The pK_a's of these groups, which are 6.3 and 6.9 in the free enzyme, point to their phenolic or imidazole nature; indeed, it is possible that these two groups are identical,

but evidence against this comes from the study of inhibition by the tartrates. The catalytically active form of fumarase may be represented by EH, in which one of these groups is protonated, although both E and EH_2 are capable of binding both substrates and inhibitors.

The values of K_i for several competitive inhibitors at pH 6.35 and 23° (Table 2-1) may be used as a rough and provisional means of evaluating the relative energies of binding, bearing in mind that these are apparent K_i's, that the enzyme exists in three different ionized states for each of which the binding is different (so that the K_i's are in a sense composite), and that the various inhibitors alter the pK_a's of the enzyme groups in different ways in the EI complexes. However, some reasonable conclusions may be drawn from these ΔF values.

TABLE 2-1

INHIBITOR CONSTANTS[a] AND RELATIVE BINDING ENERGIES FOR COMPETITIVE INHIBITORS OF FUMARASE

Inhibitor	Apparent K_i (mM)	Relative $-\Delta F$ (kcal/mole)
Adipate	100	1.35
Succinate	52	1.73
Glutarate	46	1.80
Malonate	40	1.90
D-Tartrate	25	2.16
Mesaconate	25	2.16
Maleate	11	2.64
L-α-Hydroxy-β-sulfopropionate[b]	10	2.70
D-Malate	6.3	2.97
Citrate	3.5	3.32
trans-Aconitate	0.63	4.32

[a] Values of K_i determined at pH 6.35 and 23°.

[b] The K_i for L-α-hydroxy-β-sulfopropionate was changed from 16.5 mM as given in the table (Massey, 1953 b) to correspond to the value in the curve presented (16.5 is probably a misprint for 10.5.)

(A) Since all monocarboxylates and the methyl ester of fumarate are without inhibitory activity, it must be assumed that at least two negatively charged groups are necessary for binding. However, it is evident that for the more tightly bound substances other attraction forces are involved. If we assume that these additional forces arise from hydrogen bonding be-

tween hydroxyl groups and the R_L and R_D enzyme groups, polarization of double bonds, and interactions of a third COO^- group (in the tricarboxylates), the tentative values shown in the following tabulation may be assigned for the contributions made by the various interactions to the total binding:

Two COO^- groups	1.75	kcal/mole
—OH group hydrogen bonding	0.5–1.5	kcal/mole
—C=C— polarization	1.9	kcal/mole
Additional CH_2COO^- group	0.5	kcal/mole

The hydrogen bonding and polarization values are minimal since, in part, they were derived from the K_m's of the substrates (for fumarate K_m = = 1.78 mM and $-\Delta F = 3.71$ kcal/mole, and for L-malate $K_m = 4.0$ mM and $-\Delta F = 3.24$ kcal/mole). The K_m's may not represent dissociation constants but in any case the true K_s's would be equivalent to or smaller than the K_m's, so that the binding energies for the substrates may be somewhat higher. The *cis* configuration of maleate reduces the attraction, but the value for maleate in the table should be corrected since the pK_{a_2} = 5.9 (at 23° and around 0.1 ionic strength) and only 74% of the total maleic acid would be in the form of maleate$^=$: this increases $-\Delta F$ to 2.82 kcal/mole. The difference in binding between fumarate and maleate is thus at least 0.9 kcal/mole. It is also interesting that the introduction of a methyl group into fumarate to form mesaconate brings about a 1.55 kcal/mole or greater reduction in the binding energy, resulting possibly from a steric displacement and lowered polarization interaction. The 1.9 kcal/mole estimated for electrical polarization of the double bond is not unreasonable and actually corresponds fairly closely to that calculated, using appropriate molar refractions and an interaction distance of 4 Å. A factor of unknown importance is the possible deformation of the dicarboxylates to fit the active site and the energies that would be involved with the different inhibitors.

(*B*) If these conclusions are valid, the interaction energy for fumarate is approximately half due to coulombic ion-ion forces and half due to the inductive polarization by a strong dipole. It is possible that one of the R groups on the enzyme is positively charged and the other negatively charged on the active enzyme, as suggested by Massey (1953 b). The ionic interactions serve to orient the fumarate at the active center, and the polarization not only stabilizes the complex but initiates the addition of water.

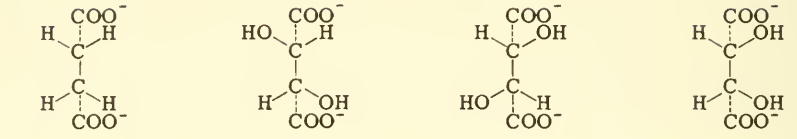
(*C*) The third COO^- group of citrate and *trans*-aconitate seems to be able to interact with an adjacent positive group on the enzyme, but rela-

tively weakly (0.3–0.6 kcal/mole). In fact, the low interaction energies of the terminal COO^- groups (0.87 kcal/mole for each group, which may be compared with the 3.3–3.6 kcal/mole binding per COO^- group of malonate or succinate on succinate dehydrogenase) might indicate that the distance between them and the enzyme cationic groups is relatively great (perhaps 12–15 Å), or could even point to a type of interaction other than ion-ion attraction.

(*D*) L-α-Hydroxy-β-sulfopropionate is bound at least 0.54 kcal/mole less tightly than L-malate, much of the affinity of this analog resulting from the OH group interaction. This would indicate that the sulfonate group is not a very good substitute for a COO^- group in this case. It would be interesting, in this connection, to have inhibition data on L-β-sulfopropionate.

(*E*) When one turns to the effects of pH on the binding of these inhibitors, it is evident that the situation is more complex than assumed from the data at a single pH (see Fig. I-14-11). Several inhibitors exhibit a progressive decline in binding with increase in the pH, but in the case of fumarate the pK_m rises between pH 7 and 8, and the pK_m-pH curve for L-malate shows several changes of slope. The inhibitor D-malate also shows an increase in binding between pH 7 and 8. Massey (1953 b) suggested that binding to different sites might be involved. However, since deviant behavior is noted with substances containing a double bond or OH group, it is possible that the affects of pH on the polarization and hydrogen bonding interactions may be involved. Succinate exhibits a linear decrease in pK_i with pH whereas fumarate behaves quite differently. Mesaconate and maleate have succinate-type pH dependences and it is possible that the polarization interaction is sterically prevented in these substances, as postulated above. Apparently some change in the active center ionization occurs between pH 7 and 8 which alters these interactions, and we shall return to this problem later when more recent inhibition data have been presented. (If the crude approach to these problems in the preceding paragraphs serves either to irritate or activate others to further theoretical or experimental study, a purpose will have been accomplished.)

One would expect the most potent competitive inhibitors of fumarase to have either a polarizable group (such as —C=C—) or a group capable of forming hydrogen bonds (such as OH). Substitution of groups at the double bond of fumarate seems to reduce the binding, and acetylene-dicarboxylate has not been studied. Thus one is left with the tartrates as possibly interesting inhibitors, and they were investigated by Wigler and Alberty (1960) in an excellent study designed to establish the more intimate nature of the catalysis. The variation of the inhibitions with pH allowed the determination of the pK_a's of the enzyme groups, the changes in these produced by complexing with the inhibitors, and the dissociation constants



of the inhibitors with the variously protonated forms of the enzyme. It was found that *meso*-tartrate is the most potent inhibitor of this group and it was concluded that this configuration of OH groups allows the formation of two hydrogen bonds with the R_L and R_D enzyme groups (Fig. 2-2). The singly protonated form of the enzyme, EH, binds the

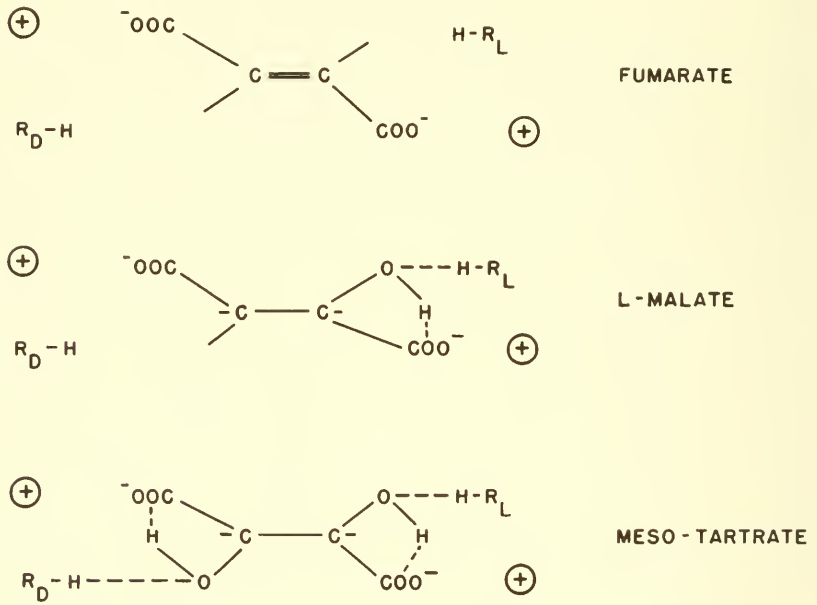
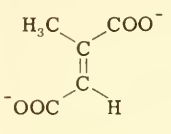


FIG. 2-2. Simplified scheme of the fumarase active site described by Wigler and Alberty (1960). The cationic, R_D, and R_L groups occur on different levels and are so located they can interact with certain isomers of substrates and inhibitors.

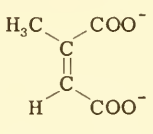
meso-tartrate most tightly. The binding energies contributed by the hydrogen bonds can be estimated from the relative interactions of succinate and the tartrates. Weak hydrogen bonds are indicated for the doubly protonated form of the enzyme, EH_2I, with D- and L-tartrates, while stronger bonds (-2.8 to -3.3 kcal/mole) are formed with *meso*-tartrate and the less protonated enzyme (see tabulation).

	ΔF for displacement of succinate (kcal/mole)		
	EH_2I	EHI	EI
D-Tartrate	−0.5	0	+0.4
L-Tartrate	−0.5	+0.8	+0.7
meso-Tartrate	−1.6	−2.8	−3.3

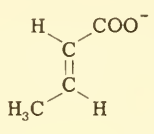
The fumarase from liver is inhibited differently from the heart enzyme, in that mesaconate (methylfumarate) is inactive whereas citraconate



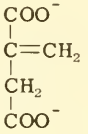
Mesaconate



Citraconate



Crotonate

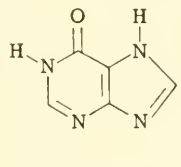


Itaconate

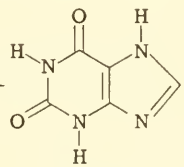
(methylmaleate) inhibits well (Jacobsohn, 1953). Itaconate inhibits less potently and crotonate even less potently (about the same as succinate). The fact that crotonate inhibits at all is interesting, in that it suggests that one COO^- is sufficient if a double bond is present. *cis*-Aconitate and *trans*-aconitate inhibit equally and weakly. DL-β-Fluoromalate inhibits competitively the conversion of malate to fumarate by fumarase (Krasna, 1961). Assuming that both optical isomers inhibit equally (which may not be true), $K_i = 28$ mM, and K_m for malate is 3.5 mM. The inhibition of malate dehydrogenase is much stronger, K_i being 0.16 mM, which may be compared to a K_m of 11 mM for malate.

INHIBITION OF XANTHINE OXIDASE BY PURINE ANALOGS AND PTERIDINES

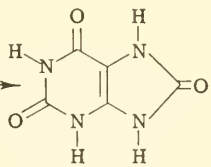
Some potent and specific inhibitors of xanthine oxidase have been discovered and have proved to be interesting not only on the enzyme level but because of the disturbances in purine metabolism produced in whole animals. Xanthine oxidase catalyzes the oxidation of hypoxanthine and



Hypoxanthine



Xanthine

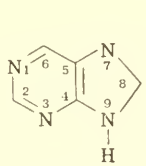


Uric acid

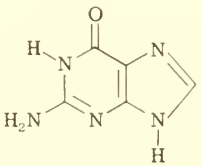
xanthine to uric acid, each step essentially involving the addition of water and the removal of two hydrogen atoms which are transferred to oxygen along a typical electron transport sequence. Hydroxypurines exhibit keto-enol tautomerism, and it is only recently that spectroscopic evidence pointing to the predominance of the keto form has been obtained (Mason, 1957). The structures of the purines and pteridines to be discussed will be written as far as possible in conformity to these results. However, in some cases it is difficult to assign the most important structure, especially in multiply substituted compounds. Ionization may also be a complicating factor. Most purines appear to be predominantly neutral at physiological pH (pK_{a_1} between 1 and 4; pK_{a_2} between 8 and 12), but some, such as uric acid, lose a proton in the acid pH range and physiologically exist as anions (e.g., pK_a for uric acid is 5.4). The site of loss of the proton is not known and it is quite possible that the anions should be considered as being equilibrium mixtures of several structures. The form in which they are written, hence, does not imply that this structure is the only one present, or even that it is necessarily the most important structure. Such considerations become important in treating the forces between these compounds and the enzyme. A final factor must be borne in mind: the most stable form in solution is possibly not the form in which the purine is bound to the enzyme surface, inasmuch as the interaction may modify the structure appreciably.

Purine Analogs

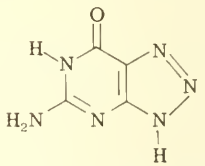
Dixon and Thurlow (1924) reported that xanthine oxidase is inhibited by various purines, such as adenine and uric acid, but no quantitative data were given. There was no further investigation until the introduction of the azapurines. 2-Azaadenine and 2-azahypoxanthine, like many purine



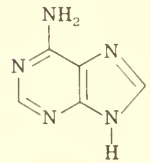
Purine



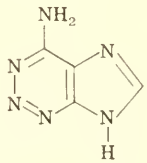
Guanine



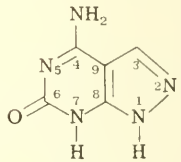
8-Azaguanine



Adenine



2-Azaadenine



Pyrazoloisoguanine

analogs, are oxidized by the enzyme in the 8-position (Shaw and Woolley, 1952). Equimolar concentrations of 2-azaadenine prolong the formation of urate from xanthine 2-fold and from hypoxanthine 4-fold, the inhibition being competitive. The kinetics in such situations may be complicated by two factors: (1) the disappearance of the inhibitor (Shaw and Woolley found, for example, that the azaadenine essentially all disappeared before much urate was formed), and (2) the inhibition produced by the product of the inhibitor oxidation. 8-Azapurine and all of its monohydroxyl and monoamino derivatives are oxidized by xanthine oxidase and the products are frequently inhibitory not only to xanthine oxidase but to other enzymes. 2-Amino-8-azapurine is converted to 8-azaguanine and hence can be used as a precursor of this inhibitor (Bergmann *et al.*, 1959).

Certain inhibitions of xanthine oxidase by purine compounds are summarized in Table 2.2 The inhibitions are not always competitive despite the close similarity of substrate and inhibitor structures. Some of the simple analogs are bound more tightly to the enzyme than are the normal substrates. 6-Chloropurine and pyrazoloisoguanine are bound particularly well and this brings up questions regarding the forces involved. Very little is known about these forces. Ionic forces must be unimportant and it is possible that hydrogen bonds, coupled with an appropriate fit of the bonding groups, play a major role. Pyrazoloisoguanine is the 4-amino-6-hydroxy derivative of pyrazolopyrimidine, and it is interesting to note that the 4-amino derivative is a very weak inhibitor relatively, as are the 4-methylamino and 1-methyl-4-amino derivatives (Feigelson *et al.*, 1957). It has been stated that there is some correlation between the potency of the xanthine oxidase inhibition and the carcinostatic activity of these and related compounds.

When inhibitory purine analogs are administered to animals it is often difficult to determine the toxic mechanisms because of the multiple possible sites for interference. The biological effects of 6-mercaptopurine seem to be related to its conversion to the ribonucleotide, which inhibits inosinic acid metabolism, rather than to any direct enzyme inhibition (Silberman and Wyngaarden, 1961). On the other hand, it has been postulated that 8-azaguanine induces a guanine deficiency by inhibiting xanthine oxidase, which operates in one guanine biosynthetic pathway (i.e., hypoxanthine → xanthine → guanine) (Feigelson and Davidson, 1956 a). It has been shown in one instance that purine metabolism can be inhibited *in vivo*. 6-Chloropurine given to rats at 80 mg/kg inhibits the formation of $C^{14}O_2$ from xanthine-6-C^{14} about 40% when administered 20 min before the xanthine (Duggan *et al.*, 1961). This is probably not due to a direct action on xanthine oxidase but to the formation of 6-chlorourate and the resulting inhibition of uricase. 6-Chloropurine also depresses the conversion of acetate to lipid, of glycine to protein, and nucleic acid synthesis.

TABLE 2-2

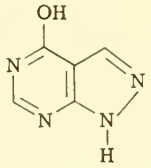
EXAMPLES OF XANTHINE OXIDASE INHIBITION BY PURINE ANALOGS

Inhibitor	Source of enzyme	Oxidizable[a]	Inhibition[b]	Potency of inhibition	Reference
Adenine	Milk	+	C	$K_i = 0.0114$ mM	Wyngaarden (1957)
	Clostridium cylindrosporum	?	M	$K_i = 0.1$ mM	Bradshaw and Barker (1960)
	Chicken kidney	?	NC	50% at 0.3 mM	Landon and Carter (1960)
6-Chloropurine	Milk	—	C	$K_i = 0.0035$ mM	Duggan and Titus (1959)
	Rat liver	—	C	33% at 1.3 mM	Duggan *et al.* (1961)
6-Chlorourate	Rat liver	—	C	91% at 0.11 mM	Duggan *et al.* (1961)
6-Mercaptopurine	Chicken kidney	—	NC	50% at 0.3 mM	Landon and Carter (1960)
	Milk	+	C	$K_i = 0.0181$ mM	Silberman and Wyngaarden (1961)
2,6-Diaminopurine	Milk	+	C	$K_i = 0.00741$ mM	Wyngaarden (1957)
2,6-Diamino-8-hydroxypurine	Milk	—	C	$K_i = 0.0106$ mM	Wyngaarden (1957)
2-Hydroxy-6-aminopurine	*Clostridium cylindrosporum*	?	C	$K_i = 2.2$ mM	Bradshaw and Barker (1960)
2-Azaadenine	Milk	+	C	50% at (I) = (S)	Shaw and Woolley (1952)
8-Azaguanine	Milk	—	NC	$K_i = 0.05$ mM	Feigelson and Davidson (1956 a)
8-Azaxanthine	*Clostridium cylindrosporum*	?	M	$K_i = 0.56$ mM	Bradshaw and Barker (1960)
Pyrazoloisoguanine	Milk	—	C	50% at 0.001 mM	Feigelson *et al.* (1957)

[a] "Oxidizable" indicates whether the inhibitor is also a substrate for xanthine oxidase.

[b] M = mixed inhibition, a situation between noncompetitive (NC) and competitive (C).

A new xanthine oxidase competitive inhibitor, 4-hydroxypyrazolo(3,4-d) pyrimidine (allopurinol, Zyloprim), is now being clinically tested in hyperuricemia and for the potentiation of the antitumor activity of the 6-substituted purines (e.g. 6-mercaptopurine) (Elion *et al.*, 1963; *Information for Investigators* report from the Burroughs Wellcome Company). It is a



4-Hydroxypyrazolo(3, 4-d)pyrimidine

very potent inhibitor, with $K_i = 0.000032$ mM, being bound around 100 times more tightly to the enzyme than is xanthine, but it is also a substrate for xanthine oxidase and its oxidation product is likewise a potent inhibitor, with $K_i = 0.000054$ mM. Mice and dogs given 100 mg/kg intraperitoneally show an increased urinary excretion of xanthine and hypoxanthine, with a decrease in allantoin, and in man a similar action has been demonstrated, serum and urinary urate being depressed. It is well tolerated by man at 200–1000 mg/day orally up to several weeks. It apparently has no antitumor activity itself but is able to potentiate the action of 6-mercaptopurine by interfering with its metabolism. Mice given 20 mg/kg intraperitoneally along with 6-mercaptopurine exhibit a reduced urinary excretion of thiourate. The value of this analog in neoplastic disease and gout is not yet known.

Inhibition of Uricase by Purine Analogs

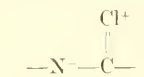
It is appropriate at this time to refer to certain studies on uricase (urate oxidase) before proceeding with the inhibition of xanthine oxidase by the pteridines. Uricase catalyzes the oxidative opening of the pyrimidine ring to form allantoin. Many methyl and ethyl derivatives of urate were tested by Keilin and Hartree (1936) on an enzyme from pig liver; they found that none is a substrate but that several inhibit quite potently. They considered the mechanism to be competitive and stated, "The fact that the methyl compounds of uric acid, although not oxidizable by the enzyme, inhibit the oxidation of uric acid shows that these methyl compounds react with the same active grouping of the enzyme molecule as uric acid itself." It is very interesting that the 1,3,7-derivative is so much more potent than the 1,3,9-derivative, particularly in view of the greater potency of the monomethyl compounds compared to the latter derivative (the urate was 10 mM in all cases) (see tabulation). 2,6,8-Trisubstituted purines were

Inhibitor	Concentration (mM)	% Inhibition
1,3,9-Trimethylurate	7.7	16
7-Methylurate	9	41
3-methylurate	9	49
1-methylurate	9	58
1,3,7-Trimethylurate	7.7	68

studied by Mahler *et al.* (1956) (see tabulation) and the results give some information on the nature of the binding to the active site. Three binding sites were recognized: (1) a cationic group binding the 2-substituent,

Substituent in position:			K_i (mM)
2	6	8	
Cl	Cl	Cl	0.0008
Cl	Cl	OH	0.0013
OH	NH_2	NH_2	0.0018
OH	OH	H	0.012
OH	OH	OH	0.025 (K_s for urate)
Cl	NH_2	OH	0.04
OH	NH_2	OH	0.15
NH_2	NH_2	OH	0.5
NH_2	OH	NH_2	No inhibition
NH_2	NH_2	NH_2	No inhibition

(2) a neutral group binding the 8-substituent, and (3) the copper which chelates with the 6- substituent and the 7-N atom. It is difficult to understand on this basis the high affinity of the trichlorourate for the enzyme, since one would predict that substitution of chlorine in the 2-position would reduce binding to the cationic group and substitution in the 6-position would interfere with the chelation. The increased binding produced by chlorine substitution might be in part the result of a stabilization of conjugative resonance and an increased hydrogen bonding (and perhaps an increased chelation of two N atoms with the copper). Also resonance with structures in which one or more of the chlorine atoms are in the form



would produce strong dipoles. The most potent inhibitor apparently is 6-chloro-2,8-dihydroxypurine (usually misnamed 6-chlorourate), which is

formed from 6-chloropurine by the action of xanthine oxidase, K_i being 0.00006 mM (Duggan and Titus, 1959). It inhibits urate degradation in the rat 75% at a dose of 20 mg/kg (Duggan *et al.*, 1961). The over all ΔF for the binding to uricase is approximately 10 kcal/mole and this would indicate the formation of some type of stable bond. It was pointed out that this analog is very stable and might be useful in studying the metabolism and disposition of urate. It should induce urate accumulation in the tissues and the effects of this might have some bearing on the manifestations of gout. The inhibition of uricase by xanthine (2,6-dihydroxypurine) in the tabulation above is interesting because it illustrates a novel effect in a multi-enzyme system. In the sequence:

$$\text{Xanthine} \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{urate} \xrightarrow{\text{uricase}} \text{allantoin}$$

the initial substrate inhibits the second enzyme in the series, causing accumulation of urate in increasing amounts as the xanthine concentration rises (e.g., in rat liver homogenates). At high concentrations the urate concentration falls due to the substrate inhibition of xanthine oxidase (Van Pilsun, 1953).

Further studies on uricase have been reported by Bergmann *et al.* (1963 a) and some of the results, including calculations of the approximate apparent relative binding energies, are presented in the following tabulation. The N-methylpurines are relatively weak inhibitors and are not included in the tabulation. It is clear that the best inhibitors contain a 2-OH group (designated as OH for convenience but the keto form is probably dominant), and this position is the most important in the binding; substitution of the 2-OH with a 2-SH group lowers the inhibitory activity markedly. The weakening of the binding by N-substitution points to the importance of the imino group for attachment. The inhibitions are generally sensitive to the pH and for most analogs increase with a rise in pH, although for 6-SH-8-OH-purine there is a decrease, and with 8-OH-purine there is no effect. The pH probably influences the tautomerism, which is quite important since the binding depends on the states of the N and OH groups. The K_i for 2,8-diOH-6-SH-purine is 0.0026 mM and for 2,6-diOH-8-SH-purine is 0.00039 mM (Bergmann *et al.*, 1963 b). The 2-OH-6,8-diSH-purine (which is 6,8-dithiourate) is a much more potent inhibitor and, although its K_i was not given, it must be at least around 0.00004 mM.

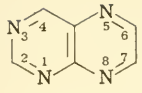
Pteridines

The inhibition of xanthine oxidase by synthetic folate, reported by Kalckar and Klenow in 1948, was soon found to be due to some impurity, and the simultaneous observation, by Lowry and Bessey, of the very po-

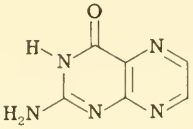
Substituent in position:			$(I)_{50}$ (mM)	Relative $-\Delta F$ (kcal/mole)
2	6	8		
OH	CH_3S	OH	0.00013	6.80
OH	SH	SH	0.0004	6.12
OH	H	Aza	0.0016	5.25
OH	SH	H	0.0027	4.94
OH	OH	SH	0.0050	4.55
OH	H	OH	0.0052	4.52
OH	OH	Aza	0.0059	4.45
OH	CH_3S	H	0.0060	4.44
OH	H	H	0.012	4.01
OH	H	OH	0.012	4.01
OH	SH	OH	0.014	3.91
OH	CH_3	H	0.017	3.78
OH	OH	H	0.018	3.76
H	CH_3S	OH	0.032	3.40
OH	OH	CH_3S	0.038	3.30
H	OH	OH	0.066	2.95
H	SH	OH	0.070	2.91
SH	OH	SH	0.080	2.84
H	H	OH	0.11	2.65
SH	SH	OH	0.15	2.44
SH	OH	H	0.19	2.30
H	OH	H	0.22	2.21
SH	OH	OH	0.25	2.13
SH	H	OH	0.50	1.70
H	SH	OH	0.50	1.70
H	SH	H	0.70	1.19

tent inhibition produced by 2-amino-4-hydroxy-6-pteridyl aldehyde, a photolytic product of folate, led to the conclusion, subsequently verified, that this was the contaminant (Kalckar *et al.*, 1948; Lowry *et al.*, 1949 a, b). For convenience we shall follow Hofstee (1949) in designating 2-amino-4-hydroxypteridine as *pterin* and the inhibitor thus as *pterin-6-aldehyde.** Pterin and many of its substituted derivatives are oxidized to varying degrees by xanthine oxidase while other derivatives are only inhibitory.

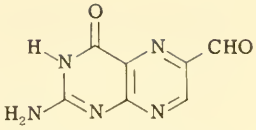
* This substance has been variably called 2-amino-4-hydroxy-6-pteridyl aldehyde, 2-amino-4-hydroxy-6-formylpteridine, 6-formylpteridine, pteridylaldehyde, 2-amino-4-hydroxy-6-pteridine carboxaldehyde, and 2-amino-4-hydroxy-6-formylpterine, in most cases without either justification or accuracy.



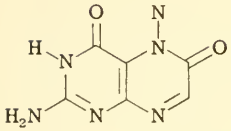
Pteridine



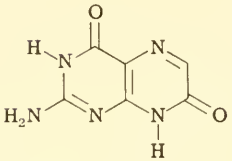
Pterin



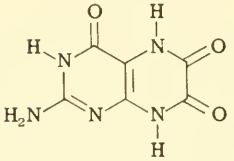
Pterin-6-aldehyde



Xanthopterin



Isoxanthopterin



Leucopterin

Actually all the photolytic oxidation products of folate are inhibitory but pterin-6-aldehyde, the primary product:

Folate → pterin-6-aldehyde → pterin-6-carboxylate → pterin → isoxanthopterin

is by far the most potent; indeed, it is one of the most potent inhibitors known.

Inhibition of xanthine oxidase by pterin-6-aldehyde is observed at a concentration of 2×10^{-4} μg/ml or roughly 10^{-9} M (Lowry *et al.*, 1949 a). Competitive inhibition with respect to both xanthine and pterin has been demonstrated, and a K_i of 6×10^{-7} mM calculated for the milk enzyme (Lowry *et al.*, 1949 b). It was shown that 35% inhibition occurs when enzyme-FAD = 9.3×10^{-6} mM, the substrate pterin = 78×10^{-6} mM, and pterin-6-aldehyde = 2.26×10^{-6} mM, this indicating that 2.26×10^{-6} mM inhibitor completely blocks 3.3×10^{-6} mM enzyme (on the basis of FAD content). It may have been that all the FAD was not catalytically active or that more than one FAD molecule was associated with one enzyme molecule. However, there is no doubt that this is a mutual depletion system and that pterin-6-aldehyde titrates the enzyme. A few other reports will be mentioned to illustrate the potency. Both the milk and rat liver enzymes are inhibited, 40–50% inhibition occurring at $5–8 \times 10^{-5}$ mM when xanthine is 0.07 mM (Kalckar *et al.*, 1950). The xanthine oxidase from *Clostridium cylindrosporum* is potently inhibited by 0.0002 mM (Bradshaw and Barker, 1960). Milk xanthine oxidase is completely inhibited by pterin-6-aldehyde at 0.0033 mM when hypoxanthine is 3.33 mM (Petering and Schmitt, 1950), and the rat intestine enzyme is inhibited completely by 0.067 mM when hypoxanthine is 6.6 mM (Westerfeld and Richert, 1952). In many experiments the substrate concentrations have been unnecessarily high since maximal rates are usually obtained at concentrations well below

0.1 mM, and hence the true potency of the inhibition has been somewhat obscured. Pterin-6-aldehyde is actually oxidized very slowly by xanthine oxidase and reversal of the inhibition develops gradually. The oxidations of aldehydes (Kalckar *et al.*, 1950) and sulfite (Fridovich and Handler, 1957) by xanthine oxidase are also inhibited by pterin-6-aldehyde, but the oxidation of NADH is not affected (Lowry *et al.*, 1949 b).

Although it has generally been stated that the inhibition by pterin-6-aldehyde is competitive, the $1/v - 1/(S)$ plots are not linear (Bradshaw and Barker, 1960), and others (Hofstee, 1949) have presented only qualitative evidence for competition. Deviations from the classic kinetics might be expected because of the mutual depletion of free enzyme and inhibitor concentrations, the difficulty in achieving true equilibrium, and the oxidative removal of the inhibitor. There is no evidence against a competitive mechanism, however, and competition has been more clearly demonstrated for some other pteridines.

The inhibition by pterin-6-aldehyde is quite specific although it must be admitted that not many enzymes have been tested. Uricase, glucose oxidase, and 3-phosphoglyceraldehyde dehydrogenase are not affected, but the quinine oxidase of liver is inhibited (Kalckar *et al.* 1950; Villela, 1963). Mouse liver guanase, using 8-azaguanine as a substrate, is inhibited but not potently when the substrate is 11 mM and 30-min preincubation is allowed (see accompanying tabulation) (Shapiro *et al.*, 1952). In fact,

Pterin-6-aldehyde (mM)	% Guanase inhibition
1	32
6	62
11	80
16	88

xanthopterin is a more potent and more rapidly acting inhibitor (Dietrich and Shapiro, 1953 b). Pterin-6-aldehyde is not carcinostatic itself, but potentiates the action of 8-azaguanine and a suppression of 8-azaguanine destruction was claimed as the mechanism, although the relatively low inhibitory potency coupled with the low doses (20 mg/kg) necessary makes it difficult to accept this explanation. Byers (1952) investigated the effects of pterin-6-aldehyde injections in rats (200 mg/kg intraperitoneally) on the tissue urate levels and found no significant changes, which might indicate a rapid destruction of the inhibitor in the animal. Daily injections of 30 μg pterin-6-aldehyde in chicks also does not alter liver xanthine oxidase activity despite the potent inhibition *in vitro* (Dietrich *et al.*, 1952).

Other pteridines, although less potent than pterin-6-aldehyde, are nev-

ertheless effective inhibitors of xanthine oxidase. The following tabulation shows the inhibitions after 20 min incubation at pH 8.5 when the substrate

Inhibitor	Concentration (mM)	% Inhibition
Pterin-6-aldehyde	0.031	100
Pteroate	0.033	100
Xanthopterin	0.033	72
Isoxanthopterin	0.066	95
Leucopterin	0.032	34
7-methylxanthopterin	0.077	91
6-Methylisoxanthopterin	0.077	89
Xanthopterin-7-carboxylate	0.050	62
Isoxanthopterin-6-carboxylate	0.075	23
Pterin-6-carboxylate	0.055	15
2,4-Diamino-6,7-dihydroxypteridine	0.040	76

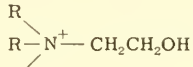
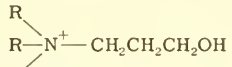
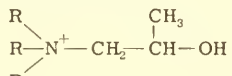
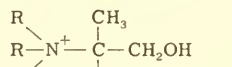
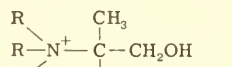
concentration is 0.063 mM (Hofstee, 1949). The potency relative to pterin-6-aldehyde is not seen here since 0.001 mM inhibits 82% under these conditions. From the K_m for xanthine of 0.02 mM (Hofstee, 1955), a K_i for pterin-6-aldehyde of 5.1×10^{-5} mM may be calculated, which is higher than was obtained by Lowry *et al.* (1949 b). The inhibition by xanthopterin appears to be completely competitive (K_m for xanthine 0.0053 mM, and K_i = 0.0016 mM), and xanthopterin-7-carboxylate is of comparable potency (Krebs and Norris, 1949). Bovine serum xanthine oxidase is inhibited 94% by 0.052 mM pterin-6-aldehyde but only 3% by xanthopterin at a comparable concentration (Villela *et al.*, 1956). There is no doubt that the aldehyde group at the 6-position confers a strong affinity for the enzyme, since when it is reduced to a CH_2OH group, oxidized to a COO^- group, or altered to a CH_3 group, the inhibitory activity is markedly reduced (Petering and Schmitt, 1950). Pterin-6-aldehyde is bound to xanthine oxidase approximately 4.9 kcal/mole more tightly than xanthopterin, and it would appear that pterin itself is bound somewhat less tightly than xanthopterin. It is tempting to relate the augmenting action of the aldehyde group to the fact that xanthine oxidase oxidizes simple aldehydes. There must be a site on the enzyme capable of reacting with aldehyde groups, and it is possible that the pterin-6-aldehyde is bound in a configuration such that the aldehyde group interacts in this manner. Support for this interpretation comes from the observation by Lowry *et al.* (1949 b) that pterin-6-aldehyde is slowly oxidized to pterin-6-carboxylate by xanthine oxidase.

CHOLINE OXIDASE

The inhibitions of liver choline oxidase reported by Wells (1954) (Table 2-3) recall the interferences exerted by these and similar analogs on cholinesterase and tissue acetylcholine receptor groups. It would appear that a certain critical distance between the N^+ group and the terminal CH_2OH

TABLE 2-3

INHIBITION OF RAT LIVER CHOLINE OXIDASE BY CHOLINE ANALOGS[a]

Series	Inhibitor R_1	R_2	R_3	Relative rate of oxidation	Equimolar % inhibition	Relative ΔF of binding (kcal/mole)
Ethanolamine	H	H	H	0	23	0
	H	Me	Me	0	53	- 0.81
	H	Et	Et	0	25	- 0.06
	Me	Me	Et	79	–	–
	Me	Et	Et	24	–	–
	Et	Et	Et	0	11	+ 0.55
3-Aminopropanol-1	H	H	H	0	25	- 0.06
	Me	Me	Me	5	15	+ 0.33
	H	Et	Et	0	18	+ 0.19
	Me	Et	Et	8	7	+ 0.88
	Et	Et	Et	0	14	+ 0.38
1-Aminopropanol-2	Me	Me	Me	66	–	–
	H	Et	Et	0	13	+ 0.43
	Me	Et	Et	14	6	+ 0.96
	Et	Et	Et	4	16	+ 0.29
2-Amino-2-methylpropanol-1						
	H	H	H	4	64	- 1.09
	Me	Me	Me	105	–	–
	Et	Et	Et	8	65	- 1.12
2-Amino-2-methylpropanediol						
	H	H	H	3	41	- 0.51
	Me	Me	Me	41	–	–
	Et	Et	Et	5	50	- 0.74

[a] Experiments done with rat liver homogenates. Choline and all analogs at 37.5 m*M*. The rates of oxidation are given relative to that for choline (100). (From Wells, 1954.)

group is necessary for substrate activity. The most reactive substrates are dimethyl or trimethyl compounds, indicating that an exact fit of the cationic head is necessary to place the hydroxyl group in position for oxidation. Substitution of groups on the C-1 atom reduces the binding whereas substitution on the C-2 atom increases the affinity even though the groups are fairly bulky. The simplest interpretation is that the cationic head anchors the molecules in position so that the CH_2OH group can react with an enzyme group on the opposite side of a hole or slit in the protein. When the analogs are too long they do not readily fit into this region, whereas groups protruding from C-2 interact by van der Waals' forces with the walls of the cavity. A three-point attachment of the cationic head is suggested by the reduction in affinity brought about by altering only one of the R groups, this perhaps tilting the molecules so that the hydrocarbon chain is not in the normal direction. The differences in binding energies between these analogs are rather small and this might indicate that dispersion forces are mainly involved, but it may also be that changes in the electrostatic interactions (resulting from the different volumes of the R groups, for example) are offset by opposite changes in the dispersion energy. Since choline must be oxidized to betaine before it can serve as a methyl donor, it is interesting that Wells demonstrated the inhibition of methionine synthesis in liver homogenates by 2-amino-2-methyl-1-propanol and its triethyl derivative. Niemer and Kohler (1957) studied analogs of choline in which one of the methyl groups is substituted by various radicals (e.g., $-CH_2CH_2OH$, $-CH_2CH_2Br$, $-CH_2CH{=}CH_2$, $-CH_2{=}{=}CH_2$, and $-CH_2COO^-$) and found 10–20% inhibition of liver choline oxidase at concentrations approximately equimolar with choline (11.5 m*M*). None of these analogs is a potent inhibitor, confirming the importance of fit at the cationic head.

INHIBITION OF NITROGEN FIXATION BY OTHER GASES

Some simple instances of competitive interference between gases in nitrogen fixation and hydrogen evolution have been observed, and are reminiscent of the suppression of hemoglobin oxygenation by carbon monoxide, nitric oxide, and other gases. The primary product of nitrogen fixation in microorganisms is probably ammonia, and the enzyme system responsible for this is generally termed *nitrogenase*. A few examples of inhibition are given in Table 2-4. Most of these have been shown to be strictly competitive. CO and NO are the most potent inhibitors while H_2 and N_2O are relatively weak. O_2 is a special case in that as pO_2 is increased from zero the nitrogen fixation accelerates, but above a certain value, depending on the organism, the rate falls off (Burris, 1956). Ethane, neon, argon, and helium have no significant effects (Molnar *et al.*, 1948).

TABLE 2-4

INHIBITION OF NITROGEN FIXATION BY VARIOUS GASES

Inhibitor	Organism	General results	Reference
O_2	*Azotobacter vinelandii*	100% inhibition at $pO_2 = 0.6$ atm	Meyerhof and Burk (1928)
		66% inhibition when pO_2 raised from 0.04 to 0.2 atm	Parker (1954)
		Competitive inhibition with $K_m = 0.01$–0.02 atm	Parker and Scutt (1958)
	Achromobacter sp.	100% inhibition at $pO_2 = 0.06$ atm	Goerz and Pengra (1961)
	Soybean nodule slices	Inhibition above $pO_2 = 0.5$ atm with $K_m = 0.025$ atm	Burris (1956)
H_2	*Azotobacter vinelandii*	Competitive inhibition with $K_m = 0.03$ atm and $K_i = 0.15$ atm	Wilson *et al.* (1942)
		35% inhibition at $pH_2 = 0.6$ atm	Molnar *et al.* (1948)
	Clostridium sp.	Competitive inhibition with $K_m = 0.03$ atm and $K_i = 0.1$ atm	Hiai *et al.* (1957)
	Soybean nodules	Marked inhibition at $pH_2 = 0.6$ atm	Bergersen (1960)
NO	*Clostridium pasteurianum*	100% inhibition at $pNO = 0.001$–0.01 atm.	Mozen (1955)
N_2O	*Azotobacter vinelandii*	45% inhibition at $pN_2O = 0.6$ atm	Molnar *et al.* (1948)
		Competitive inhibition with $K_m = 0.02$ atm and $K_i = 0.08$ atm	Repaske and Wilson (1952)
		Competitive inhibition with K_i about 10× greater than K_m	Wilson and Roberts (1954)
	Soybean nodules	100% inhibition at $pN_2O = 0.6$ atm	Bergersen (1960)
CO	Soybean nodules	100% inhibition at $pCO = 0.002$ atm	Bergersen (1960)

Inhibition of *Azotobacter* growth by N_2O occurs if N_2 is the sole source of nitrogen, but does not if ammonia (Repaske and Wilson, 1952) or nitrate (Mozen *et al.*, 1955) is present, indicating that the site of the block is previous to these substances. Likewise, in *Clostridium* H_2 inhibits uptake of N_2 but not of ammonia (Hiai *et al.*, 1957). These inhibiting gases are occasionally utilized. For example, N_2O is assimilated by *Azotobacter*, although slowly, and this is inhibited by N_2 and H_2 (Burris, 1956). It is quite likely that the competition in all these cases, with the possible exception of O_2, is at the nitrogenase active site binding N_2. Nitrogenase is a metalloflavoprotein containing molybdenum and it is reasonable that these gases are bound to the metal, the catalysis of N_2 reduction being of similar type to those mediated by various inorganic metal preparations (which are also inhibited by other gases).

It will be necessary before discussing mechanisms of inhibition in greater detail to consider the enzyme hydrogenase, which has recently been closely linked to nitrogen fixation, and its inhibitions. This enzyme catalyzes the reduction of some unknown primary acceptor by molecular H_2 and the reduced acceptor then transfers the hydrogen atoms to other acceptors, such as dyes, NAD, or eventually oxygen. It has been postulated that it may in some instances participate in the reduction of N_2. The inhibition by O_2 is primarily due to oxygenation of the enzyme:

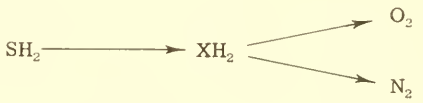
$$E + nO_2 \rightleftarrows E(O_2)_n$$

and this inhibition is reversible upon removal of the O_2 by dialysis (Krasna and Rittenberg, 1954; Fisher *et al.*, 1954). Prolonged exposures to O_2 lead to progressive inactivation of bacterial hydrogenase (Shug *et al.*, 1956). Although n has generally been assumed to be 1, Atkinson (1956) obtained rather complex data possibly indicating a value of 2 for the *Hydrogenomonas facilis* enzyme. The hydrogenase-catalyzed evolution of H_2 in *Rhodospirillum rubrum* (Lindstrom *et al.*, 1949) and soybean root nodules (Hoch *et al.*, 1960) is inhibited by N_2. Although this might be attributed in part to a diversion of the flow of hydrogen atoms to the reduction of N_2, Bregoff and Kamen (1952) observed that 1 mole of N_2 prevents the release of several moles of H_2. One of the difficulties in assuming a direct competition between H_2 and N_2 for the hydrogenase active site is the fact that, despite the inhibition of H_2 evolution by N_2, the exchange reaction whereby HD is formed from D_2 and a hydrogen donor is actually accelerated by N_2 (Hoch *et al.*, 1960). The H_2 inhibition of nitrogen fixation was previously claimed to be competitive, but Parker and Dilworth (1963) found that H_2 causes a lag in the N_2 uptake at low pN_2, whereas in cells of *Azotobacter vinelandii* adapted to H_2 the lag is abolished. Taking the lag phase into account, the inhibition is not competitive; from the reciprocal

plot presented, although the point scatter is marked, the inhibition might be uncompetitive. The inhibition of nitrogen fixation in soybean root nodules by O_2 is complex, due to both plant and bacterial components of the respiration, but is competitive when the O_2 is above 80% (Bergersen, 1962). N_2O is a somewhat more potent inhibitor of H_2 evolution than is N_2, but the most potent is NO, complete and irreversible inhibition being produced by concentrations of 1% or greater (Shug *et al.*, 1956). The hydrogenase in cell-free extracts of *Proteus vulgaris* is inhibited 87% by 0.002% or 0.00004 m*M* NO, and the inhibition at these low concentrations is partially reversible (Krasna and Rittenberg, 1954). The NO is neither oxidized nor reduced by the enzyme.

Although the configurations, electronic structures, and physical properties of these simple gases must be important in determining the interaction with nitrogenase and hydrogenase, it is difficult to establish correlations. Some structural and physical properties that might relate to the interactions of these molecules are given in Table 2-5. Comparing the effects of H_2, N_2O, ethane, and the rare gases on *Azotobacter* nitrogen fixation, Molnar *et al.* (1948) concluded there is no correlation with the van der Waals constants and doubted if any mechanism could be based on physical properties alone. Wilson and Roberts (1954) postulated that N_2O is inhibitory because the N—N distance is close to that in N_2; since N_2O is linear, the oxygen might neither interfere nor be involved in the binding. If the binding is to metal groups on the enzymes, the degree of interaction would depend more on the types of bond possible and hence on the electronic structures of both the gases and the metal, as it is in the interactions of O_2, CO, and NO with hemoglobin and cytochrome oxidase.

The inhibition of nitrogen fixation by O_2 has been explained as a competition between N_2 and O_2 as terminal acceptors for electrons originating in the oxidation of substrates by various dehydrogenases, nitrogen fixation

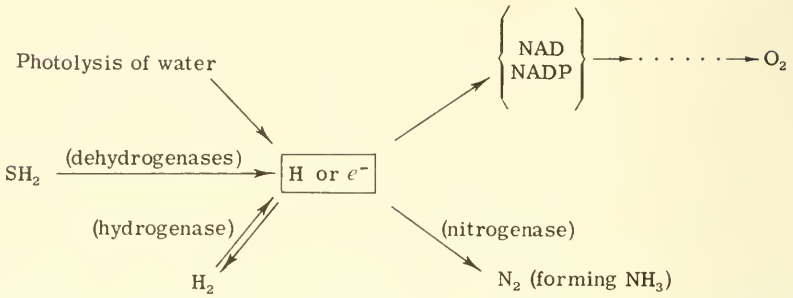


being considered as a form of respiration (Parker, 1954; Parker and Scutt, 1958, 1960). It is likely that the interrelationships between N_2 and H_2 metabolism, and the inhibitions on these systems, must be considered in the light of a hydrogen or electron pool with all the possible pathways for formation and utilization of hydrogen atoms. The scheme below, modified from Gest *et al.* (1956), may serve as a means of visualizing some of these pathways. Some of the inhibitions observed are due to competition for

TABLE 2-5

SOME PHYSICAL PROPERTIES OF GASES INTERACTING WITH NITROGENASE AND HYDROGENASE

Property	Nitrogen	Oxygen	Hydrogen	Nitrous oxide	Nitric oxide
Principal structure	$:N \equiv N:$	$:O \overset{\cdot\cdot}{=} O:$	H — H	$\left\{ \begin{matrix} :\ddot{N}=\overset{+}{N}=\ddot{O}: \\ :N\equiv\overset{+}{N}-\ddot{\underset{\cdot\cdot}{O}}:^{-} \end{matrix} \right\}$	$:N \overset{\cdot\cdot}{\equiv} O:$
Bond distance (Å)	1.094	1.20	0.74	$\left\{ \begin{matrix} N—N & 1.126 \\ N—O & 1.186 \end{matrix} \right\}$	1.151
Mean dimensions (van der Waals' surfaces) (Å)					
Length	4.09	4.00	3.14	5.21	4.05
Width	3.00	2.80	2.40	2.93	2.90
Molar refraction (R_o) (ml/mole)	4.402	3.965	2.037	7.420	4.306
Interaction constants in:					
$\varphi = Ad^{-9} - Bd^{-6}$					
A (erg-cm^9) $\times 10^{81}$	9.90	7.70	0.391	35.6	5.23
B (erg-cm^6) $\times 10^{60}$	199	179	12.1	679	171
Van der Waals' constants					
a (atm-cm^2) $\times 10^{-6}$	1.35	1.36	0.245	—	—
b (ml/mole)	39.6	31.9	26.5	—	—
Coefficient of viscosity (η) (g/cm-sec) $\times 10^4$	1.67	1.93	0.850	1.36	1.79

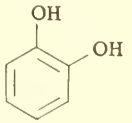


available hydrogen atoms, while others are based on direct competition at the enzyme active sites.

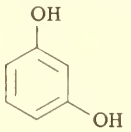
PHENOL OXIDASES

These enzymes usually hydroxylate monophenols in the *ortho* position and further oxidize these to *o*-quinones. They have often been named according to the particular substrate chosen: e.g., catechol oxidase (catecholase), cresolase, tyrosinase, phenolase, *o*-diphenol oxidase, or polyphenol oxidase. Different specificities are observed with enzymes from various sources. The role such enzymes play in tyrosine metabolism will be discussed in the following section.

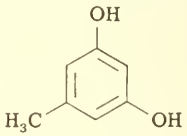
The competitive inhibition of potato catechol oxidase by resorcinol was first observed by Richter (1934). He noticed that the enzymes from various sources exhibited quite different susceptibilities to resorcinol, those from elder (*Sambucus*) and lilac (*Syringa*) being more sensitive than the potato enzyme, and those from mushroom (*Polyporus*) and mealworm (*Tenebrio*) less sensitive. The respiration of apple skin is inhibited about 35% by 50 m*M* resorcinol and thus Hackney (1948) studied an extracted catechol



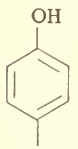
Catechol



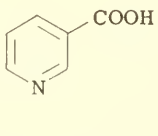
Resorcinol



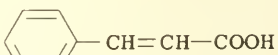
Orcinol



p-Cresol



Nicotinic acid

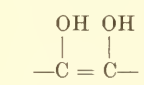


Cinnamic acid

oxidase. Although the inhibition of this enzyme is reduced by increasing catechol concentration, her data do not correspond to pure competitive inhibition and the derived K_i seems to be in error, as pointed out by Warner (1951). A potato enzyme oxidizing *p*-cresol is inhibited by resorcinol, phloroglucinol, and orcinol (see tabulation), the inhibition being completely

Inhibitor (10 m*M*)	% Inhibition (*p*-cresol = 10 m*M*)
Resorcinol	78
Phloroglucinol	43
Orcinol	20

reversible by dialysis and apparently competitive (Schneider and Schmidt, 1959). 4-Chlororesorcinol is a much more potent inhibitor of the potato enzyme, oxidizing either catechol or *p*-cresol, and it has been claimed that the initial inhibition is competitive, although progressive inactivation occurs (Heymann *et al.*, 1954). A K_i of 0.024 m*M* was calculated. However, the double reciprocal plots seem to me to be of the perfectly noncompetitive type. Bonner and Wildman (1946) postulated that the bulk of spinach leaf respiration passes through a polyphenol oxidase. *p*-Nitrophenol is a rather potent inhibitor of this respiration, 1 m*M* inhibiting 94%, and of the polyphenol oxidase, whether it is oxidizing catechol or *p*-cresol. On the other hand, *o*-nitrophenol is only a weak inhibitor of both. It was felt that *p*-nitrophenol might well be an analog of naturally occurring substrates, and *o*-nitrophenol an analog of *o*-phenols against which the enzyme is inactive. It is interesting, finally, to note that dihydroxymaleate is an inhibitor of catechol oxidase (Florkin and Duchateau-Bosson, 1939), and it was suggested that the



grouping complexes with the enzyme in a manner similar to the analogous catechol grouping. Most of this work on inhibiting phenolic compounds is unsatisfactory from the quantitative standpoint and clear-cut proofs of competitive inhibition are lacking. Nevertheless, effective analog inhibition for this class of enzymes has been demonstrated.

We shall now turn to a more potent, more interesting, and more thoroughly studied type of inhibitor, namely, the benzoates. The inhibition of mushroom catechol oxidase by benzoate itself was attributed to a competition with the substrate for the active center, although no direct evidence for this was adduced (Ludwig and Nelson, 1939; Gregg and Nelson, 1940), but good competitive kinetics for the inhibition by *m*-hydroxybenzoate

were observed later (Warner, 1951), with K_i 2.5 mM for the potato enzyme and 0.6 mM for the mushroom enzyme. An excellent investigation by Kuttner and Wagreich (1953) of the inhibition of a catechol oxidase from *Psalliota campestris* provides data from which some ideas of the mechanism may be obtained. Some of the inhibition data are presented in Table 2-6, and have been used to calculate the apparent relative binding energies. However, these inhibitors are mostly weak acids and the degree

TABLE 2-6

INHIBITION OF A PHENOL OXIDASE FROM *Psalliota campestris* WITH CATECHOL (1.82 mM) AS SUBSTRATE (AT pH 5.2 AND 25°)

Inhibitor	Concentration (mM)	% Inhibition	Relative $-\Delta F$ of binding[a] (kcal/mole)
Benzoate	0.012	50	6.96
p-Chlorobenzoate	0.023	50	6.56
p-Methylbenzoate	0.04	50	6.23
o-Chlorobenzoate	0.21	50	5.21
p-Methoxybenzoate	0.26	51	5.10
Nicotinate	0.34	48	4.88
o-Hydroxybenzoate	0.38	50	4.84
p-Nitrophenol	0.48	50	4.70
p-Hydroxybenzoate	0.49	50	4.69
trans-Cinnamate	0.81	52	4.43
Phenylacetate	0.77	50	4.40
o-Methylbenzoate	1.0	50	4.25
o-Nitrophenol	1.1	50	4.19
Benzoate methyl ester	0.65	30	3.94
Hydroquinone	2.2	50	3.76
p-Nitrobenzoate	0.72	19	3.55
Orcinol	3.2	50	3.53
Resorcinol	4.5	50	3.32
o-Nitrobenzoate	0.72	8	2.95
o-Methoxybenzoate	8.0	49	2.94

[a] The relative energies of binding to the enzyme were calculated assuming competitive inhibition and without taking into account the state of ionization. (Data from Kuttner and Wagreich, 1953.)

of ionization at the experimental pH of 5.2 varies. It was found that the inhibition by benzoate decreases with rise in the pH (see tabulation) and

pH	% Inhibition by benzoate (0.0123 m*M*)	% Un-ionized
5.2	56	9.0
5.8	28	2.5
6.4	8	0.6
7.0	0	0.2

similar results were obtained with some substituted benzoates. This was interpreted to mean that the un-ionized form of the inhibitor is the active one, for example that benzoic acid is the inhibitor and not the benzoate ion. The relative binding energies have been recalculated (Table 2-7) on this basis and a somewhat different order of potency is obtained. This il-

Table 2-7

Inhibition of Mushroom Phenol Oxidase Corrected for the State of Ionization of the Inhibitors [a]

Inhibitor	pK_a	(HA)/(A_t)	Corrected relative $-\Delta F$ of binding (kcal/mole)
Benzoate	4.203	0.0914	8.44
o-Chlorobenzoate	2.943	0.0055	8.39
p-Chlorobenzoate	3.978	0.0565	8.32
o-Hydroxybenzoate	3.001	0.0063	7.95
p-Methylbenzoate	4.371	0.129	7.47
o-Nitrobenzoate	2.173	0.00094	7.24
p-Methoxybenzoate	4.470	0.157	6.24
o-Methylbenzoate	3.909	0.0487	6.10
p-Nitrobenzoate	3.425	0.0165	6.07
p-Hydroxybenzoate	4.559	0.186	5.71
Nicotinate	4.854	0.311	5.59
o-Methoxybenzoate	4.092	0.0724	4.56

[a] Relative binding energy corrected on the basis that the un-ionized forms of the inhibitors are active. The ionization constants were obtained from Dippy (1939); inhibition data are given in Table 2-6 (Kuttner and Wagreich, 1953.)

lustrates the importance of taking ionization into account in the comparison of inhibitors, as discussed earlier in the chapter. It must be emphasized that the absolute values of the binding energy are meaningless; it is only the differences between the $-\Delta F$ values that are significant.

Before discussing the implications of these results we will examine the data reported by Krueger (1955) on the inhibition of a mushroom enzyme oxidizing *p*-cresol (Table 2-8). Confirming the work of Kuttner and Wag-

TABLE 2-8

INHIBITION OF *p*-CRESOL OXIDATION BY MUSHROOM TYROSINASE[a]

Inhibitor	Concentration (m*M*)	% Inhibition		Relative $-\Delta F$ of binding (kcal/mole)
		pH 5.3	pH 7.0	
Benzoate	4*	90	27	5.20
Oxalate	4*	83	—	4.78
Cyclohexanecarboxylate	16	84	—	3.57
Phenylacetate	4	54	0	3.51
Fluoride	40*	72	14	2.99
Butyrate	40	73	10	2.60
Bromide	40*	56	5	2.56
Iodide	40*	54	8	2.51
Benzamide	4*	8	—	2.33
Lactate	20	40	0	2.16
Terephthalate	4	11	—	2.12
Phthalate	4	10	—	2.05
Chloride	40	45	5	1.85
Formate	4	6	—	1.71
	20	24	—	
	40*	10	0	
Acetate	20	21	—	1.68
	40	42	6	
Trimethylacetate	40	30	4	1.46
Chloroacetate	20	16	0	1.39
Benzenesulfonate	40	20	0	1.13
Trichloroacetate	20	8	—	0.91

[a] Concentration of *p*-cresol was 4.63 m*M* except where indicated by asterisks, in which cases it was 9.26 m*M*. The relative binding energies were calculated for pH 5.3 and were not corrected for ionization. (Data from Krueger, 1955.)

reich, a marked decrease in the inhibition with an elevation of the pH from 5.3 to 7 is observed, but Krueger interpreted this as indicating an ionizing group on the enzyme with a pK_a around 6 and possibly an imidazole group. One reason for assuming the ionizing group to be on the enzyme is the pH effect on inhibitions by the inorganic anions; however, the inhibition by these ions may be through a different mechanism than the benzoates, and indeed Krueger found noncompetitive kinetics for chloride. The substrates for the enzyme are, of course, un-ionized and this might favor the concept of the acid form of the inhibitors being active and the important ionizing group on the inhibitors. It is impossible at the present time to decide which is the correct interpretation and hence the significance of the pH effects. Ionizing groups on both enzyme and inhibitors might also be considered. It should be added that the following were found to be without effect: sulfate (40 m*M*), nitrate (40 m*M*), pyrophosphate (40 m*M*), pyruvate (20 m*M*), succinate (20 m*M*), maleate (20 m*M*), fumarate (20 m*M*), and ethyl benzoate (4 m*M*).

The lack of information on the exact catalytic mechanism involved in these enzymes, and particularly our ignorance of the state and role of copper, make it difficult to understand the binding of the inhibitors. There are two copper ions at the active center but we do not know if they complex with oxygen, or the substrates, or both, or whether one copper complexes with oxygen and the other with the substrates. Some of the ionic inhibitions might be due to the formation of complexes with the copper; such complexes might be more difficult to form at higher pH's because of competition with hydroxyl ions. The strong inhibition by oxalate might be due to chelation of the copper, but it is odd that pyrophosphate does not inhibit since it also chelates copper well. It is also surprising that sulfate and nitrate do not inhibit at all, unless the ionic size is as critical as Krueger believes.

It would appear that inhibitory activity is related to the presence of a carboxyl group (excepting the inorganic ions). Benzoate ester and benzamide are bound much less tightly than benzoate (around 3 kcal/mole difference). The weak action of benzenesulfonate might be explained in three ways; (1) if the un-ionized form of the acid is necessary for activity, there would be less in the case of benzenesulfonic acid than with benzoic acid, (2) the sulfonate group might be too bulky, according to Krueger (although it is certainly not much larger than the carboxyl group), and (3) the sulfonate group does not have the ability to form bonds with the copper or hydrogen bonds with an enzyme group. Separation of the carboxyl from the benzene ring reduces the binding, as in phenylacetate or cinnamate. The second requirement for potent inhibitory activity is a benzene ring. This may be seen by comparing acetate and phenylacetate, benzoate and nicotinate, and benzoate and cyclohexanecarboxylate; in

the last example the increased binding of benzoate may be due to its greater polarizability. In general the substitution of groups on the benzene ring reduces the affinity for the enzyme. This may be due to steric interference with the approach of the ring or to inductive effects on the carboxyl group's interaction with the enzyme. It is rather odd that an *ortho* chlorine does not disturb binding much while an *ortho* methoxy group reduces the binding some 4 kcal/mole.

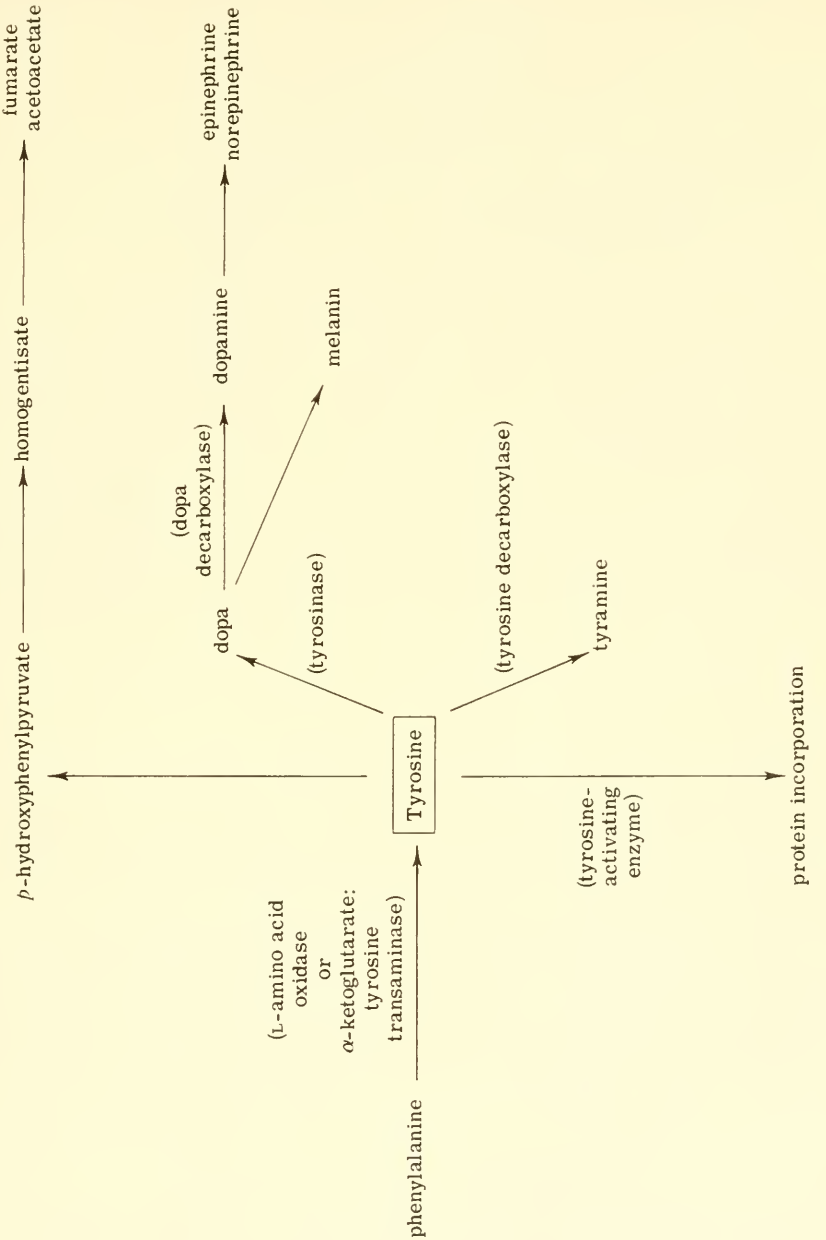
The forces binding the substrates and inhibitors to these enzymes are thus vague at the present time. It is possible that hydrogen bonds between the OH or COOH groups and the enzyme are important, and it is equally possible that bonds to the copper ions are involved. One might conceive of the inhibitor's COOH group reacting with either the two copper ions, or with a copper ion and a vicinal —NH— group. Copper ions are able to catalyze the oxidation and hydroxylation of phenols nonenzymically, and it might be interesting to study the inhibition of such reactions by some of the compounds active in the enzymic reaction.

TYROSINE METABOLISM

Many interesting and practically important examples of analog inhibition are to be found in the general field of amino acid metabolism, and we shall begin the discussion of this subject by considering the inhibitions of the various pathways of tyrosine metabolism. Tyrosine may be hydroxylated to form dihydroxyphenylalanine (dopa), oxidatively deaminated or transaminated to form *p*-hydroxyphenylpyruvate, decarboxylated to form tyramine, or activated prior to incorporation into proteins; inhibition of all of these reactions by analogs has been reported. The scheme on page 303 indicates the major pathways of tyrosine metabolism. Interference with these reactions might be expected to bring about physiological changes due to the acceleration or suppression of active amine synthesis, and also to affect melanin formation.

Tyrosinase (Phenol Oxidase)

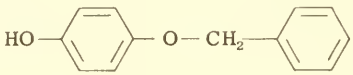
These enzymes hydroxylate tyrosine in the *ortho* position to form dopa and further oxidize dopa to dopa-quinone. The enzymes discussed in the previous section generally possess this activity. However, mammalian tyrosinases are much more specific than the plant enzymes and oxidize tyrosine and dopa more rapidly than other phenols. Inhibitions of the enzymes with tyrosine as the substrate will be considered in this section, but the results with *p*-cresol or catechol as substrate are probably generally applicable to tyrosinase activity. The high inhibitory potency of 4-chlororesorcinol on potato tyrosinase, as determined by the rate of formation of


phenylalanine
(L-amino acid oxidase or α-ketoglutarate: tyrosine transaminase)
Tyrosine
p-hydroxyphenylpyruvate
homogentisate
fumarate
acetoacetate
dopa
(dopa decarboxylase)
dopamine
epinephrine
norepinephrine
(tyrosinase)
melanin
(tyrosine decarboxylase)
tyramine
(tyrosine-activating enzyme)
protein incorporation

melanin from tyrosine, was noted by Kull *et al.* (1954), for example (see following tabulation). Other substitutions in the 4-position usually reduce

Inhibitor	Lowest inhibitory concentration (m*M*)
4-Chlororesorcinol	0.000069
Resorcinol monobenzoate	0.0047
m-Aminophenol	0.0092
4-*n*-Hexylresorcinol	0.103
Naphthoresorcinol	0.125
Orcinol	0.141
p-Aminophenol	0.183
Resorcinol	0.183
Phloroglucino'	6.17
2-Nitroresorcinol	65

or abolish the inhibitory activity. The formation of melanin involves several steps and the inhibitions observed are not necessarily entirely on tyrosinase. Hydroquinone was found to be a weak inhibitor but the monobenzyl ether of hydroquinone is as potent as resorcinol. This latter substance,



Monobenzone (Benoquin)

known also as monobenzone (Benoquin), is an inhibitor of melanin formation in the skin when applied topically, can produce leucoderma in Negroes, and is used in various conditions of melanosis. Some have thought that it releases hydroquinone after penetration into the skin but this is questionable in view of its own inhibitory activity.

An active tyrosinase occurs in the Hardin-Passey mouse melanoma and is probably responsible for pigment formation. It is competitively inhibited by various tyrosine analogs (Lerner *et al.*, 1951). The values of K_i shown in the tabulation were calculated on the basis of a K_m of 0.60 m*M*

Inhibitor	K_i (m*M*)
N-Acetyl-L-tyrosine	0.140
N-Formyl-L-tyrosine	0.177
3-Amino-L-tyrosine	0.314
3-Fluoro-L-tyrosine	1.25

obtained from the reciprocal plots. 3-Nitro-L-tyrosine and *O*-methyl-L-tyrosine are not inhibitory. The effects of 3-substitution may be mediated through inductive effects on the 4-OH group and its interaction with the enzyme, whereas *N*-substitution must lead to an altered position of binding to prevent oxidation. L-Phenylalanine and phenylpyruvate inhibit competitively the tyrosinase from melanoma, inhibit the incorporation of tyrosine-C^{14} into melanin, and depress the respiration of tumor tissue with tyrosine as the substrate (Boylen and Quastel, 1962). The high concentrations of these inhibitors in phenylketonuria might be responsible for the reduced pigment formation in these individuals.

Tyrosine : α-Ketoglutarate Transaminase

The effects of numerous analogs on the formation of *p*-hydroxyphenylpyruvate from tyrosine and α-ketoglutarate by a dog liver transaminase were reported by Canellakis and Cohen (1956 b); some of the results are given in Table 2-9. Certain of these analogs are transaminated (e.g., the 3-substituted tyrosines) and the inhibitory activity varies inversely with their abilities to act as substrates. The rapid transamination of 3-fluorotyrosine may partly explain its toxic effects and inhibition of growth, since fluorofumarate or fluoroacetoacetate may be formed. Comparing the hydroxyl-substituted phenylalanines, it is seen that a *m*- or *p*-hydroxyl is necessary for tight binding, the contribution to the binding energy being over 2 kcal/mole. The K_m for L-tyrosine is 0.71 m*M*, so that its relative binding energy is at least − 4.47 kcal/mole, and that of *m*-hydroxy-DL-phenylalanine is at least − 4.75 kcal/mole (− 5.18 kcal/mole if only the L-isomer is active), which may be compared with the − 2.55 kcal/mole for L-phenylalanine. Hydroxyl groups in the *o*-positions, on the other hand, do not augment binding very much. The tighter the binding between a basic hydroxyl and an acidic enzyme group, the greater the inhibitory activity; ring substituents modify the electronic character or basicity of the phenolic group. A carboxylate group is necessary for strong binding, as may be seen by comparing tyrosine with tyramine, and *p*-hydroxybenzoate with *p*-cresol. The α-amino group may also be involved in the binding (− ΔF for *p*-hydroxyphenylacetate is 2.87 kcal/mole), but lacking data on *p*-hydroxyphenylproprionate it is not possible to evaluate this accurately. The strong inhibition by epinephrine is rather surprising in the light of the absence of a carboxylate group, but the β-hydroxyl or *N*-methyl group may contribute to make up for this deficiency. A similar study on the rat liver enzyme has been reported by Jacoby and La Du (1964).

p-Hydroxyphenylpyruvate Oxidase

The further oxidation of the product of tyrosine transamination is catalyzed by an enzyme from dog liver and is inhibited markedly by phenyl-

TABLE 2-9

INHIBITION OF TYROSINE: α-KETOGLUTARATE TRANSAMINASE FROM DOG LIVER[a]

Inhibitor	(I)/(S)	% Inhibition	Relative $-\Delta F$ of binding (kcal/mole)
3-Amino-DL-tyrosine	1	85	6.00
3,4-Dihydroxy-L-phenylalanine	2	70	5.03
Epinephrine	2	70	5.03
m-Hydroxy-DL-phenylalanine	2	60	4.75
2,5-Dihydroxy-L-phenylalanine	2	35	4.13
3-Fluoro-DL-tyrosine	2	20	3.65
3,5-Dibromo-L-tyrosine	2	10	3.15
o-Hydroxy-DL-phenylalanine	2	7	2.91
p-Hydroxybenzoate	10	26	2.87
p-Hydroxyphenylacetate	10	26	2.87
p-Aminobenzoate	10	21	2.70
L-Tryptophan	10	21	2.70
p-Nitro-DL-phenylalanine	2	5	2.69
L-Phenylalanine	2	4	2.55
o-Hydroxybenzoate (salicylate)	10	17	2.53
p-Nitrobenzoate	10	15	2.44
3,5-Diiodo-L-tyrosine	2	3	2.36
D-Tyrosine	2	3	2.36
p-Cresol	10	13	2.34
Tyramine	10	9	2.09

[a] Relative binding energies were calculated on the basis of competitive inhibition with $K_m = 0.71$ mM for L-tyrosine. No correction for ionization was made. It should be noted that the different isomers of the DL-compounds may have different activities, so that the binding energy of the active form should be increased. (Data from Canellakis and Cohen, 1956 b.)

pyruvate, essentially complete inhibition occurring with 0.4 mM in the presence of 2 mM substrate (Zannoni and La Du, 1959). The inhibition is negligible for the first 5 min but then increases to become complete at around 20 min. This might be due to protection by the substrate and preincubation experiments would have been informative. An equally good inhibitor is *m*-hydroxyphenylpyruvate but phenylacetate, 2,5-dihydroxyphenylpyruvate, *p*-hydroxyphenyllactate, *p*-hydroxybenzoate, and homogentisate are not inhibitory.

Other Pathways of Tyrosine Metabolism

Little is known about the effects of analogs on tyrosine decarboxylation, although this might well be an important site to block if one wished

to reduce the tissue tyramine concentration. Mardashev and Semina (1961) found that the tyrosine decarboxylase from *Streptococcus fecalis* is inhibited 20% by 8.3 m*M* cysteine and 25% by 8.3 m*M* homocysteine when substrate concentration is 2.8 m*M*. This is a general phenomenon seen with several amino acid decarboxylases and is presumably due to the formation of complexes of the inhibiting amino acids with pyridoxal phosphate.

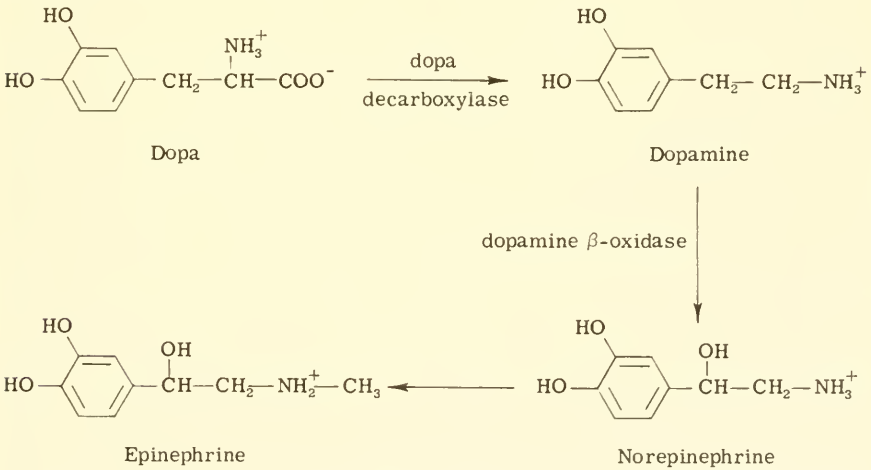
The tyrosine-activating enzyme of pig pancreas catalyzes the first

$$\text{Amino acid} + \text{ATP} + \text{E} \rightarrow \text{E-amino acyl-AMP} + \text{PP}$$

step in the incorporation of amino acids into proteins. This also would be a very interesting step to investigate from the standpoint of analog inhibition, but our present information is meager. Schweet and Allen (1958) found that 3-fluoro-L-tyrosine activated 50% the rate of L-tyrosine and does not inhibit. L-tyrosinamide inhibits weakly but tyramine inhibits the phosphate exchange reaction 80% at 0.2 m*M*, this inhibition being reduced by increase in substrate concentration. The importance of the *p*-hydroxyl group is indicated by the fact that no inhibition is seen with phenylethylamine.

Dihydroxyphenylalanine (Dopa) Decarboxylase

This enzyme is on the pathway leading from tyrosine to the important catecholamines, epinephrine and norepinephrine, and lately has been the subject of much investigation because of the possible clinical applications of producing a selective block at this step. Inhibitors have indeed been found which are effective *in vivo*, reduce amine formation, and lower the blood pressure. The sequence of reactions for the formation of amines from dopa is the following:



A block of dopa decarboxylase would thus decrease the rate of formation of these three physiologically active amines in the tissues.

Of historical interest are the following observations on the analog inhibition of this enzyme: dopamine (Blaschko, 1942), epinephrine (Schapira, 1946), various aromatic amines (tryptamine, tyramine, phenylethylamine, etc.) (Polonovski *et al.*, 1946), and the various hydroxy, methoxy, and dimethoxy derivatives of phenylethylamine, the dimethoxy analogs being the most potent (Gonnard, 1950).

These studies were extended in important ways by Sourkes (1954), who discovered the potent inhibiting activity of certain α-methylphenylalanines (Table 2-10). Especially inhibitory is α-methyldopa and this sub-

TABLE 2-10

INHIBITION OF DOPA DECARBOXYLASE FROM PIG KIDNEY CORTEX [a]

Inhibitor	Concentration (mM)	% Inhibition	Relative $-\Delta F$ of binding (kcal/mole)
α-Methyldopa	0.01	22	5.94
	0.1	71	
	0.5	98	
α-Methyl-3-hydroxy-PA	0.05	45	5.57
	0.5	74	
	5	95	
α-Methyl-3-hydroxy-4-methoxy-PA	4.3	92	4.86
α-Methyl-3,4-dimethoxy-PA	0.01	16	4.65
	0.1	20	
N-Acetyl-3,4-dimethoxy-PA	3.6	44	3.32
N-Methyldopa	0.8	11	3.12
	1.7	30	
Diiodotyrosine	2.3	15	2.68
3,4-Dimethoxy-PA	5	25	2.59
2,4-Dimethoxy-PA	5	22	2.49
3-Methoxy-4-hydroxy-PA	5	18	2.33
α-Methyl-3-methoxy-PA	5	18	2.33
α-Methyltyrosine	5	16	2.25
α-Methyl-PA	2	6	2.13
N-Methyl-3-methoxy-4-hydroxy-PA	1.6	0	—

[a] Concentration of DL-dopa 4 mM, pH 6.8, preincubation with inhibitor 15 min. PA = phenylalanine, and dopa = 3,4-dihydroxyphenylalanine. Relative binding energies calculated on the basis of competitive inhibition, which may not be strictly true; in any event, these values give a better indication of the relative inhibitory potency than the per cent inhibition at different concentrations. (Data from Sourkes, 1954.)

stance has been thoroughly studied biochemically and pharmacologically during recent years. It is interesting that these analogs have very little inhibitory activity toward tyrosine decarboxylase and that α-methyltyrosine does not inhibit dopa decarboxylase strongly, both facts pointing to the importance of the 3-hydroxyl group in the binding to the enzyme. This is also seen by comparing α-methylphenylalanine and its hydroxylated derivatives: The addition of a 4-hydroxyl has little effect, whereas a 3-hydroxyl increases the binding energy over 3 kcal/mole. A 3-methoxy group seems to be ineffective.

The inhibition by α-methyldopa was shown to be pseudoirreversible by varying the enzyme concentration and using the graphic procedure of Ackermann and Potter (1949). At concentrations of 0.01–0.03 mM, the inhibition being 15–25%, the behavior is fairly reversible, but at concentrations of 0.1 mM or above there is marked nonlinearity of the curves. As pointed out by Sourkes, these data indicate merely that K_i is low and the affinity for the enzyme is high. The binding might be to the apoenzyme, to a great extent through the phenolic groups, or the inhibition could be the result of reaction with pyridoxal phosphate. The former mechanism was favored by Sourkes on the basis of the following evidence against a reaction with the coenzyme. (1) The inhibition is reversible by dialysis. (2) The rate of nonenzymic reaction of α-methyldopa with pyridoxal phosphate is too slow at inhibiting concentrations to be significant. (3) Increase in pyridoxal phosphate concentration does not alter the inhibition significantly. (4) Analysis for pyridoxal phosphate at the end of inhibition experiments showed no loss. (5) Tyrosine decarboxylase is also a pyridoxal phosphate enzyme and is not inhibited. None of this evidence is completely conclusive and it is possible that α-methyldopa can form a reversible complex with pyridoxal phosphate on the enzyme surface, so that increase in coenzyme concentration would not be effective and analysis for total coenzyme would not detect the small amount combined. 5-Hydroxytryptophan decarboxylase is also potently inhibited by α-methyldopa (it is possible that the decarboxylases for dopa, 5-hydroxytryptophan, tryptophan, tyrosine, and phenylalanine in mammalian tissues represent a single enzyme) and S. E. Smith (1960 a) has investigated the mechanism, using the mouse brain enzyme. Plots of $1/(S)$ against $1/v$ showed pure competitive inhibition with respect to substrate at higher coenzyme concentrations (above 0.01 mM), but at low coenzyme concentrations the inhibition becomes noncompetitive with substrate. In contrast to dopa decarboxylase, increase of coenzyme concentration leads to a reduction in the inhibition (Fig. 2-3). Smith inclines to a coenzyme inactivation mechanism but admits that the inhibition is incompletely explained. If α-methyldopa forms a complex with pyridoxal phosphate on the enzyme surface, which it can do because it is decarboxylated slowly, it might be considered to be an in-

hibitor which enters into the catalytic sequence of reactions but is not able to complete the process readily. This mechanism is also suggested by the results of Lovenberg *et al.* (1963) on kidney aromatic amino acid decarboxylase, using tryptophan as the substrate. When α-methyldopa is preincubated with the enzyme in the absence of pyridoxal-P the inhibition is noncompetitive and potent, but when pyridoxal-P is added during the preincubation period the inhibition is competitive with respect to substrate

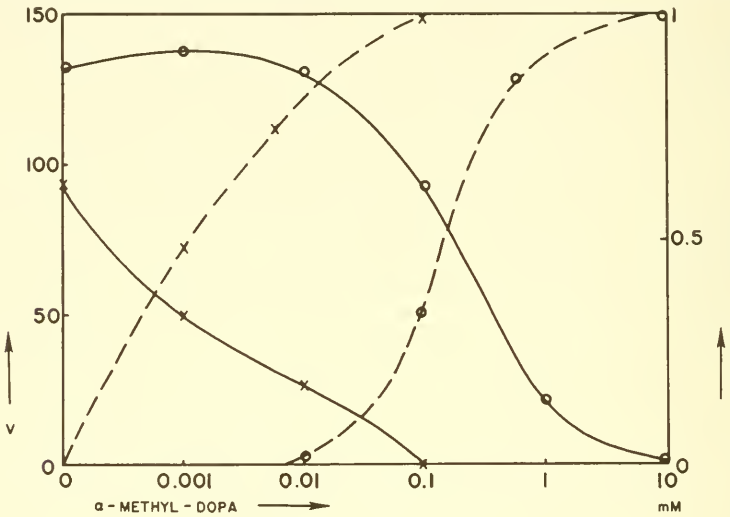


FIG. 2-3. Inhibition of mouse brain 5-hydroxytryptophan decarboxylase by α-methyldopa. The solid curves show the rate of formation of serotonin in μg/g/hr, and the dashed curves show the fractional inhibition. The curves X-X-X-X show the results without addition of pyridoxal-P, and the curves O-O-O-O show the results after addition of 0.008 m*M* pyridoxal-P. (From S. E. Smith, 1960 a.)

and is less potent. If no preincubation is done and α-methyl dopa is added with the substrate, competitive inhibition is observed. The data suggest that α-methyldopa interacts specifically with the enzyme-pyridoxal-P complex (the enzyme as isolated contains tightly bound pyridoxal-P), and the protection or reversal of the inhibition by exogenous pyridoxal-P may be due to the reactivation of the enzyme-bound coenzyme. The reaction of the α-methyldopa with pyridoxal-P may involve the cyclization of a Schiff base (Mackay and Shepherd, 1962).

Another comprehensive study of dopa decarboxylase was made by Hartman *et al.* (1955), who determined the inhibitory activities of some 200 compounds. The results, some of which are presented in Table 2-11, enable

TABLE 2-11

INHIBITION OF DOPA DECARBOXYLASE FROM PIG KIDNEY CORTEX[a]

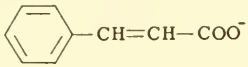
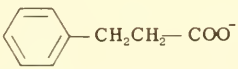
Inhibitor	Concentration (m*M*)	% Inhibition	Relative - ΔF of binding (kcal/mole)
Cinnamates 			
3-Mercapto-	0.1	78	6.90
3, 4-Dihydroxy- (ethyl ester)	0.3	90	6.78
2, 6-Dihydroxy-	0.2	84	6.70
3, 4-Dihydroxy-	0.4	74	5.90
2-Hydroxy-3, 5-dibromo-	0.4	65	5.63
3-Hydroxy- (methyl ester)	0.4	60	5.50
2-Hydroxy- (methyl ketone)	0.2	31	5.19
3-Hydroxy- (methyl ketone)	0.2	30	5.15
3-Hydroxy-	0.4	37	4.92
α-Methyl-3-hydroxy-	0.4	31	4.75
3-Hydroxy-6-sulfonate-	0.4	31	4.75
α-Ethyl-3-hydroxy-	0.4	27	4.64
3-Hydroxy- (amide)	0.4	19	4.35
2-Hydroxy-	2	52	4.30
2, 4-Dichloro-	2	41	4.03
2-Chloro-	2	36	3.91
4-Hydroxy-	0.4	7	3.65
3-Nitro-	2	15	3.19
3-Amino-	2	10	2.91
2, 4-Dihydroxy-	2	9	2.83
2, 3-Dimethoxy-	2	8	2.75
4-Chloro-	2	8	2.75
Unsubstituted	2	0	–
Hydrocinnamates 			
3-Hydroxy-2, 4, 6-triiodo-	0.2	90	7.04
α-Methyl-3-hydroxy-2, 4, 6-triiodo-	0.4	91	6.68
3, 4-Dihydroxy-	0.44	63	5.52
α-Methyldopa	0.2	37	5.35
4-Hydroxy-	2	9	2.83
3-Hydroxy-	2	0	–

TABLE 2-11 (*continued*)

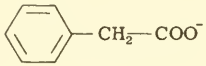
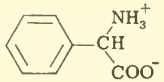
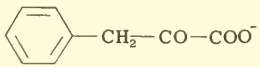
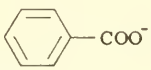
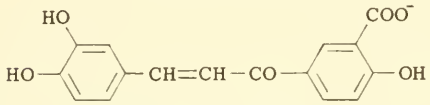
Inhibitor	Concentration (m*M*)	% Inhibition	Relative - ΔF of binding (kcal/mole)
α-Hydroxy-	2	0	–
Unsubstituted	2	0	–
Phenylacetates 			
3, 4-Dihydroxy-	2	88	5.49
2, 5-Dihydroxy-	2	28	3.68
3, 4-Dimethoxy-	2	18	3.32
3-Hydroxy-	2	11	2.97
Unsubstituted	2	0	–
Phenylglycines 			
3, 4-Dihydroxy-	2	16	3.24
3-Hydroxy-	2	0	–
4-Hydroxy-	2	0	–
Unsubstituted	2	0	–
Phenylpyruvates 			
3, 4-Dihydroxy-	0.2	60	5.92
3-Hydroxy-	1	72	5.26
4-Hydroxy-	2	60	4.50
2, 5-Dihydroxy-	2	60	4.50
Unsubstituted	2	60	4.50
Benzoates 			
2-Hydroxy-3, 5-diiodo-	0.2	47	5.60
2-Hydroxy-3, 5-dibromo-	0.2	33	5.24
3, 5-Dibromo-	0.2	10	4.33
3, 4, 5-Trihydroxy-	2	42	4.07
3-Hydroxy-4, 6-dibromo-	2	36	3.91
2-Hydroxy-5-amino-	2	28	3.68
2-Hydroxy-4-nitro-	2	26	3.62

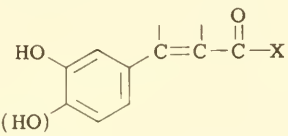
TABLE 2-11 (*continued*)

Inhibitor	Concentration (m*M*)	% Inhibition	Relative - ΔF of binding (kcal/mole)
2, 5-Dihydroxy-	2	21	3.44
2, 4, 6-Trihydroxy-	2	16	3.24
3, 4-Dihydroxy-	2	0	–
2, 4-Dihydroxy-	2	0	–
Miscellaneous			
5-(3, 4-Dihydroxycinnamoyl)salicylate	0.002	87	9.69



[a] Concentration of dopa 2 m*M* and pH 6.8. (Data from Hartman *et al.*, 1955.)

one to speculate further about the nature of the binding to the active center. Several inhibitors more potent than α-methyldopa were found. The basic structure for inhibition was written as:



where X is OH, O-alkyl, alkyl, or aryl. It is rather surprising that the negatively charged carboxylate group is not necessary, esters and amides being as potent as the acids, and it may be that the CO group is critical. The positively charged amino group is also not necessary, since 3,4-dihydroxyhydrocinnamate is a good inhibitor, and this would make it likely that the binding of the inhibitors is not too much dependent on pyridoxal phosphate. The importance of the 3- and 4-hydroxyls is again evident and all the potent inhibitors have phenolic groups; apparently only the sulfhydryl group can replace the hydroxyl. Halogens have the ability to augment binding when they are the only substituents but particularly when a hydroxyl group is also present; 3,5-dibromobenzoate and 2,4-dichlorocinnamate are bound reasonably well (at least 2 kcal/mole more than the unsubstituted compounds). The only unsubstituted inhibitor is phenylpyruvate, which must be significant, although of what is not clear. The interaction of the side chain must be complex and involve different types of forces. If one compares all the 3,4-dihydroxy derivatives, it is seen that

inhibitory activity increases with the length or bulk of the side chain. Also it may be noted that the linear cinnamate derivatives are generally more potent than the hydrocinnamates. One must conclude that the most important binding groups are the hydroxyls, the ring, the side-chain carbonyl, and any more terminal groups, which just about includes all of the molecule. The specificity of these inhibitors may well be quite high, since 3-hydroxycinnamate and 3,4-dihydroxycinnamate (caffeate) were tested on tyrosine decarboxylase, glutamate decarboxylase, histidine decarboxylase, and succinate dehydrogenase, and found to be without effect. Tyrosinase is inhibited somewhat, especially by caffeate.

There is evidence in patients with phenylpyruvic oligophrenia of a disturbance in tyrosine metabolism (hypopigmentation), and it is possible that the phenyl acids which are abnormally high might be inhibiting some step or steps in these pathways. Fellman (1956) therefore studied the effects of such substances on the dopa decarboxylase from beef adrenal medulla. The order of inhibitory potency is phenylpyruvate > phenyllactate > phenylacetate > phenylalanine. Phenylpyruvate inhibits 77% when equimolar (3.3 m*M*) with L-dopa. The low plasma epinephrine levels found in these patients thus might be due to such an inhibition, but another point of attack would have to be adduced for a suppression of melanin formation. It is interesting that this enzyme is inhibited quite strongly by norepinephrine and dopamine, whereas epinephrine exerts no effect (Fellman, 1959). The susceptibilities of dopa decarboxylases from various tissues are obviously different, since the results of Fellman are often different from those of previous workers.

α-Methyldopa and related analogs can effectively inhibit decarboxylases *in vivo*, thereby interfering with amine formation and modifying tissue function. Direct evidence for an *in vivo* inhibition was obtained by intramuscular injection of α-methyldopa into guinea pigs and demonstration of a marked depression of the decarboxylation of both dopa and 5-hydroxytryptophan in isolated kidney 15–30 min afterward (Westermann *et al.*, 1958). Indeed, the inhibition of 5-hydroxytryptophan decarboxylation is complete and after 90 min is 83%. More indirect evidence has been obtained by showing that these analogs prevent the pharmacological actions of dopa and 5-hydroxytryptophan, these actions being dependent on decarboxylation of these substances to dopamine and serotonin, respectively. Injection of dopa leads to a rise in the blood pressure which is probably primarily due to dopamine (although some norepinephrine and epinephrine may also be formed). This pressor response can be blocked by several decarboxylase inhibitors, including 5-(3-hydroxycinnamoyl)salicylate (Pogrund and Clark, 1956) and α-methyldopa (Dengler and Reichel, 1958). There is no effect on the response to dopamine or norepinephrine. The increase in cardiac contractility induced by dopa is also completely blocked

by α-methyldopa. 5-Hydroxytryptophan causes bronchoconstriction in guinea pigs and central excitation in mice (if brain monoamine oxidase is blocked), these effects being due to serotonin, and pretreatment of the animals with α-methyldopa prevents these actions (Westermann *et al.*, 1958).

Decarboxylase inhibition should lead to a decrease in the tissue concentrations of certain amines and this has been demonstrated. The degree of reduction will depend on the relative rates of formation and metabolism of the amines, as well as on the magnitude of the decarboxylase inhibition (which will depend in part on the penetration of the analogs into the tissues), since we are dealing with steady-state multienzyme systems. Injection of α-methyldopa (200 mg/kg) into dogs leads to a lowering of serotonin in the caudate nucleus (1.03 to 0.43 μg/g at 3 hr), a more prolonged lowering of norepinephrine (2.41 to 1.77 μg/g at 24 hr) (Goldberg *et al.*, 1960), and a fall of total catecholamines in the brain stem (0.17 → 0.08 μg/g), heart (0.59 → 0.26 μg/g), and spleen (0.94 → 0.43 μg/g) (Stone *et al.*, 1962). In the mouse, brain serotonin is reduced but norepinephrine is unaffected (S. E. Smith, 1960 a). The urinary amines in four hypertensive patients were decreased by α-methyldopa (1–4 g per day): the reductions were 81% for tyramine, 63% for serotonin, and 55% for tryptamine (Oates *et al.*, 1960).

Altering these amine levels should produce physiological disturbances. It has been found that α-methyldopa lowers the blood pressure and causes sedation in the dog (Goldberg *et al.*, 1960), decreases coordinated activity and produces miosis in mice (S. E. Smith, 1960 a), and in a variety of animals induces a syndrome similar to that produced by reserpine (including hypothermia), a drug releasing amines from the tissues (S. E. Smith, 1960 b). α-Methyldopa is being studied clinically for the reduction of hypertension and a preliminary report (Oates *et al.*, 1960) indicated its effectiveness, doses of 1–4 g/day for 1 week leading to a fall in supine blood pressure from 187.0/115.4 to 173.4/108.1 and in standing blood pressure from 177.7/119.3 to 138.6/98.0, the controls being given a placebo in a double-blind study.

It is thus clear that α-methyl-dopa can inhibit certain amino acid decarboxylases *in vivo*, can alter amine levels in tissues, and can produce physiological disturbances that could reasonably be attributed to the inhibition. Recently, however, more detailed studies of tissue amines have indicated that other mechanisms are possibly operative. Injections of α-methyl-3-hydroxyphenylalanine into guinea pigs lead to a reduction in brain amines (Fig. 2-4), the degree of lowering and the duration of the effect depending on the amine. α-Methyldopa acts similarly but is slightly more potent. Simultaneously there is an inhibition of amino acid decarboxylase (Fig. 2-5). Cardiac norepinephrine is even more potently reduced

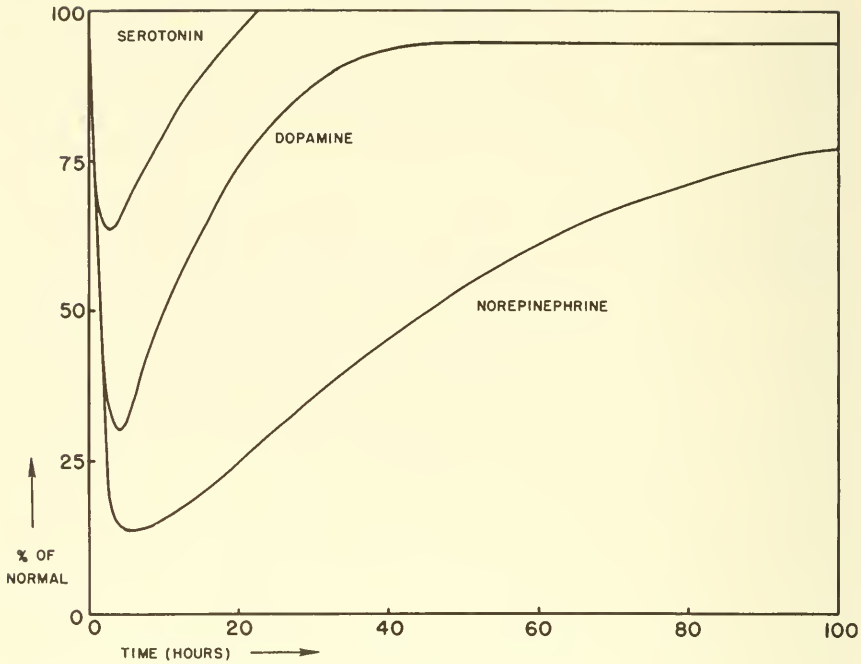


FIG. 2-4. Effects of α-methyl-*m*-tyrosine on brain amine concentrations in the guinea pig following an intraperitoneal dose of 400 mg/kg. (From Hess *et al.*, 1961.)

and remains at a lower level for a longer time than in brain. The results on serotonin and dopamine levels in the brain correspond as expected to the time course of decarboxylase inhibition, but the prolonged depletion of norepinephrine is difficult to explain on this basis. Since dopamine levels return to normal long before norepinephrine, there must be either an inhibition of the β-hydroxylation of dopamine to norepinephrine or an interference with the tissue binding of norepinephrine. Hess *et al.* (1961) showed that these analogs inhibit β-hydroxylation only at relatively high concentrations, which might have been produced soon after injection but certainly would not occur several hours later, and thus inclined to the second explanation. The lack of inhibition of dopamine β-oxidase has been confirmed by Creveling *et al.* (1962)

The time course for catecholamine depletion in mouse brain and heart following administration of these analogs is similar to that following reserpine, except the return toward normal in the brain is somewhat faster. Porter *et al.* (1961) examined different analogs for ability to reduce norepinephrine in the brain and heart, and compared these results with their effectiveness in inhibiting decarboxylation of 5-hydroxytryptophan in kidney (Table 2-12). Some lack of correlation between the two activities is

TABLE 2-12

EFFECTIVE DOSES OF ANALOGS IN INHIBITING KIDNEY DECARBOXYLASE AND LOWERING BRAIN AND HEART NOREPINEPHRINE IN MICE [a]

Analog	ED_{50} (mg/kg)		
	Inhibition of renal decarboxylase	Depletion of norepinephrine	
		Brain	Heart
L-α-Methyl-3,4-dopa	2.63	32	21
L-α-Methyl-2,3-dopa	1.35	>100	>100
L-α-Methyl-3-hydroxy-PA	11.7	12	1
L-α-Methyldopamine	No inhibition	>100	5
L-α-Methyl-3-hydroxyphenylethylamine	No inhibition	33	0.7

[a] Analogs injected intraperitoneally. Kidney decarboxylase activity determined 45 min after injection. Doses required to half deplete tissues of norepinephrine in last two columns. Since it has been shown that only the L-isomers are active, results are given on this basis for more convenient comparison. (Data from Porter *et al.*, 1961.)

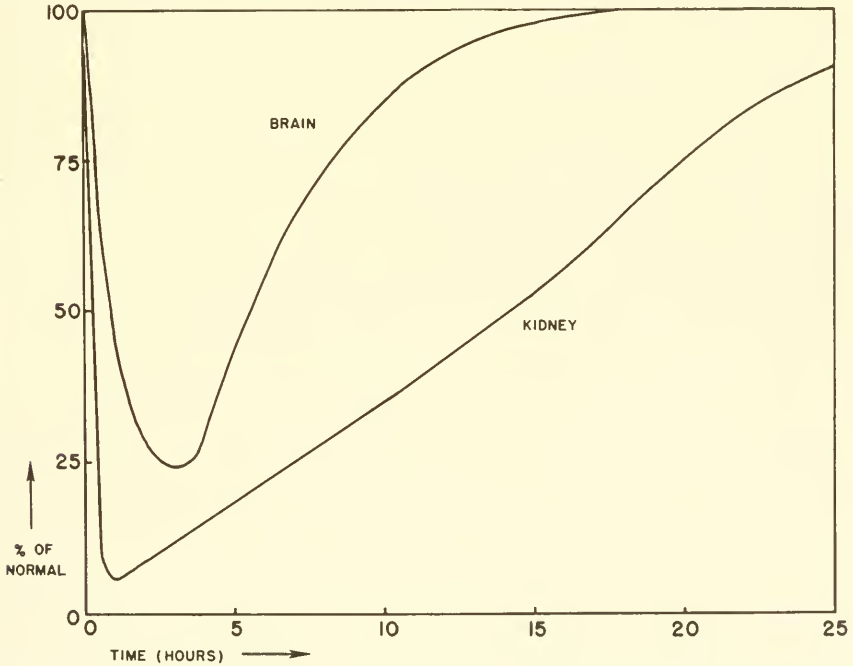


FIG. 2-5. Inhibition of amino acid decarboxylase in guinea pig tissues by α-methyl-*m*-tyrosine injected intraperitoneally at 400 mg/kg. (From Hess *et al.*, 1961.)

evident and led to the conclusion that an action other than decarboxylase inhibition is involved, this probably being an interference with the binding of amines in the tissues. Since the α-methylamino acid analogs can be slowly decarboxylated to the corresponding α-methylamines in the body, and since these amines have the ability to deplete norepinephrine, it was suggested that at least part of the tissue amine lowering is due to displacement by the α-methyl analogs of the amines. This mechanism has been subscribed to by several recent workers. Maître and Staehelin (1963) administered α-methyldopa to rats and guinea pigs and found the cardiac catecholamine levels to be insignificantly altered. They detected an increase in the α-methylnorepinephrine level, however, and a corresponding decrease in norepinephrine, indicating the displacement of the normal catecholamine with its analog. Muscholl and Maître (1963) further demonstrated that α-methylnorepinephrine stored in the sympathetic nerve endings is released by nerve stimulation and is active on various adrenergic receptors. Pletscher *et al.* (1964) after injecting α-methyldopa into rats, found marked reduction of brain serotonin several hours later and felt that inhibition of the decarboxylase could not explain the results. They inclined to the view that α-methyldopa must also release or displace stored amines, and might also interfere with the uptake of amino acids by the brain. However, S. E. Smith (1963) had shown that α-methyldopa is only a very weak inhibitor of 5-hydroxytryptophan uptake in brain slices (50% inhibition at 6.7 m*M*), although it inhibits the decarboxylase potently (50% inhibition at 0.00056 m*M*). It may be noted that other analogs may inhibit uptake more than decarboxylation. Day and Rand (1964) showed that α-methyldopa can restore the activity in animals whose catecholamine levels have been depleted by treatment with reserpine, presumably by the formation of α-methylnorepinephrine, which is generally only 1/9–1/2 as pharmacologically potent as norepinephrine; this does not provide direct evidence for the mechanism of inhibition by α-methyldopa, but clearly shows that it forms an active amine analog.

The principles involved in the interpretation of these results are important in the general field of analog inhibition and the disturbances produced in tissue function, and, furthermore, the foregoing experiments might be carelessly construed as invalidating the decarboxylase inhibition mechanism; thus some critical comments may not be out of place.

(1) It is unfortunate that Porter *et al.* (1961) did not determine decarboxylase inhibition in brain and heart for comparison with amine depletion in these tissues, since the inhibition in kidney may be quite different. In the first place, the penetration of the analogs into the three tissues may vary. Indeed, Hess *et al.* (1961) found that α-methyldopa concentrations in brain, heart, and kidney are in the ratio 1:1.66:3.28 at 1 hr and 1:1.8:9.8 at 5 hr after injection. The concentrations of the other analogs in the tis-

sues are not known, but it was demonstrated that α-methyldopa does not penetrate into brain. In the second place, the decarboxylases from the various tissues may have different susceptibilities to the analogs, as we have noted above. Perhaps cardiac decarboxylase is resistant to α-methyldopa while the renal enzyme is more sensitive.

(2) Monoamine oxidase inhibitors were administered during norepinephrine depletion, and norepinephrine levels immediately rose (Hess *et al.*, 1961). It was concluded that "biosynthesis of norepinephrine can still occur in animals treated with α-methylamino acids." All these results mean is that inhibition of the decarboxylase was not complete. In any sequence

$$A \xrightarrow{(1)} B \xrightarrow{(2)} C$$

an inhibition of (1) will lower B and an inhibition of (2) will raise B (see Chapter I-7).

(3) It is stated that the decarboxylase step is the most rapid in the over-all sequence and therefore cannot be rate-limiting (Hess *et al.*, 1961). The conclusion was that the decarboxylase must be inhibited very strongly for any effect to be observed. First, it is very difficult to establish that

$$\text{Tyrosine} \xrightarrow[slow]{very} \text{dopa} \xrightarrow[fast]{very} \text{dopamine} \xrightarrow[slow]{} \text{norepinephrine}$$

these are the relative rates of the reactions *in vivo*, where the concentrations and states of the enzymes are quite different than when isolated from the cells. Second, the rate of formation of norepinephrine in a steady state is controlled by the first reaction (or a previous reaction) since these reactions are virtually irreversible. An inhibition of dopa decarboxylase will not alter the rate of norepinephrine formation as long as a steady state is maintained; the concentration of dopamine will also be unchanged in the steady state. However, it has been demonstrated that dopamine concentration falls, indicating that a departure from a steady state has occurred. One must also consider the other possible metabolic pathways for dopamine (e.g., oxidation and *O*-methylation), since this is a divergent sequence. A certain depression of the decarboxylation need not be reflected in the same depression of norepinephrine formation, even under nonsteady-state conditions; the latter can be either greater or less than the inhibition of dopamine formation. In the case of serotonin formation, the decarboxylation is the last step, and whether it will be inhibited or not will depend on the degree to which 5-hydroxytryptophan concentration can rise to overcome the block. In any event, it has been shown that the *in vivo* inhibition of decarboxylase by these analogs can be very high and sometimes complete.

(4) The fact that certain α-methyl analogs of the catecholamines can deplete tissues of the amines, although they do not inhibit the decarboxylase, is not evidence against a decarboxylase inhibition mechanism for the α-methylamino acids, but indicates another mechanism, which may play a role in the prolonged lowering of norepinephrine levels without necessarily being involved in the initial rapid fall in tissue amines. The relatively rapid return of serotonin and dopamine levels to normal (Fig. 2-4) suggests that there is no generalized disturbance in tissue amine binding, but that the effect is specifically on norepinephrine. The most satisfactory position at the present time might be the following: the initial marked fall in tissue amines brought about by the α-methyl analogs is primarily due to an inhibition of decarboxylation (perhaps supplemented at peak concentrations by inhibition of other steps, such as β-hydroxylation), and further disturbances in amine binding are progressively produced by the α-methylamines formed from the inhibitors, so that even when the decarboxylase is normally active again the tissues cannot concentrate certain of the normal catecholamines.

Dopamine β-Hydroxylase

This enzyme catalyzes the synthesis of norepinephrine from dopamine and, as we have seen, its inhibition by analogs could be both theoretically and practically important. Hess *et al.* (1961) found that α-methyl-3-hydroxyphenylalanine does not inhibit at 2 mM but inhibits 50% at 4 mM. The concentration of α-methyldopa 1 hr after injection is given as 376 μg/g in the heart and this could mean a concentration around 750 μg/ml of intracellular fluid (assuming extracellular fluid has a low concentration at this time). This is approximately equivalent to 4 mM so that appreciable inhibition might occur. Inhibition data indicate that 3-methyl-3-hydroxyphenylalanine concentrations in the tissues are roughly the same as α-methyldopa concentrations. Until more is known about the nature of the inhibition of this enzyme, it might be safe to conclude that it plays some role in the effects of the α-methyl analogs of phenylalanine.

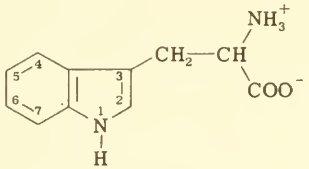
Various amines can inhibit this enzyme (Goldstein and Contrera, 1961). When dopamine concentration is 0.26 mM, the following inhibitions are observed: tyramine at 2.9 mM (75%), β-phenylethylamine at 3.3 mM (45%), amphetamine at 5.9 mM (35%), and 3-methoxydopamine at 4.8 mM (15%). None of these inhibitors appears to be potent enough to be practically important in reducing norepinephrine synthesis and, furthermore, these amines are so pharmacologically active that their use is limited. Benzyloxyamine, and particularly the p-hydroxyl derivative, inhibit this enzyme rather potently, 0.01 mM of the latter blocking almost completely after 90 min (van der Schoot *et al.*, 1963), this being attributed to the isosteric relation between phenethylamines and benzyloxyamines.

TRYPTOPHAN METABOLISM

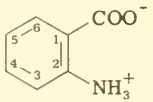
Tryptophan is involved in several important metabolic pathways, forming active substances as well as being incorporated into proteins, so that many attempts to block these pathways specifically with analogs have been made. Growth inhibition and physiological disturbances are readily produced by many of these analogs. (See scheme on page 322).

Synthesis of Tryptophan

L-Tryptophan is a potent feedback inhibitor of the conversion of 5-phosphoshikimate to anthranilate, an early reaction in tryptophan biosynthesis, and 5-methyltryptophan also inhibits, although not so strongly, a phenomenon (i.e., inhibition of a biosynthetic step by an analog) termed *false feedback inhibition* by Moyed (1960). It is likely that this mechanism explains the bacteriostatic activity of this analog. The condensation of anthranilate and 5-phosphoribosyl-1-pyrophosphate is not inhibited by 5-methyltryptophan, but 6-fluorotryptophan is inhibitory. A later reac-

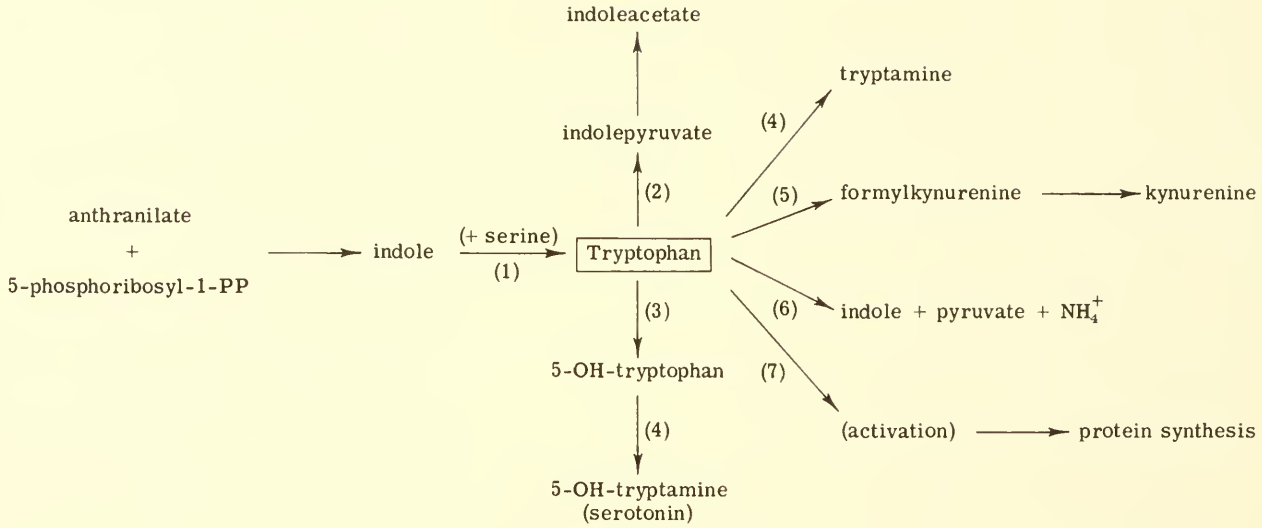


Tryptophan



Anthranilate

tion in this sequence, the conversion of anthranilic deoxyribonucleotide to indoleglycerol-3-phosphate, is inhibited by a variety of anthranilate derivatives, especially the 3- and 4-methyl analogs (Gibson and Yanofsky, 1960). The final reaction, the condensation of indole and serine to form tryptophan, catalyzed by tryptophan synthetase, is a major site of the attack by 4-methyltryptophan, which is a bacterial growth inhibitor (Trudinger and Cohen, 1956). The 5- and 6-methyl indoles are fairly potent competitive inhibitors, with K_i values near 0.1 mM (Hall *et al.*, 1962). They are also antibacterial. The growth depression of *E. coli* is counteracted by tryptophan (Fig. 2-6). At least two sites for the inhibition have been demonstrated. Tryptophan synthetase is inhibited competitively, but there is also a block of the much earlier formation of anthranilate. There are no effects on the immediate metabolism of anthranilate or on tryptophanase, which indeed readily splits the analog to 4-methylindole. The bacteriostatic action is probably due mainly to suppression of tryptophan synthesis rather than to a disturbance of tryptophan utilization. Thus several steps in the biosynthesis are susceptible to analogs and it is quite possible that other



(1) tryptophan synthetase (desmolase)
(2) L-amino acid oxidase
(3) tryptophan hydroxylase
(4) tryptophan decarboxylase
(5) tryptophan pyrrolase
(6) tryptophanase
(7) tryptophan-activating enzyme

unstudied reactions are likewise inhibited. A more indirect mechanism is the inhibition of the synthesis of tryptophan synthetase in growing cells, whereby tryptophan formation is further reduced.

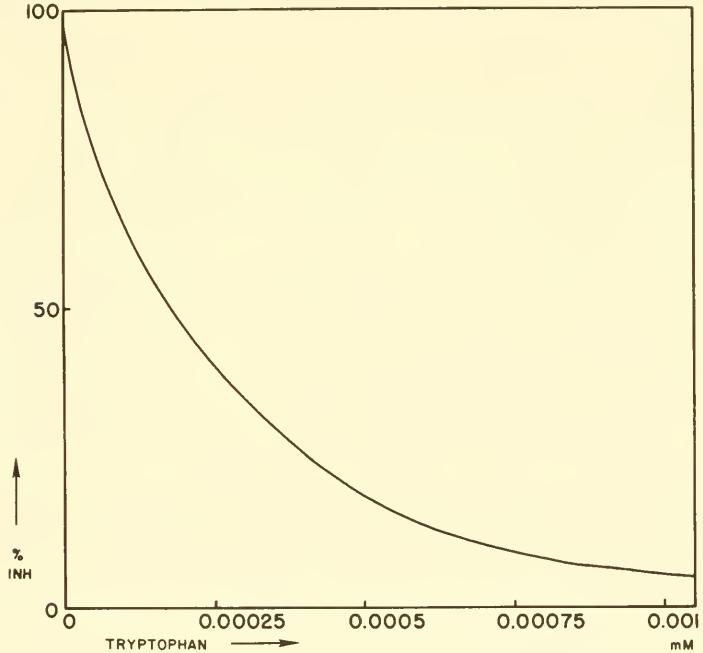


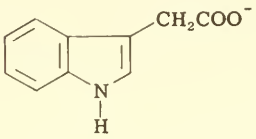
FIG. 2-6. The inhibition of *E. coli* growth by 4-methyltryptophan at 0.01 m*M* and its antagonism by increasing tryptophan concentrations. (From Trudinger and Cohen, 1956.)

Tryptophanase

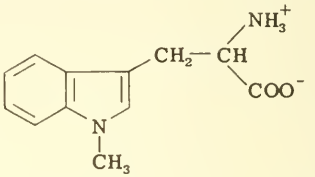
This bacterial and fungal enzyme may be involved in the fermentation of tryptophan and is responsible for the putrefactive reaction in the intestines. It is potently and competitively inhibited by the product indole and less potently by some analogs (as shown in the accompanying tabula-

Inhibitor	Relative $-\Delta F$ of binding (kcal/mole)
Indole	5.40
β-3-Indolylpropionate	4.05
3-Indolylacetate	3.76
β-3-Indolylethylamine	2.55
DL-β-1-Methyl-3-indolylalanine	1.44

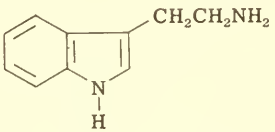
tion) (Gooder and Happold, 1954). The importance of the indole N in binding is indicated by the weak inhibition with the last analog (tryptophan with the indole N methylated) and the failure of indene (the hydrocarbon



3-Indolylacetate



β-1-Methyl-3-indolylalanine



β-3-Indolylethylamine

analog of indole) to inhibit, while the importance of the carboxylate group is reflected in the weak inhibition by the indolylethylamine. The potency of indole may be attributed to the fact that it may not have to be oriented in a manner necessary for reaction of the side chain.

Tryptophan Pyrrolase (Tryptophan Peroxidase)

This enzyme initiates one of the most important catabolic pathways of tryptophan and is readily inhibited by certain analogs. Hayaishi (1955 b) found the *Pseudomonas* enzyme to be sensitive to the hydroxytryptophans, and calculated the values of K_i shown in the following tabulation. Since

Substance	K_i or K_s (mM)	Relative $-\Delta F$ of binding (kcal/mole)
5-Hydroxytryptophan	0.002	8.06
7-Hydroxytryptophan	0.12	5.55
L-Tryptophan	0.4	4.81

5-hydroxytryptophan is normally formed from tryptophan on the pathway to serotonin, its potent inhibition of the pyrrolase suggests that it may play a role in regulating tryptophan metabolism. The enzyme from rat liver is also inhibited by 5-hydroxytryptophan and even more potently

by serotonin (Frieden *et al.*, 1961). The K_i's in the following tabulation indicate 3-indolylacrylate to be the most effective inhibitor. Other inhibitions observed when (S) = (I) = 3 mM are: indole 69%, tryptazan 50%,

Inhibitor	K_i (mM)	Relative — ΔF of binding (kcal/mole)
3-Indolylacrylate	0.012	6.99
Serotonin	0.067	5.93
5-Hydroxytryptophan	0.094	5.71
3-Indolylbutyrate	0.16	5.39
Tryptamine	0.20	5.25
3-Indolylpropionate	0.29	5.02
3-Indolylacetate	0.91	4.32
β-Methyltryptophan	1.1	4.20
D-Tryptophan	1.6	3.97
6-Fluorotryptophan	2.0	3.83
5-Fluorotryptophan	2.2	3.77

5-methyltryptophan 38%, and 6-methyltryptophan 33%. The analogs with altered side chains are competitive while the others are mainly noncompetitive. The roughly equivalent binding of tryptamine and 3-indolylpropionate, and of serotonin and 5-hydroxytryptophan, might indicate that the binding is primarily with the indole ring, the side chains contributing little, and this is substantiated by the fact that indole is bound approximately as well as 3-indolylpropionate. The stronger binding of 3-indolylacrylate compared to 3-indolylpropionate (about 2 kcal/mole) is thus difficult to account for unless there is a modification of the interaction of the indole N. Tryptophan pyrrolase is an inducible enzyme in the rat but none of these analogs is active, although Sourkes and Townsend (1955) found α-methyltryptophan to induce after subcutaneous injection.

Tryptophan Hydroxylase (Phenylalanine Hydroxylase)

Excessive feeding of phenylalanine leads to low blood serotonin, a low excretion of 5-hydroxyindoleacetate, and a decrease in brain serotonin, and this has usually been attributed to an inhibition of 5-hydroxytryptophan decarboxylase. However, no accumulation of 5-hydroxytryptophan has been demonstrated and it is possible that the site of the inhibition might be earlier, perhaps on the hydroxylation of tryptophan (Freedland *et al.*, 1961). Hydroxylating preparations from rat liver are indeed quite potently inhibited by L-phenylalanine, and also by phenylpyruvate and phenyllactate (K_m for L-tryptophan is 29 mM, and K_i for L-phenylalanine is 0.22 mM).

It is likely that the same enzyme is responsible for the hydroxylation of both phenylalanine and tryptophan, since the K_i for phenylalanine is close to the K_m when it is the substrate; the affinity for tryptophan is, however, much less. These results may help to explain some of the changes observed in phenylpyruvic oligophrenia (see pages 329 and 429).

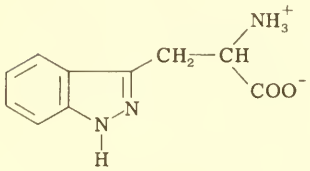
Tryptophan-Activating Enzyme

An activating enzyme from pancreas is specific for tryptophan with respect to other normal amino acids but can activate certain analogs of tryptophan (Sharon and Lipmann, 1957). The analogs tested fall into three categories:

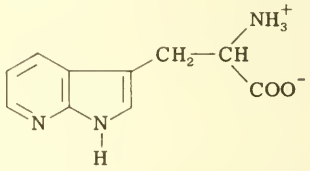
Group I are activated (tryptazan, azatryptophan, 5-fluorotryptophan, and 6-fluorotryptophan).

Group II are inhibitory (tryptamine, D-tryptophan, β-methyltryptophan, 5-hydroxytryptophan, 5-methyltryptophan, and 6-methyltryptophan).

Group III are inactive (indole, indoleacetate, 6-methyltryptazan, and *N*-acetyltryptophan).



Tryptazan

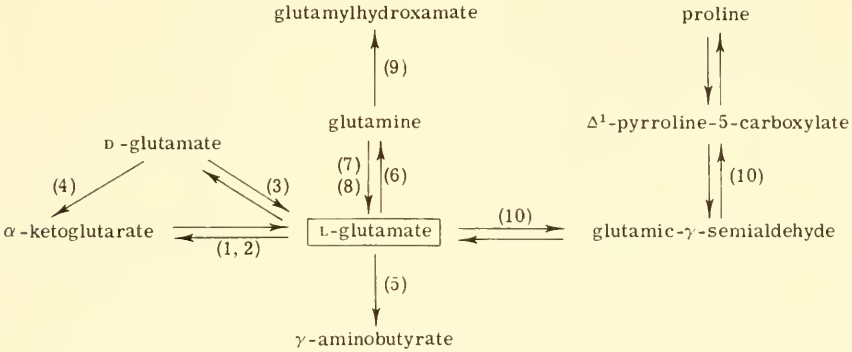


Azatryptophan

Reciprocal plots were said to indicate competitive inhibition but actually do not show pure competition, and it might better be designated as partially competitive inhibition. For analogs to be activated they must match the size of tryptophan, and the introduction of bulkier groups prevents reaction with ATP but allows binding and inhibition. Azatryptophan and tryptazan are both incorporated into proteins. In *E. coli*, azatryptophan permits synthesis of proteins and nucleic acids but many of the enzymes are not in the normal forms (Pardee and Prestidge, 1958). The synthesis of adaptive enzymes, such as β-galactosidase, is inhibited very rapidly, and phage formation is blocked more readily than bacterial growth. The induced synthesis of maltase in yeast is also very potently suppressed by tryptazan, incorporation of all amino acids being simultaneously blocked (Halvorson *et al.*, 1955). 5-Methyltryptophan and 6-methyltryptazan inhibit moderately, while 6-methyltryptophan is inactive.

GLUTAMATE METABOLISM

Glutamate occupies a central position in many important metabolic pathways and serves to link amino acid metabolism with the tricarboxylic acid cycle. Its relationship to the biochemically active glutamine and the physiologically active γ-aminobutyrate (GABA) makes possible specific inhibitions of glutamate reactions of great interest. The reactions catalyzed by enzymes studied with respect to analog inhibition are shown in the following scheme.



(1) L-glutamate dehydrogenase
(2) glutamate transaminases
(3) glutamate racemase
(4) D-glutamate oxidase
(5) glutamate decarboxylase
(6) glutamine synthetase
(7) glutaminase
(8) formylglycinamidine phosphoriboside synthetase
(9) γ-glutamyl transferase
(10) Δ^1-pyrroline-5-carboxylate dehydrogenase

Glutamate Decarboxylase

Several analogs of glutamate inhibit its utilization by *Lactobacillus arabinosus*, and thus a study of decarboxylase from *E. coli* was undertaken by Roberts (1953). The two most potent inhibitors are α-oximinoglutarate and α-methylglutamate, but the former is probably active by virtue of its hydrolysis to hydroxylamine which inactivates pyridoxal phosphate. The latter analog inhibits competitively when added with the substrate, but if it is preincubated with the enzyme the inhibition becomes progressively more noncompetitive and difficulty reversible. The rates of combination with the enzyme and dissociation from the enzyme are very slow. The methyl group interferes with the normal binding of the molecule so that decarboxylation does not occur, but by some unknown mechanism brings about a type of binding that is very strong, a behavior seen with some other α-methylamino acids. The decarboxylation of glutamate in rat brain homogenates is also inhibited competitively by aspartate (K_m

$= 21$ mM, and $K_i = 23$ mM) (Wingo and Awapara, 1950), which is rather surprising because of the shorter intercarboxylate distance.

Glutamate decarboxylase from the squash *Curcurbita moschata* is inhibited competitively by a variety of organic acids (see accompanying tabulation), and the results are of some interest with regard to structure and

Inhibitor (13.6 mM)	% Inhibition
Monocarboxylates	
Formate	17
Acetate	42
Propionate	10
n-Butyrate	18
Isobutyrate	8
n-Valerate	25
Isovalerate	15
n-Caproate	24
Isocaproate	20
Dicarboxylates (saturated)	
Oxalate	0
Malonate	0
Succinate	0
Glutarate	24
Adipate	49
Pimelate	58
Suberate	37
Dicarboxylates (unsaturated)	
Fumarate	0
Maleate	14
Citraconate	0
Mesaconate	7
Itaconate	11
Tricarboxylates	
cis-Aconitate	29
trans-Aconitate	18

intercarboxylate distance (Ohno and Okunuki, 1962). Glutamate concentration was 27.2 mM in all cases. Many amino acids examined are weakly inhibitory or without effect. The maximal inhibition by pimelate in its series probably indicates that a cambering of the molecule is necessary for binding of the two carboxylate groups, or possibly that the cationic groups of the enzyme are farther apart than in glutamate. The relatively high

inhibition by acetate is surprising; if the monocarboxylates interact with the cationic groups, one might expect propionate or butyrate to be more inhibitory.

The problem of the abnormal brain development in phenylpyruvic oligophrenia prompted an investigation of the effects of the phenyl acids on brain glutamate decarboxylase by Hanson (1958); the results are presented in the tabulation below in which they are compared with those of Tashian

Inhibitor	Relative $-\Delta F$ of binding (kcal/mole)[a]	
	Hanson (1958)	Tashian (1961)
p-Hydroxyphenylacetate	3.15	4.16
o-Hydroxyphenylacetate	—	4.26
Phenylpyruvate	2.62	2.62
Phenylacetate	2.53	3.73
p-Hydroxyphenylpyruvate	1.75	3.83
Phenylalanine	0.60	—
Phenyllactate	0.53	—

[a] Relative $-\Delta F$'s of binding adjusted so that value for phenylpyruvate is the same in each series.

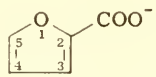
(1961), who also used rat brain. There are some rather striking differences, part of which may be due to the procedures used since these analogs, although stated to be competitive, present deviations from classic kinetic formulations (and for this reason the binding energies calculated from apparent K_i's are probably not very reliable). If these analogs, which are present in high concentrations in the blood enter the brain readily, it is possible that they depress the formation of γ-aminobutyrate (GABA) which may be essential for normal neurological development. In branched-chain ketonuria (maple sugar urine disease) various keto and hydroxy fatty acids accumulate in the body, which Tashian (1961) showed also inhibit glutamate decarboxylase (relative $-\Delta F$'s of binding for α-hydroxyisovalerate, α-ketoisovalerate, and the corresponding isocaproates from 3.58 to 4.50 kcal/mole on the scale above). The enzyme from *E. coli*, on the other hand, is relatively insensitive to any of these analogs.

L-Glutamate Dehydrogenase

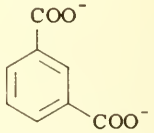
The oxidative deamination of L-glutamate in beef liver homogenates is catalyzed by a NAD-linked dehydrogenase, the inhibition of which at pH 8.4 was thoroughly studied by Caughey *et al.* (1957). The accompanying tabulation shows the K_i values for competitive analogs, from which the

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
5-Bromofuroate	0.059	6.00
5-Chlorofuroate	0.063	5.96
5-Nitrofuroate	0.17	5.35
m-Iodobenzoate	0.46	4.74
m-Bromobenzoate	0.54	4.65
Isophthalate	0.56	4.62
Glutarate	0.58	4.60
α-Ketoglutarate	0.73	4.45
m-Chlorobenzoate	1.02	4.25
D-Glutamate	2.0	3.83
m-Nitrobenzoate	3.4	3.51
Trimesate	4.0	3.40
Fumarate	6.8	3.08
Succinate	11	2.78
Adipate	16	2.55

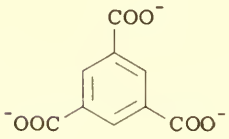
relative binding energies have been calculated. Inhibitors were classed as competitive if the interaction constant α is greater than 10; trimesate and D-glutamate are only partially competitive, with $\alpha = 1.7$. It was postulated



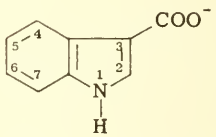
Furoate



Isophthalate



Trimesate



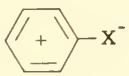
Indole-3-carboxylate

that the enzyme contains two cationic groups at a separation optimal for interaction with the carboxylate groups of glutamate, glutarate, and isophthalate. The effects of some other inhibitors for which the K_i's were not calculated are shown in the following tabulation; most of these are

relatively weak (fumarate is included for comparison with values in the preceding table). L-Glutamate was 2 m*M* in each case. The following are not inhibitory at 10 m*M*: L-glutamine, L-diethylglutamate, β-methylglutarate, citrate, *o*- and *p*-hydroxybenzoate.

Inhibitor	Concentration (m*M*)	% Inhibition
Benzoate	10	12
Furoate	10	26
Phthalate	10	17
Terephthalate	6.7	16
Isophthalate	2	50
trans-Aconitate	8	20
Indole-2-carboxylate	7.5	21
Indole-3-carboxylate	8.3	38
m-Hydroxybenzoate	10	27
Pyridine-2,6-dicarboxylate	10	27
Fumarate	10	29

In the various substituted benzenes the *meta* compounds are invariably the most potent inhibitors, presumably because the intergroup distances are close to the enzyme intercationic separation. The dipoles of the halogen and nitro compounds may interact with the cationic group since they may be represented as:



However, there is no correlation of inhibitory activity with dipole moment. One might think that the dipole-cation interaction would be weaker than the carboxylate-cation interaction, but the hydration of the inhibitors must also be considered. Less water needs to be displaced when the dipoles approach the enzyme cationic group. It was pointed out that all good inhibitors are reasonably planar and the presence of bulky groups protruding lowers the affinity. The particularly good binding of the furoates may be related to some interaction of the ring *O* with the enzyme. The reverse reaction from α-ketoglutarate to glutamate is inhibited less than the forward reaction by glutarate, isophthalate, and 5-bromofuroate, and the inhibitions are noncompetitive.

The L-glutamate dehydrogenase from cockroach muscle mitochondria

is similarly inhibited (see accompanying tabulation), and here glutarate also appears to present a relatively good fit to the active site (Mills and Cochran, 1963). Glutamate was 15 mM in all cases. The reverse reaction catalyzed by the glutamate dehydrogenases (both NAD- and NADP-

Inhibitor (3 mM)	% Inhibition
Succinate	15
Fumarate	15
Malate	15
Glutarate	65
Adipate	30
D-Glutamate	55
L-Aspartate	20
D-Aspartate	10

linked) from *Fusarium oxysporum* is also inhibited by glutarate, the K_m for α-ketoglutarate being 2.1 mM, and the K_i for glutarate 1.52 mM (Sanwal, 1961).

Glutaminase

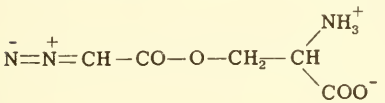
The deamidation of glutamine is inhibited by the product glutamate and this is not a reversal of the equilibrium but a competition for the active center, as first pointed out by Krebs (1935). L-Glutamate and D-glutamate inhibit guinea pig kidney glutaminase equally (98% at 25 mM when glutamine is 8.7 mM). Inhibition by glutamate has been confirmed for the enzyme from guinea pig kidney (van Baerle *et al.*, 1957), pig kidney (Klingman and Handler, 1958), dog kidney (Sayre and Roberts, 1958), and rat brain (Blumson, 1957). The inhibition has generally been found to be noncompetitive with respect to glutamine, but oddly is competitive with phosphate on the phosphate-activated glutaminase from dog kidney. The ammonium ion is, however, strictly competitive with glutamine on both the pig and dog kidney enzymes. Another type of glutaminase (called glutaminase II), which is transaminating in the presence of pyruvate and is obtained from guinea pig kidney, is not inhibited by even 100 mM glutamate (Goldstein *et al.*, 1957). Sayre and Roberts (1958) pictured the active center as containing two cationic groups, one binding the phosphate and one the glutamine carboxylate group; the negatively charged phosphate also interacts with the positive α-amino group of glutamine. Since the active enzyme is the phosphate complex, it is easy to see why phosphate would antagonize inhibitions produced by certain substances (e.g., dyes such as bromosulfalein or bromcresol green which complex with both enzyme cationic sites), but it is difficult to understand why glutamate inhibits

competitively with respect to phosphate. It was stated that for a substance to compete with glutamine it should have affinity for the enzyme-phosphate complex, and it would seem that glutamate may fall into this category.

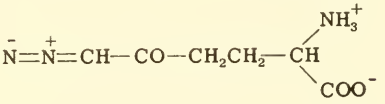
Few other analogs have been tested on this enzyme. Krebs (1935) observed a mild inhibition by DL-β-hydroxyglutamate (22% at 80 mM when glutamine 40 mM), and Girerd *et al.* (1958) reported inhibition by ethyl D-glutamate and DL-β-methylglutamate. Because of the postulated role of glutaminase in renal function, these two latter analogs, along with L-glutamate and bromosulfalein, were tested *in vivo*. These inhibitors reduce diuresis in rats around 50% at 50 mg/kg subcutaneously, whereas a group of five less potent glutaminase inhibitors actually increase diuresis.

Formylglycinamidine Phosphoriboside Synthetase

Glutamine participates in purine biosynthesis by contributing its amide N. Azaserine and 6-diazo-5-oxo-L-norleucine (DON) are potent inhibitors of inosinate biosynthesis and lead to the accumulation of formylglycinamide phosphoribotide (FGAR). These substances may be considered as analogs of glutamine and have been shown to inhibit formylglycinamide ribonucleotide amidotransferase competitively with respect to glutamine



Azaserine



DON

(Levenberg *et al.*, 1957). The K_m for glutamine is 0.615 mM and the K_i's for azaserine and DON are 0.034 mM and 0.0011 mM, respectively. Once azaserine binds to the enzyme, however, an irreversible reaction occurs, due perhaps to an alkylation of the enzyme. French *et al.* (1963 a) pointed out that 50% inhibition can be obtained with a (S)/(I) ratio of 2100 with DON. Phosphoribosyl-PP amidotransferase, another enzyme catalyzing the transfer of the amide nitrogen of glutamine, is also inhibited competitively by DON, with a K_i of 0.019 mM, and much more weakly by azaserine (Hartman, 1963 b). A slow covalent binding of DON to the enzyme occurs following the initial reversible attachment, and this is accelerated by the presence of phosphoribosyl-PP and Mg^{++} on the enzyme, indicating that the active site for the reaction of glutamine, or the binding of DON, is partly dependent on the other substrate and the cofactor. Blocking of an SH group prevents the attachment of DON to the enzyme, suggesting that this SH group is catalytically functional in the nitrogen transfer and the irreversible binding of DON, as French *et al.* (1963 b) concluded from their work with azaserine on the formylglycinamide ribonucleotide amidotransferase.

Glutamate Transaminases

This enzyme apparently possesses two cationic groups properly separated to interact with glutamate and the other dicarboxylates, because it is inhibited best by glutarate of all the saturated dicarboxylates, as shown in the accompanying tabulation (aspartate = 1.7 mM and α-ketoglutarate = 6.7 mM) (Jenkins *et al.*, 1959). This is one instance in which the α-methyl analog has no affinity for the enzyme. Very similar results were obtained

Inhibitor (40 mM)	% Inhibition	Relative $-\Delta F$ (kcal/mole)
Malonate	0	<0.17
Succinate	31	1.49
Glutarate	72	2.56
Adipate	62	2.29
Pimelate	0	<0.17
Suberate	0	<0.17
Maleate	78	2.76
α-Methylaspartate	35	1.60
α-Methylglutamate	0	<0.17

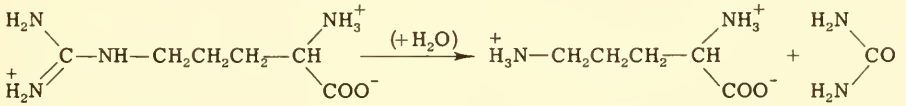
by Velick and Vavra (1962), glutarate inhibiting the most potently of the saturated dicarboxylates; from their values for K_i one can calculate that glutarate is bound 0.85 kcal/mole more tightly than succinate, and 0.32 kcal/mole more tightly than adipate. Of the three phthalates, *o*-phthalate is the most inhibitory ($K_i = 5$ mM), *m*-phthalate intermediary ($K_i = 8$ mM), and *p*-phthalate the least active ($K_i = 10$ mM). The results of the studies above were obtained on pig heart transaminase, and from the limited data reported by Goldstone and Adams (1962) it would appear that the enzyme from rat liver is different in that glutarate is about 10 times more potent than maleate as an inhibitor. The alanine:α-ketoglutarate transaminase from rat liver is inhibited moderately and competitively by certain amino acids, such as leucine and valine, and also by maleate, but no data were given for comparison with the aspartate: α-ketoglutarate transaminase (Segal *et al.*, 1962). Fluorooxalacetate inhibits the latter enzyme from heart competitively with respect to oxalacetate (and to α-ketoglutarate and aspartate when the reverse reaction is run), K_m being 3.5 mM for α-ketoglutarate and 0.5 mM for aspartate, and K_i being 0.1 mM (Kun *et al.*, 1960). It is also slowly transaminated to fluoroaspartate which likewise inhibits the enzyme.

Other Enzymes in Glutamate Metabolism

Analog inhibition has been reported for several other enzymes involved in the metabolism of glutamate but quantitative studies from which structure-action relationships may be derived have not been made. Some of these results are summarized in Table 2-13. An interesting inhibitor is the convulsant isolated from agenized flour, methionine sulfoximine, which is inhibitory to the incorporation of methionine into proteins in bacteria and brain. Since these actions are to a great extent antagonized by methionine, this substance has generally been considered as a methionine antagonist, but glutamine also is antagonistic. Sellinger and Weiler (1963) have shown that methionine sulfoximine inhibits brain glutamine synthetase competitively with respect to glutamate, some inhibition being seen at 0.01 mM and around 50% inhibition at 1 mM with low glutamate concentrations (K_i = 0.05–0.064 mM). The relation between this inhibition and the convulsant action is not clear but it was postulated that methionine sulfoximine interferes in some vague manner with glutamine synthesis in an intracellular compartment in the brain.

ARGININASE

The hydrolysis of arginine:



Arginine Ornithine Urea

is catalyzed by the Mn^{++}-activated enzyme arginase and is a step in the urea cycle. Arginase is inhibited by the product ornithine, as first shown by Gross (1921) and confirmed by Bach and Williamson (1942), who found that the inhibition is much more marked in liver extracts than in slices (50% inhibition given by 5.3 mM ornithine in extracts and by 15.9 mM in slices when arginine is 3.56 mM). Edlbacher and Zeller (1936) noted that several amino acids inhibit, but ornithine is the most potent with lysine running a close second.

These inhibitions were compared and subjected to quantitative treatment by Hunter and Downs (1945) in the publication wherein the first use of the single-curve plot (type F) was made (see Chapter I-5). Ornithine and lysine inhibit competitively (K_i = 4.1 mM and 4.8 mM, respectively) but other amino acids are usually only partially competitive (since the DL-forms of the inhibitors used and only the L-isomers are active, it is likely that these constants should be halved). Calculations of relative

TABLE 2-13

INHIBITION OF MISCELLANEOUS ENZYMES IN GLUTAMATE METABOLISM BY ANALOGS

Enzyme	Source	Analog	Results	Reference
D-Glutamate oxidase	Octopus hepatopancreas	L-Glutamate	Competitive: $K_m = 6$ mM and $K_i = 120$ mM	Rocca and Ghiretti (1958)
Glutamine synthetase	*Lactobacillus arabinosus*	DL-α-Methyl-glutamate	Competitive: inhibition marked when (I)/(S) = 5	Ayengar and Roberts (1952)
		β-Hydroxy-glutamate	Competitive	Ayengar and Roberts (1952)
	Ehrlich ascites carcinoma cells	δ-Hydroxylysine	Competitive: 2 mM inhibits around 35–40%	Rabinovitz *et al.* (1957)
	Green peas	D-Glutamate	Competitive: is also a substrate with $K_m = 2$ mM	Varner (1960)
Δ^1-Pyrroline-5-carboxylate dehydrogenase	Ox liver	δ-Aminovalerate	0.33 mM inhibits 67%	Strecker (1960)
		δ-Valerolactam	0.33 mM inhibits 15%	
		L-Hydroxyproline	3.3 mM inhibits 78%	
		γ-Aminobutyrate	3.3 mM inhibits 65%	
		L-Proline	3.3 mM inhibits 65%	
		β-Alanine	3.3 mM inhibits 15%	
γ-Glutamyltransferase	Sheep brain	DL-α-Methyl-glutamate	Competitive: (I)/(S) of 2 inhibits 25% and of 20 inhibits 70%	Lichtenstein *et al.* (1953)
		D-Glutamate	Competitive: (I)/(S) of 1.25 inhibits 25% and of 5 inhibits 45%	

interaction energies from the constants given are shown in the following tabulation. Two types of inhibition may be possible: (1) competitive inhibition by diamino compounds that are probably oriented as the substrate, and (2) partially competitive or noncompetitive inhibition by monoamines

Inhibitor	Relative $-\Delta F$ of binding (kcal/mole)
L-Ornithine	3.82
L-Lysine	3.72
L-Norvaline	2.53
L-Isoleucine	2.48
L-Valine	1.98
L-Cysteine	1.94
L-Leucine	1.81
L-α-Aminobutyrate	1.40
L-Phenylalanine	1.09
L-Norleucine	0.87
L-Proline	0.78
L-Aspartate	0.67
L-Alanine	0.61
L-Citrulline	0.47
L-Serine	0.25
L-Tryptophan	0.21
L-Histidine	−0.82
Glycine	−0.99

that may be oriented otherwise. Inhibitory activity increases in the straight-chain series from glycine to norvaline; each additional methylene group contributes 1.17 kcal/mole to the binding energy, a value similar to that found in other series, and undoubtedly due to dispersion forces. It is odd that there is a sudden and marked drop in the affinity on adding another methylene group to form norleucine. As pointed out by Hunter and Downs, it is difficult to establish structural correlation in the series of substituted alanines (shown in the accompanying table); why, for example, is cysteine bound so much more tightly than serine? Substitution in the α-amino group (carbamyl or formyl) always reduces the activity; hence this probably constitutes one binding group. The experiments were done at pH 8.4 and therefore all carboxyl groups were essentially completely ionized, but there would be some variation between the inhibitors with respect to the fraction of the amino groups protonated (pK_a's for these inhibitors run from 8.2 to 10.6).

Substituent group	Relative $-\Delta F$ of binding (kcal/mole)
—Imidazole	−0.82
—Indole	0.21
—OH	0.25
—H	0.61
$-COO^-$	0.67
—Phenyl	1.09
$-CH_3$	1.40
—SH	1.94

The relative sensitivities to these amino acids probably vary with the source of the arginase. For example, the mouse liver enzyme is inhibited somewhat more strongly by L-lysine than L-ornithine, and even D-lysine is a weak inhibitor (inhibitions at 1 m*M* are 47% for L-lysine, 42% for ornithine, and 8% for D-lysine) (Nadai, 1958). Johnstone (1958) stated that there is evidence in intact ascites cells that ornithine can interfere with the transport of arginine into the cells, as well as inhibit arginase intracellularly, so that this additional site of inhibition must be borne in mind.

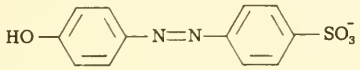
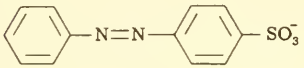
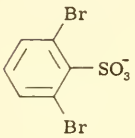
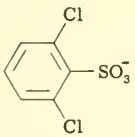
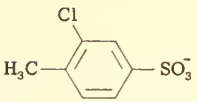
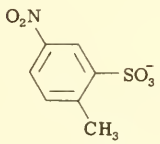
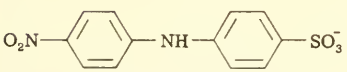
An attempt was made by Sen (1959) to determine the effects of L-lysine *in vivo* in order to evaluate its possible use in uremia. Bilaterally nephrectomized dogs show an increase of blood urea at a rate of 15–16 mg/day and die in 80–84 hr. When L-lysine is injected daily and intravenously at a dose of 1 g, the blood urea rise only 3–4 mg/day and the animals survive for 274–278 hr. Thus it would seem possible to reduce urea formation *in vivo* with this competitive inhibitor, but the clinical benefit of this remains to be tested.

L-AMINO ACID OXIDASES

These enzymes from snake venoms and mammalian tissues oxidize the L-isomers of the monoamino-monocarboxylate amino acids, the substrate used often being L-leucine. The snake venom enzyme was shown by Zeller and Maritz (1944) to be inhibited by various aromatic sulfonates. Some of the results are shown in Table 2-14, from which it may be seen that *p*-nitrodiphenyl sulfonate is the most potent inhibitor studied. The inhibitions seem to be competitive and a complex between the sulfonate group and an enzyme amino group was postulated. Benzoate is a rather weak inhibitor of rat kidney L-amino acid oxidase, 10 m*M* inhibiting 28% (Blanchard *et al.*, 1944). However, the ammonium ion is a surprisingly good inhibitor, 12 m*M* producing 69% depression of activity.

Table 2-14

Inhibition of Snake Venom L-Amino Acid Oxidase by Sulfonates[a]

Inhibitor	Sulfonate (m*M*)	L-Leucine (m*M*)	% Inhibition
	2.9	0.01	81
	2.9	0.01	76
	2.9	0.01	66
	2.9	0.01	55
	2.9	0.01	50
	0.33	1.67	31
	0.07	0.01	21

[a] From Zeller and Maritz (1944).

The L-amino acid oxidase from the hepatopancreas of *Cardium tuberculatum* is more specific than the venom or kidney oxidases, since many L-amino acids are not oxidized but are inhibitory (Roche *et al.*, 1959). The inhibitions by 16.7 m*M* L-leucine are shown in the following tabulation. At pH 7.6 the inhibitions are competitive, but not at pH 9.2. Apparently this enzyme can complex with both L- and D-isomers although in no case is a direct comparison possible.

Amino acid	% Inhibition at:	
	pH 7.6	pH 9.2
DL-Alanine	89	71
D-Alanine	39	—
L-Serine	54	73
Glycine	51	56
L-Glutamate	26	—
L-Proline	24	74
L-Valine	24	30
L-Threonine	20	14
L-Aspartate	13	12
D-Histidine	12	—
D-Leucine	5	—

D-AMINO ACID OXIDASE

D-Amino acid oxidase is also inhibited by certain amino acids, but no thorough studies have been reported. The enzyme from pig kidney oxidizing D-leucine is inhibited by DL-leucinamide, DL-leucylglycine, glycyl-DL-leucine, and DL-leucylglycylglycine, but not glycylglycine (Heimann-Hollaender and Lichtenstein, 1954). It is interesting that the oxidation of D-phenylalanine is inhibited by DL-*N*-ethylphenylalanine and the oxidation of D-leucine by DL-*N*-ethylleucine, since *N*-substituted amino acids are usually not inhibitory for any enzymes acting on amino acids. D-Lysine is a good inhibitor of this enzyme oxidizing D-alanine (K_m = 3.3 m*M*, and K_i = 5 m*M*), but L-lysine is completely inactive (Murachi and Tashiro, 1958). The D-amino acid oxidase from pig kidney with glycine as a substrate is competitively inhibited by L-leucine (K_i = 1 m*M*) (Neims and Hellerman, 1962). Pyruvate not only competes with D-alanine (K_i = 43 m*M*) but accelerates the photodecomposition of FAD (Yagi and Natsume, 1964). However, the most interesting and best studied inhibition of D-amino acid oxidase is that of benzoate and its derivatives, and the opportunity will be taken in this section of discussing not only the actions of the benzoates on this enzyme but also on other enzymes and metabolism in general.

Benzoates and Related Compounds on D-Amino Acid Oxidase

The oxidation of D-alanine in slices and homogenates of rat liver and kidney was shown to be markedly inhibited by 1 m*M* benzoate by Klein and Kamin (1941). A preparation of D-amino acid oxidase from pig kidney was thus obtained and found to be inhibited 79% by 0.1 m*M*, this being reversible upon dialysis. Several substituted benzoates are also inhibitory but all are less potent than benzoate; benzamide is inactive. The inhibitions of a lamb kidney D-amino acid oxidase by benzoate and *p*-amino-benzoate were shown by Hellerman *et al.* (1946) to be competitive with substrate. The rate of spontaneous inactivation of the apoenzyme is reduced by either substrate or FAD, and benzoate was shown by Burton (1951 a) to have a comparable action, indicating combination with the active center.

Before discussing the more detailed mechanism of the inhibition we shall turn to three studies providing information on the structural requirements for inhibition. Bartlett (1948) compared many substituted benzoates (Table 2-15) and found only four to be more potent inhibitors than benzoate,

TABLE 2-15

INHIBITION OF PIG KIDNEY D-AMINO ACID OXIDASE BY SUBSTITUTED BENZOATES [a]

Substituent	Relative $-\Delta F$ of binding (kcal/mole)		
	ortho	*meta*	*para*
F	4.26	—	6.25
Cl	3.27	6.81	5.39
Br	2.11	6.40	5.05
I	2.84	5.39	3.90
OH	4.55	5.20	3.27
NH_2	4.26	4.26	2.84
NO_2	2.52	5.12	4.90
CH_3	2.11	6.25	5.12
OCH_3	2.11	3.90	3.78
COOH	1.85	2.11	2.84
None	—	5.57	—

[a] The substrate is DL-alanine at 30 m*M* and the pH 7.6. Binding energies calculated from concentrations for 50% inhibition. (Data from Bartlett, 1948.)

pure competitive inhibition with respect to substrate being observed. J. R. Klein (1953, 1957) demonstrated inhibition, often potent, by various aromatic carboxylates (Table 2-16), and Frisell *et al.* (1956) provided further

TABLE 2-16

INHIBITION OF PIG KIDNEY D-AMINO ACID OXIDASE BY AROMATIC ACIDS[a]

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
2-Chloromethyl-5-hydroxy-1,4-pyrone	0.004	7.68
Kojate	0.021	6.65
m-Toluate	0.022	6.62
Pyrrole-2-carboxylate	0.026	6.52
Benzoate	0.048	6.14
Furan-2-acrylate	0.052	6.08
p-Toluate	0.08	5.82
Nicotinate	0.11	5.63
Cinnamate	0.56	4.62
Furan-2-carboxylate	0.60	4.58
1,2-Pyrone-5-carboxylate (coumalate)	0.60	4.58
Indole-3-acetate	2.3	3.74
Hydrocinnamate	5.5	3.21
Phenylacetate	6.4	3.12
1,4-Pyrone-2,6-dicarboxylate (chelidonate)	15	2.59
o-Toluate	29	2.18

[a] The substrate is DL-alanine and the pH 8.0–8.3. (Data from J. R. Klein, 1953, 1957.)

data on aliphatic and heterocyclic carboxylates (Table 2-17). Some of the conclusions regarding relations between structure and inhibition derived from these investigations will be summarized.

(1) A negatively charged anionic group is necessary for activity. This is seen from the lack of inhibition by benzamide, nicotinamide, and cinnamamide. It is likely that the COO^- group of the inhibitors reacts with the enzyme cationic site normally reacting with the amino acid COO^- group. A SO_3^- group can replace the COO^- but is less effective.

(2) Klein has emphasized the importance of a positive charge at a distance from the COO^- group approximating the separation in amino acids. Most of the potent inhibitors can be written in structures possessing such a positive charge by virtue of resonance effects. Benzoate, for example, resonates between the following structures:

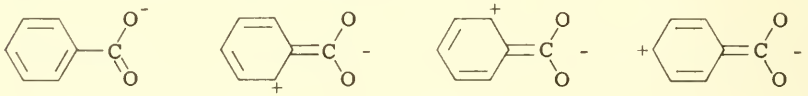


TABLE 2-17

INHIBITION OF LAMB KIDNEY D-AMINO ACID OXIDASE[a]

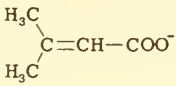
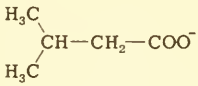
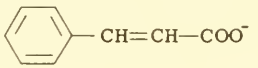
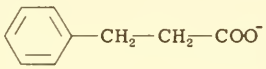
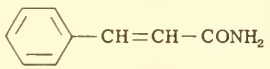
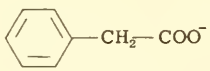
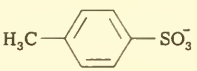
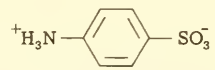
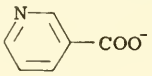
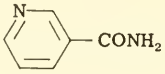
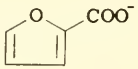
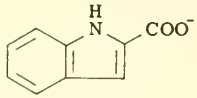
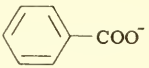
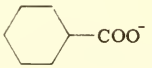
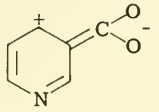
Inhibitor (3 m*M*)	Structure	% Inhibition
Crotonate	$CH_3-CH=CH-COO^-$	99
Butyrate	$CH_3-CH_2-CH_2-COO^-$	0
Dimethylacrylate		70
Isovalerate		0
Fumarate and maleate	$^-OOC-CH=CH-COO^-$	0
Cinnamate		100
Hydrocinnamate		55*
Cinnamamide		0
Phenylacetate		15
p-Toluenesulfonate		34
Sulfanilate		3

TABLE 2-17 (*continued*)

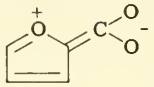
Inhibitor (3 m*M*)	Structure	% Inhibition
Nicotinate		65
Nicotinamide		6
2-Furoate		100
Indole-2-carboxylate		100
Benzoate		100
p-Methoxybenzoate		100
o-Methoxybenzoate		67
p-Carboxybenzoate		74
o-Carboxybenzoate		3
m-Carboxybenzoate		20
Cyclohexanecarboxylate		53*
Hydroquinone		73
Tribromophenol		40
p-Aminophenol		30
Triiodophenol		19
Catechol		19
3, 5-Dihydroxyphenol		5
2, 3-Dihydroxyphenol		0
Resorcinol		0

[a] The substrate is D-alanine at 6.25 m*M* and the pH 8.3. The inhibitions marked with an asterisk are at least partially due to contamination with the unsaturated compounds. (Data from Frisell *et al.*, 1956.)

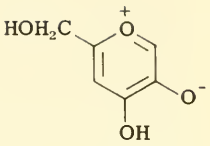
and some of the other inhibitors may be written as:



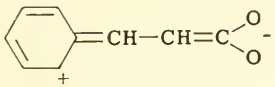
Nicotinate



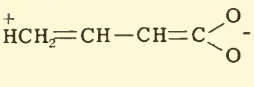
2-Furancarboxylate



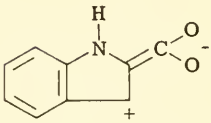
Kojic acid



Cinnamate

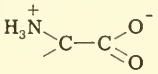


Crotonate

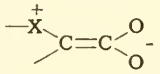


Indole-2-carboxylate

Such structures would be less possible or impossible for hydrocinnamate, 1,4-pyrone-2,6-dicarboxylate, cyclohexanecarboxylate, phenylacetate, and some of the other weaker inhibitors. The orientation relative to the substrate, according to Klein, would be represented as:



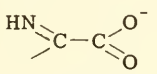
Substrate



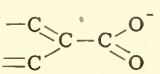
Inhibitor

where X is carbon, oxygen, or nitrogen.

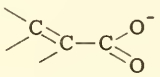
(3) Frisell and his co-workers, on the other hand, emphasized the importance of a double bond near the carboxylate group. Saturation of crotonate, cinnamate, dimethylacrylate, and benzoate definitely reduces the inhibition. They postulated that this double bond might correspond in position on the enzyme to the double bond of the iminoketonic form of the dehydrogenated product, and thus according to their theory the structural correspondence would be:



Product



Benzoate



Aliphatic carboxylate

(4) These two theories are not incompatible, since we have seen that the presence of a positive charge generally depends on resonance, which in turn requires double bonds and either conjugation or hyperconjugation.

(5) The different potencies of the substituted benzoates would be explained on the basis of the effects such groups would have on resonance and the magnitude of the positive charge, but in addition other factors must play a role, for example the dipole moments of the ring X bonds and the possible interactions of the substituent groups themselves. One might expect *ortho* groups to decrease the binding and it is true that all are less inhibitory than benzoate. Bartlett pointed out that the inhibition increases markedly with the electronegativity of the halogens. It is very interesting to attempt to interpret the results in Table 2-15 but without more knowledge about the nature of the binding any hypotheses must be vague for the time being.

(6) It is unlikely that the degree of ionization of the carboxyl group is important here because all of this work was done between pH 7.6 and 8.3 where a negligible fraction is undissociated. The pK_a's of all the substituted benzoates tested run from 2.85 to 4.65. However, the series of phenols studied by Frisell *et al.* (1956) may have to be considered in terms of the ionization of the OH groups when relating structure to activity.

(7) The K_i's for some selected inhibitors were determined by Frisell *et al.* (1956) and from these the relative binding energies may be estimated (see accompanying tabulation). The 1.45 kcal/mole difference between cinnamate and benzoate (it is 1.52 kcal/mole from Klein's data) may be attributed to a more satisfactory location of the positive charge in benzoate. The lesser affinity of the enzyme for nicotinate compared with benzoate (0.51 kcal/mole) could be due to the reduction in resonance brought about by the asymmetry of the former. If the 1.94 kcal/mole difference between pyrrole-2-carboxylate and furan-2-carboxylate is related to the different amounts of positive charge on the nitrogen and oxygen atoms, this would not be surprising since pyrrole should resonate more effectively. Finally, if the positive charge theory is valid, the 3.02 kcal/mole difference between benzoate and phenylacetate might indicate the amount of binding contribution from the positive charge. The positive charge may, of course, be stabilized somewhat by an enzyme anionic site with which it interacts.

Source of enzyme	Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
Lamb kidney	Cinnamate	0.076	5.85
	Crotonate	0.038	6.28
Pig kidney	Cinnamate	0.22	5.20
	Benzoate	0.021	6.65
	Indole-2-carboxylate	0.0034	7.77

Although the concept that these inhibitors bind to the enzyme and compete with the substrate is very reasonable, some recent evidence may indicate that the situation is a little more complex. The values of K_i for benzoate should be the same for every substrate, according to the classic treatment, but Klein (1956, 1960) has found that they are not, although all the $1/v-1/(S)$ plots are definitely competitive. The k_i's may vary as much as almost 3-fold (see tabulation below). One possibility is that in

Substrate	Relative V_m	K_m (mM)	K_i (mM)
Alanine	1.00	6.3	0.059
Proline	1.66	5.8	0.070
Phenylalanine	1.39	14.0	0.092
Valine	0.60	4.6	0.096
Isoleucine	0.79	4.1	0.104
Methionine	1.32	5.3	0.163

the aqueous extracts of pig kidney there are different oxidases for each substrate but Klein prefers to assume that the inhibitors may react with the ES complex to release the substrate:

$$ES + I \rightleftarrows EI + S$$

The equilibrium constant $K = (ES)(I)/(EI)(S)$ depends on the substrate used and in the usual rate equation for competitive inhibition, $(I)K_s/K_i$ would be replaced by $(I)/K$. Since $K = K_i/K_s$, the determined values of K should be inversely proportional to K_s. They are not inversely proportional to K_m, but is $K_m = K_s$ in this case? It may be noted that this mechanism is not quite the same as uncoupling inhibition, since there an ESI complex is formed and the substrate is not forced off. I must admit that I cannot easily visualize how a competitive inhibitor can actively displace an enzyme-bound substrate molecule.

Although Hellerman *et al.* (1946) reported the inhibition of D-amino acid oxidase by benzoate to be independent of FAD concentration, there is more recent evidence that certain aromatic carboxylates and phenols not only can compete with substrate, but can also either compete with FAD or complex directly with FAD (Yagi *et al.*, 1957, 1959, 1960). The constants for each of these reactions are given for a few of these inhibitors in the following tabulation. Only the carboxylates compete with substrate, while the phenols act by the other two mechanisms; the substances with both COO^- and OH groups react in all three ways, although competition with the substrate is the most important. Yagi and his group have recently

Inhibitor	K_i (mM)		
	Competition with substrate	Competition with FAD	Complex with FAD
Benzoate	0.0145	—	—
Salicylate	0.35	1.05	0.65
p-Aminosalicylate	6.35	15	75
p-Aminobenzoate	0.305	7.25	—
p-Nitrobenzoate	—	0.85	—
Aniline	—	9.15	—
Phenol	—	56.5	32
m-Aminophenol	—	28	110
p-Nitrophenol	—	5.0	0.5
2,4-Dinitrophenol	—	3.9	0.02
2,6-Dinitrophenol	—	2.3	0.04
2,4,6-Trinitrophenol	—	0.64	0.012

crystallized the complex of apoenzyme, FAD, and benzoate, and found equimolar amounts of each component present.

It would be interesting to have more data on the effects of these inhibitors on L-amino acid oxidases. Benzoate inhibits the L-amino acid oxidases from rat kidney (Blanchard *et al.*, 1944) and snake venom (Zeller and Maritz, 1945), but does not inhibit the enzyme from *Neurospora* even at 10 mM (Burton, 1951 b).

Benzoate on Other Enzymes and Metabolism

The endogenous ammonia formation and respiration of rat kidney slices are inhibited 52% and 66%, respectively, by 25 mM benzoate (Herner, 1944). One might assume that the former might be attributed to inhibition of amino acid oxidases, but this is unlikely because benzamide, phenylacetate, and β-phenylpropionate are even more potent inhibitors than benzoate. The deamination of various L- and D-amino acids in kidney is, however, strongly inhibited by benzoate, at least in part competitively.

The inhibition of respiration by benzoate was first observed by Griffith (1937) in slices and minces of various tissues in the presence of glucose, but no analysis of the site of action has been published, although in connection with recent studies on the salicylates some effects on mitochondria have been investigated. Benzoate inhibits the oxidations of citrate, α-ketoglutarate, and succinate in rat kidney homogenates but is invariably less active than salicylate (E. H. Kaplan *et al.*, 1954); and neither the O_2 uptake nor the P : O ratio is affected markedly in rat liver mitochondria, although salicylate uncouples strongly (Brody, 1956). Endogenous phosphor-

ylation in liver mitochondria is inhibited 55% by 10 m*M* benzoate (Weinbach, 1961). One can conclude from this limited material that benzoate is certainly a weak inhibitor of cycle oxidations and phosphorylations. Bosund (1959, 1960 a, b) has investigated the effects of benzoate on the metabolism of glucose and pyruvate in *Proteus vulgaris* in attempting to elucidate the mechanisms for the bacteriostatic activity. There is no interference with glucose metabolism to the acetate level, and acetate was found to accumulate. The respiratory quotient is increased from 1.24 to 1.82 by benzoate during the oxidation of pyruvate and the O_2/pyruvate ratio is decreased. The oxidation of pyruvate in yeast is quite strongly inhibited by benzoate (50% at 0.4 m*M*), especially at low pH's where penetration is better, but acetate oxidation is less sensitive. It is quite possible that these inhibitions play a role in the suppression of growth, which for yeast requires 5 m*M* benzoate at pH 5.1 and 60 m*M* at pH 6. It is clear that much more work must be done before the mechanisms of respiratory inhibition are understood.

Benzoate can also interfere in lipid metabolism, as demonstrated many years ago by Jowett and Quastel (1935 a, b), but the mechanism is still unknown. In liver slices it was claimed that benzoate at around 0.5–2 m*M* inhibits specifically the oxidation of fatty acids, and the oxidation of crotonate 63% at 1 m*M*. There is progressively less effect on the higher fatty acids, little inhibition of decanoate being observed. It is possible that the benzoate ring simulates the aliphatic chains of butyrate or crotonate enabling it to compete with these substrates for some enzyme; it would be interesting to know if benzoate can participate in any of these reactions (e. g., if benzoyl-CoA is formed) and deplete the systems of some cofactor. Benzoate is a weak inhibitor of tyrosinase (Ludwig and Nelson, 1939), chymotrypsin (Foster and Niemann, 1955 b), *p*-aminobenzoate acetylation (Koivusalo and Luukkainen, 1959), and NADPH dehydrogenase (Kasamaki *et al.*, 1963); it does not effect shikimate dehydrogenase (Balinsky and Davies, 1961 b) or D-glutamate oxidase (Mizushima and Izaki, 1958) at 1 m*M*, or α-ketoisocaproate decarboxylase at 4 m*M* (Sasaki, 1962).

Kojic Acid

The potent inhibition of D-amino acid oxidase by kojic acid is interesting in light of the central nervous system effects observed in dogs, rabbits, and rats, namely, ataxia, excitement, and convulsions (Friedemann, 1934). Kojic acid was first isolated by Saito in 1907 from *Aspergillus oryzae* and has since been found in many species of *Aspergillus*. It is a weak antibiotic, inhibiting growth of most bacteria at 2–15 m*M*, but is particularly active against *Leptospira*, complete growth inhibition being observed at 0.007 m*N* (Morton *et al.*, 1945). Toxic effects are produced in dogs by 150 mg/kg and in mice by 250 mg/kg when injected parenterally; the LD_{50} for mice is

1.5–2.0 g/kg. Leucocytic activity and phagocytosis are not affected by 18 mM kojic acid. A biochemical study was undertaken by Klein and Olsen (1947), who found that the respiration of muscle and heart mince is resistant to kojic acid, whereas 10 mM suppresses the respiration of liver 40%, kidney 20%, and brain 15%. The convulsant dose corresponds to a tissue concentration around 4–50 mM. The oxidation of both L- and D-amino acids in liver homogenates is quite strongly inhibited in a competitive fashion: for example, 50% inhibition is given by 0.04 mM for L-methionine and by 0.12 mM for L- and D-phenylalanine. Xanthine oxidation is also inhibited (50% at 0.7 mM). It was suggested that kojic acid may be an inhibitor of flavin enzymes, and it is possible that some direct complexing with FAD may occur. Nevertheless, FAD does not influence the inhibition of D-amino acid oxidase. There is no inhibition of the oxidation of succinate, tyramine, L-proline, choline, or urate at 5 mM kojic acid. Although the metabolic effects are interesting, it is impossible to correlate any of these inhibitions with either the central effects in animals or the bacteriostatic activity.

ANALOG INHIBITION OF THE METABOLISM OF VARIOUS AMINO ACIDS

Many reports indicate the influence of analogs on various enzymes concerned with amino acid metabolism, but in most cases insufficient work has been done to draw clear conclusions about the mechanism of the binding to the enzymes. It will suffice to present some of the results in Table 2-18. Probably many of these inhibitions are competitive but they have been so indicated only when graphical analysis has shown this to be true. A few of these inhibitions may be significant in feed-back control or in the general regulation of amino acid metabolism.

Generally speaking, there are certain types of amino acid analog that have proved to be effective inhibitors. McIlwain (1941) pointed out that aminosulfonate analogs of amino acids are frequently bacteriostatic and that this inhibition is reduced by adding the normal amino acids. If staphylococci are trained to be independent of exogenous amino acids, the α-aminosulfonates no longer inhibit. However, the exact sites of action of these analogs have not been determined. Umbreit (1955 b) has discussed the general inhibitory properties of the α-methylamino acids and pointed out that the inhibitions are often competitive only for a short interval if both substrate and inhibitor are present, whereas they are noncompetitive if the inhibitor is added first. This is a matter of terminology; the inhibitions are probably competitive but appear to be noncompetitive because of the very high affinities of some analogs for the enzymes (an inhibition can be competitive even though irreversible but the substrate must be given an

opportunity to compete). A third group of interesting analogs is the halogen-substituted amino acids, which are often potent inhibitors of protein synthesis and growth. The fluoroamino acids are particularly active. The *m*-, *o*-, and *p*-fluorophenylalanines all inhibit the formation of the adaptive maltase in yeast, the last being the most potent (Halvorson and Spiegelman, 1952). On the other hand the *p*-chloro- and *p*-bromophenylalanines do not inhibit. The incorporation of phenylalanine and other amino acids into ascites cell proteins is competitively inhibited by *o*-fluorophenylalanine; this is in part due to a depression of transport into the cells and in part due to block of some unknown steps in the incorporation (since labeled phenylalanine accumulates in cells) (Rabinovitz *et al.*, 1954). There is also an inhibition of protein synthesis in rat liver *in vitro* by the fluorophenylalanines, this leading to a net breakdown of tissue proteins since the constant balance of synthesis and degradation is disturbed (Steinberg and Vaughan, 1956). α-Amino-β-chlorobutyrate is an analog of valine and inhibits valine incorporation into rabbit reticulocyte proteins, including hemoglobin; it was suggested that the analog enters a precursor protein which is unable to assume the proper configuration of hemoglobin and thus there is accumulation of protein intermediates (Rabinovitz and McGrath, 1959). As pointed out previously, some of these analogs are incorporated into cell proteins. *p*-Fluorophenylalanine-C^{14} is incorporated into the proteins of muscle, blood, and liver when fed to rabbits, this being a replacement of phenylalanine (Westhead and Boyer, 1961). The replacement of phenylalanine in aldolase is 25% and in 3-phosphoglyceraldehyde dehydrogenase 16%, and in each case the enzyme activities are normal. Despite this appreciable incorporation, the rabbits suffer no obvious biochemical or physiological disturbances, so that mammals may well differ from microorganisms in the response to this analog.

Feedback inhibition in the biosynthetic pathways of amino acids is an important aspect of regulation but we can touch only briefly on this problem. An interesting example of this has been studied in connection with the synthesis of histidine, since the enzyme inhibited is the first in the pathway and catalyzes a reaction not involving substrates structurally similar to histidine (Martin, 1963). This enzyme is phosphoribosyl-ATP pyrophosphorylase and the reaction catalyzed is:

$$5'\text{-P-ribosyl-PP} + \text{ATP} \rightleftarrows \text{N-1-(5'-P-ribosyl)-ATP} + \text{PP}$$

Histidine is a surprisingly potent and specific inhibitor with $K_i = 0.1$ mM. Related compounds inhibit weakly or not at all; 2-methylhistidine, for example, exhibits weak inhibition with $K_i = 2.4$ mM. The inhibition varies with the pH but maximal inhibition is exerted at physiological pH. $HgCl_2$ at 0.03 mM does not inhibit the enzyme but blocks almost completely the inhibition by histidine. This coupled with the fact that the inhibition by

TABLE 2-18

MISCELLANEOUS ANALOG INHIBITIONS OF ENZYMES INVOLVED IN AMINO ACID METABOLISM [a]

Enzyme	Source	Analog	Type of inhibition	(S) (mM)	(I) (mM)	% Inhibition	K_i (mM)	Reference
Leucine decarboxylase	*Proteus vulgaris*	L-α-Aminoisobutyrate		58	58	0		Haughton and King (1961)
		L-α-Aminobutyrate		58	58	3		
		L-Lysine		58	58	8		
		N-Acetyl-L-leucine		58	58	23		
		Isobutylamine	C	58	58	35		
		Isovalerate	C	58	58	85	1.8	
		Valerate	C	—	—	—	2.9	
		Butyrate		—	—	—	17	
Histidine decarboxylase	Guinea pig kidney	Dopa		17	0.02	60		Werle and Koch (1949)
		Catechol		17	0.02	22		
				17	0.2	57		
		Pyrogallol		17	0.2	58		
		Tryptophan		17	2	26		
				17	10	71		
		Tyrosine		17	2	14		
		Indole		17	2	21		
				17	10	68		
		Imidazole		17	50	18		
	Rabbit kidney	Aminoguanidine		10	0.1	0		Waton (1956)
				10	1	40		

	Rat peritoneal mast cells	DL-5-Hydroxytryptophan		—	0.4	25	Rothschild and Sehayer (1959)
				—	0.8	38	
				—	1.6	50	
				—	3.2	76	
		DL-Tryptophan		—	0.8	16	
				—	3.2	22	
		DL-Tyrosine		—	3.2	49	
		DL-Phenylalanine		—	3.2	11	
	Guinea pig kidney	5-Hydroxytryptophan		0.64	0.065	50	Robinson and Shepherd (1961)
		α-Methyl-5-hydroxy-tryptophan		0.64	0.0075	50	
		Histidine hydrazide		0.64	0.085	50	
	Rat hepatoma	5-Hydroxytryptophan		0.64	7.5	50	Robinson and Shepherd (1961)
		α-Methyl-5-hydroxy-tryptophan		0.64	0.1	50	
		Histidine hydrazide		0.64	0.2	50	
Histidase	Rat liver	Imidazole		12	2	20	Edlbacher *et al.* (1940)
		Imidazoleacetate		12	24	45	
		Guanidine		12	24	47	
		Methylguanidine		12	24	26	
		Dimethylguanidine		12	24	28	
		Lysine		12	48	31	
		Ornithine		12	48	20	
Arginine deiminase	*Streptococcus faecalis*	Canavanine	C	1.67	0.33	14	Oginsky and Gehrig (1952)
				1.67	1.67	32	
				1.67	8.3	55	
				1.67	33.3	82	

TABLE 2-18 (*continued*)

Enzyme	Source	Analog	Type of inhibition	(S) (m*M*)	(I) (m*M*)	% Inhibition	K_i (m*M*)	Reference
L-Alanine dehydrogenase	*Bacillus cereus*	D-Alanine	C	—	—	—	23.1	O'Connor and Halvorson (1961a)
		D-Cysteine	C	—	—	—	0.36	
		Glycine	C	—	—	—	42	
		D-Serine	C	—	—	—	51	
		D-Isoleucine	C	—	—	—	125	
		D-Valine	C	—	—	—	250	
		D-Norvaline	C	—	—	—	280	
Phenylalanine hydroxylase	Rat liver	DL-Alanine		2.4	5	0		Udenfriend and Cooper (1952)
		Cyclohexyl-DL-alanine	C	2.4	2.4	26		
				2.4	16	43		
		β-2-Thienylalanine		2.4	12	66		
				2.4	20	74		
Phenylalanine-activating enzyme	*E. coli*	β-Phenylserine	C	0.06	0.3	22		Conway *et al.* (1964)
				—	0.6	55		
				—	1.5	74		
				—	3	80		
		p-Fluorophenylalanine	C	0.06	0.6	17		
				—	1.5	64		
				—	3	72		
		β-2-Thienylalanine	C	0.06	6	33		
				—	12	48		

		2-Pyridylalanine	C	0.06	12	45		
		p-Aminophenylalanine	C	0.06	12	37		
		p-Tolylalanine	C	0.06	6	46		
Phenylalanine deaminase	*Hordeum vulgare*	L-Tyrosine	C	10	0.2	36		Koukol and Conn (1961)
				10	0.6	57		
		Dopa		10	6	22		
		trans-*p*-Coumarate	C	10	0.2	45		
				10	0.6	64		
		β-Phenyl-DL-serine		10	6	22		
Glycine *N*-acylase	Beef liver	Hippurate	NC?	—	0.58	50	0.00004	Schachter and Taggart (1954)
		p-Hydroxyhippurate		—	0.5	65		
		p-Aminohippurate		—	0.5	32		
Δ^1-Pyrroline-5-carboxylate reductase	Beef liver	L-Proline	C	0.16	0.1	55	0.019	Adams and Goldstone (1960a)
		D-Proline	C	0.16	5	9	11.9	
		L-Hydroxyproline	C	0.16	1	9	2.4	
		Imidazole	C	0.16	1	55	0.19	
Δ^1-Pyrroline-5-carboxylate dehydrogenase	Beef liver	L-Hydrixyproline	C	—	—	—	0.7	Adams and Goldstone (1960b)
		L-Proline	C	—	—	—	2.6	
		Imidazole	C	—	—	—	29	
Aspartase	*Propionibacterium peterssonii*	Fumarate	C	—	100	80		Ellfolk (1954)
		Ethanedisulfonate	C	—	100	75		
		Sulfoacetate	C	—	100	20		
		D-Aspartate		—	100	0		
Aspartate: α-ketoglutarate transaminase	Pig kidney	Hydroxyaspartate	C	—	—	—		Garcia-Hernandez and Kun (1957)
		2,3-Diaminopropionate	NC	—	—	—		

TABLE 2-18 (*continued*)

Enzyme	Source	Analog	Type of inhibition	(S) (m*M*)	(I) (m*M*)	% Inhibition	K_i (m*M*)	Reference
Aspartokinase	*E. coli*	L-Lysine	NC	10	10	32		Stadtman *et al.* (1961)
		L-Threonine	C	10	10	38		
		L-Homoserine		10	10	10		
Glutaminase	Rat liver	DON		67	1.1	50		Ghosh *et al.* (1960)
		Azaserine		67	92	12		
Glutamine: fructose-6-phpsphate transamidase	Rat liver	DON		—	0.006	60		Ghosh *et al.* (1960)
	E. coli	DON		—	0.11	80		
Betaine: homocysteine transmethylase	Pigeon liver	Dimethylglycine	C?	—	—	—		Sloane and Boggiano (1960)
Thetin: homocysteine transmethylase	Mouse liver	Betaine	C	10[b]	100	70	64	Maw (1959)
		Triethylsulfonium	C	10[b]	100	35		
		Sulfocholine		10[b]	100	32		
		Diethylthetin		10[b]	100	25		
		Choline		10[b]	100	24		
		Trimethylammonium	C	10[b]	100	14		

	Rat liver	DL-Homoserine	C	—	—	—		Fromm and Nordlie (1959)
Cysteine desulfurase	Dog liver	Glutarate		—	10	38		Fromageot and Grand (1944)
		Glutamate		—	10	47		
		Aspartate		—	10	39		
Serine deaminase	Rat liver	L-Homocysteine		—	—	—		Selim and Greenberg (1960)
		L-Cysteine		—	—	—		
Homoserine kinase	*E. coli*	L-Threonine	C	1.25	10	55	5	Wormser and Pardee (1958)
		DL-α-Aminobutyrate	C	1.25	10	49		
		L-Cysteine	C	1.25	10	37		
		Butyrate		1.25	10	31		
		L-Isoleucine	C	1.25	10	26		
		L-Arginine	C	1.25	10	24		
		L-Lysine	C	1.25	10	24		
		L-Histidine	C	1.25	10	21		
L-Threonine dehydrase	Sheep liver	L-Serine	C	—	—	—		Nishimura and Greenberg (1961)
L-Threonine synthetase	*Neurospora crassa*	Phosphothreonine		13.5	10	35		Flavin and Slaughter (1960)
		D-Homoserine		13.5	5	0		

[a] C = competitive and NC = noncompetitive. DON = 6-diazo-5-oxo-L-norleucine.

[b] This concentration for dimethylthetin. DL-Homocysteine was 30 m*M*.

histidine is noncompetitive indicates that the histidine site is different from the catalytically active site. ATP and 5′-P-ribosyl-PP protect the enzyme against inactivation by trypsin, and histidine eliminates this protection, so it is possible that histidine modifies the enzyme configuration by combining with the feedback site.

Aminooxyacetate

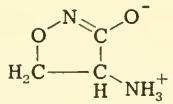
An interesting analog studied recently in connection with the physiologically important γ-aminobutyrate (GABA) is aminooxyacetate ($^{+}H_3N$—O—CH_2COO^{-}). This substances inhibits the GABA: α-ketoglutarate transaminase of *E. coli* very potently (40% at 0.0033 m*M* and 100% at 0.33 m*M* when both substrates are 27 m*M*), so it was tested on a similar enzyme from brain and found to inhibit as strongly (Wallach, 1960). Aminooxyacetate in doses of 6–50 mg/kg elevates the brain GABA in several species as much as 4- to 5-fold, peak levels being reached about 6 hr after administration and high levels remaining up to 24 hr (Wallach, 1961 a; Schumann *et al.*, 1962). Reinvestigation of the transaminase inhibition led to a K_i of 0.0075 m*M* (K_m is 9.66 m*M* for α-ketoglutarate and 27.6 m*M* for GABA); the inhibition is competitive with respect to both substrates. The alanine: α-ketoglutarate transaminase from rat liver and heart is also potently inhibited, around 60–80% by 0.0001 m*M* aminooxyacetate at pH 6.8 (Hopper and Segal, 1964). Indeed, this transaminase seems to be more sensitive than either the GABA:α-ketoglutarate or aspartate:α-ketoglutarate transaminase.

Inasmuch as GABA has been implicated in certain convulsive disorders (e. g., GABA formation in epileptic brains is apparently depressed), aminooxyacetate was administered to animals made convulsive with thiosemicarbazide and methionine sulfoximine (DaVanzo *et al.*, 1961). Anticonvulsant activity was observed but there is some doubt if this is correlated with the brain GABA levels since the time relations are not correct. The effects of aminooxyacetate on central nervous system function are complex. There is first a progressive depression and muscular relaxation with loss of certain reflexes, but at high doses tonic-clonic convulsions occur (DaVanzo *et al.*, 1964 a). Pyridoxal-P antagonizes these convulsions and it was postulated that oxime formation occurs between aminooxyacetate and pyridoxal-P. Pyridoxal-P does not reverse the transaminase inhibition in the brain (Wallach, 1961 b). Wallach also suggested that a depletion of succinic semialdehyde, which arises from GABA by transamination, might also play a role in the convulsant action. Since a pyridoxal deficiency is produced in rats by the administration of aminooxyacetate, DaVanzo *et al.* (1964 b) postulated another possible mechanism of action, namely, the inhibition of pyridoxal kinase, inasmuch as McCormick and Snell (1961) had shown this enzyme to be rather potently inhibited by the condensation

product of aminooxyacetate and pyridoxal. It may be that aminooxyacetate should be classed as a carbonyl reagent which reacts with pyridoxal-P, rather than strictly as an amino acid analog, but it is sometimes difficult to distinguish these actions. Nevertheless, the effects of aminooxyacetate on GABA levels in the tissues will undoubtedly make it a useful compound for the study of the physiological role of GABA.

Cycloserine

D-Cycloserine (orientomycin, Oxamycin) is a tuberculostatic antibiotic isolated from *Streptomyces* which can be considered as a cyclic form of aminooxyalanine or *O*-aminoserine. It is written in the zwitterion state be-

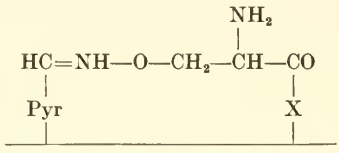


Cycloserine

cause there is some evidence that this is the inhibitory form (Neuhaus and Lynch, 1964). Its actions are similar to aminooxyacetate in many respects but differ occasionally in interesting ways. D-Cycloserine inhibits the growth of many bacteria and this is often well antagonized by D-alanine, and competitively inhibits the incorporation of D-alanine into a uridine nucleotide necessary for the synthesis of cell wall material. The growth of mycobacteria is 50% suppressed, for example, by D-cycloserine at 0.03–0.045 m*M* and this is reversed by D-alanine but not by L-alanine (Zygmunt, 1963). L-Cycloserine is also inhibitory to certain bacteria and this is antagonized by L-alanine. It is interesting that *S. aureus* can develop a 50-fold resistance to D-cycloserine, no cross-resistance with other antibiotics being observed (Howe *et al.*, 1964). In animals it produces sedation, lethargy, muscular relaxation, ataxia, an accentuated startle response, and, above all, epileptic convulsions (Dann and Carter, 1964; Holtz and Palm, 1964). In these respects it at least superficially acts like aminooxyacetate and, furthermore, these effects are antagonized by pyridoxal.

D-Cycloserine inhibits certain enzymes dependent on pyridoxal-P, such as the transaminases and glutamate decarboxylase, and it has been postulated that it simply reacts with pyridoxal-P to form a substituted oxime. However, this is not the usual reaction in which a Schiff base is produced, but involves an opening of the cycloserine ring. The inhibition of GABA: α-ketoglutarate transaminase is initially competitive with respect to GABA (K_i is 0.25 m*M* for the enzyme from cat brain and around 0.6 m*M* for the enzyme from *E. coli*), but a secondary progressive irreversible inhibition also occurs (Dann and Carter, 1964). This may be related to the hypothesis of Khomutov *et al.* (1961) that the decyclicized cycloserine forms an oxime

bond with enzyme-bound pyridoxal-P, and also an acyl bond with a cationic group at the active site, these two groups thus being connected by a bridge would prevent access to the site. The L-asparagine:α-ketoglutarate

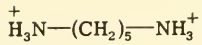


transaminase is inhibited by L-cycloserine with respect to L-asparagine (K_i = 0.0001 mM) but D-cycloserine is a much weaker inhibitor (K_i = 1 mM) (Braunstein *et al.*, 1962). L-Cycloserine likewise competitively inhibits the L-alanine: α-ketoglutarate transaminase (K_i = 0.008 mM). This taken with previous work indicates that the cycloserines inhibit specifically those enzyme attacking substrates of the same optical isomerism. Another possible site of action of D-cycloserine in bacteria would be the D-alanyl-D-alanine synthetase, a block of which would prevent the incorporation of D-alanine into cell wall material. Alanylalanine is often able to reverse the cycloserine inhibition of bacterial growth, sometimes more effectively than alanine. One example of this is the inhibition of the proliferation of agents of the psittacosis group in chick embryo yolk sac (Moulder *et al.*, 1963). Chick embryos infected with the mouse pneumonitis organism, for example, are well protected by D-cycloserine at 0.004–0.008 mM, and, of all the possible reversors tested, only alanylalanine is effective. The D-alanyl-D-alanine synthetase of *Streptococcus fecalis* is inhibited competitively with respect to D-alanine (K_i = 0.022 mM), and Neuhaus and Lynck (1964) felt that this enzyme may well be the major site of inhibition in certain bacteria. It is unfortunate that cycloserine and aminooxyacetate have not been accurately compared in any study.

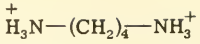
DIAMINE OXIDASE (HISTAMINASE)

Enzymes in this group exhibit different degrees of substrate specificity depending on the source, but most oxidatively deaminate diamines of the type $^{+}H_3N—(CH_2)_n—NH_3^{+}$ (with maximal rates when n is around 5) and histamine, in all cases primary amines being attacked. The usual substrate in most studies has been cadaverine (n = 5).

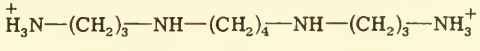
The diamines have been written as cations because the pK_a's are usually between 8.5 and 10.5. The amidines and guanidines exist almost entirely as cations at physiological pH since the pK_a's for these groups are around 13 to 14. The structures have been written in a rather unconventional way



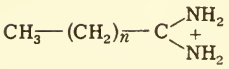
Cadaverine



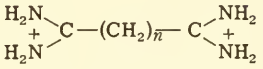
Putrescine



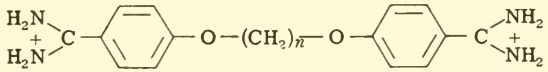
Spermine



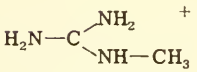
Monoamidines



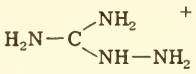
Diamidines



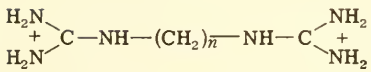
Dibenzamidines



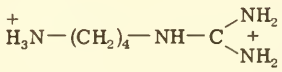
Methylguanidine



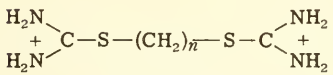
Aminoguanidine



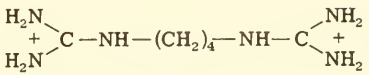
Diguanidines



Agmatine

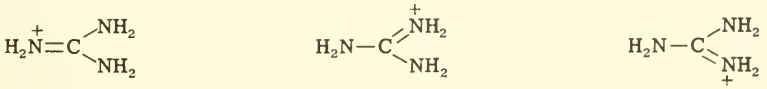


Diisothioureas



Arcaine

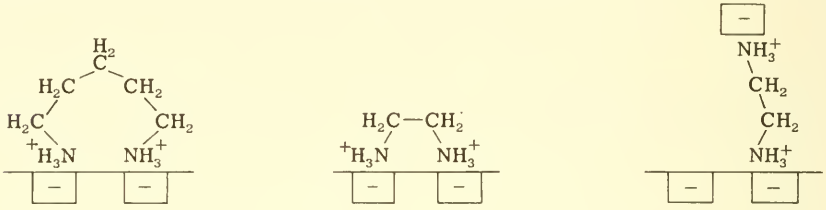
to indicate the equivalence of the C—N bonds and the states of the amino groups, since there is resonance between forms such as the following for the guanidinium ion:



Alkyl substitution does not reduce the resonance appreciably.

Aliphatic monoamines, such as amylamine, are not substrates nor are

they readily bound to the enzyme since they inhibit weakly (Zeller, 1940). The short aliphatic diamines are very poor substrates but inhibit quite well; thus both ethylenediamine and trimethylenediamine inhibit the oxidation of cadaverine by pig kidney diamine oxidase (Zeller, 1938). If the amino groups of these inhibitors react with the same anionic enzyme sites as does cadaverine, these anionic groups must be fairly close so that cadaverine would have to assume a very bowed configuration. On the other



hand, the anionic groups may be separated by a distance corresponding to the amino groups in cadaverine and the inhibitors react with only one of the anionic sites ,the other amino group interacting with some anionic site outside the active center (as in (c)).

Certain guanidine derivatives are more potent inhibitors. Guanidine itself is rather weak, inhibiting cadaverine (2 m*M*) oxidation 42% at 10 m*M*, but methylguanidine inhibits 63% at 1 m*M* (Zeller, 1938). Although Zeller noted that in the pig kidney preparation methylguanidine did not inhibit histamine oxidation very well, Waton (1956) found marked inhibition of histaminase activity in cat kidney, 0.01 m*M* inhibiting 42% and 0.1 m*M* 75%. The oxidations of putrescine and agmatine are both well inhibited by methylguanidine (Zeller, 1940). An even more potent inhibitor, however, is aminoguanidine. Apparently the diamine oxidases differ in sensitivity to aminoguanidine; 50% inhibition is given by 0.00005 m*M* for the enzyme from pig kidney (Schuler, 1952), by 0.001 m*M* for the enzyme from cat kidney (Waton, 1956), by 0.01 m*M* for the enzyme from rabbit liver (Kobayashi, 1957), and the enzyme from mouse liver is not inhibited even by 0.1 m*M*. The nature of the inhibition is not clear, inasmuch as aminoguanidine is also a derivative of hydrazine and might act by attacking carbonyl groups: hydrazine and semicarbazide are, indeed, potent inhibitors of diamine oxidase (Schuler, 1952; Waton, 1956). A further complication is that aminoguanidine hydrolyzes to form semicarbazide and eventually hydrazine. It behaves chemically more like a hydrazine than a guanidine, and reacts with carbonyl groups without being hydrolyzed (Lieber and Smith, 1939). It is also possible that the $NHNH_2$ group simulates the CH_2NH_2 substrate group, as in the monoamine oxidase inhibitors, and forms a tight bond to the enzyme. *In vivo* inhibition of diamine oxidase by amino-

guanidine was demonstrated by Schayer *et al.*, (1954) by injecting labeled cadaverine into mice and determining the $C^{14}O_2$ respired (Fig. 2-7). It is much more effective than isoniazid or agmatine. The metabolism of histamine should also be blocked by aminoguanidine. This was shown in three psychiatric patients by administering labeled histamine and finding that

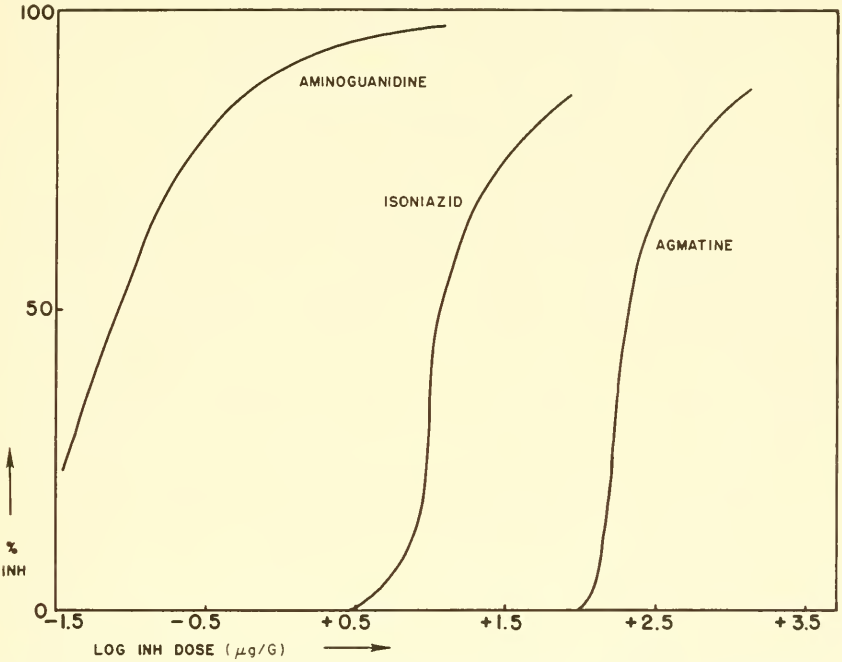


FIG. 2-7. Effects of diamine oxidase inhibitors on the oxidation of cadaverine in mice, as determined by the formation of $C^{14}O_2$ from labeled cadaverine. (Data from Schayer *et al.*, 1954.)

urinary imidazoleacetate-C^{14} is reduced by aminoguanidine at 0.1–1 mg/kg (Lindell *et al.*, 1960). A larger fraction of the histamine is excreted as methylhistamine, demonstrating a diversion of metabolic pathways by this inhibitor.

Diamine oxidase is not strongly inhibited by monoamidines, but diamidines, diguanidines, and diisothioureas of the proper chain lengths are quite potent inhibitors (Blaschko *et al.*, 1951). The data for these series are summarized in Fig. 2-8. The correlation between chain length and inhibition is not nearly so clear as for monoamine oxidase (Blaschko and Duthie, 1945; Blaschko and Himms, 1955), and in some cases, as the diamidines, there is surprisingly little variation of inhibition with chain length. For diamidines of $n = 10$–16, one wonders if the binding is actually to

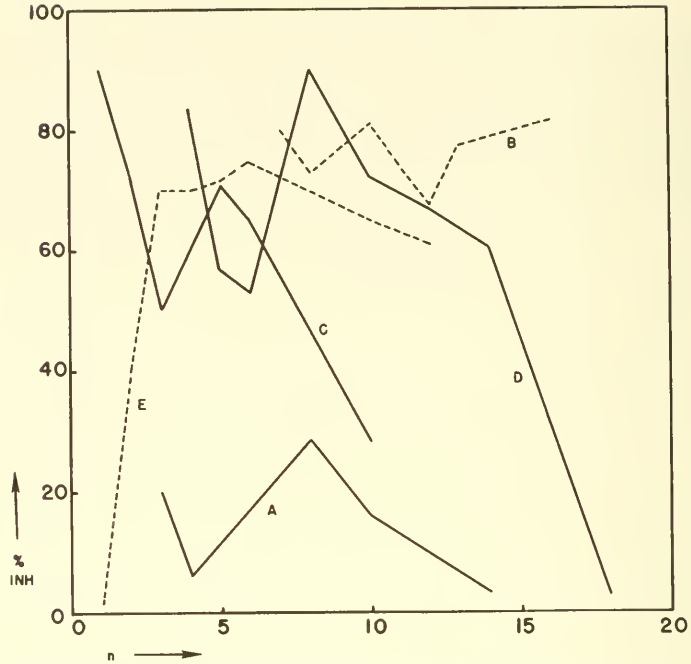
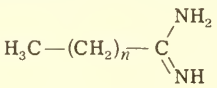
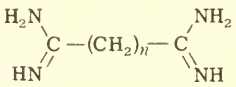


FIG. 2-8. Inhibition of pig kidney diamine oxidase with cadaverine (5 mM) as the substrate and all the inhibitors at 1 mM. (From Blaschko *et al.*, 1951.)

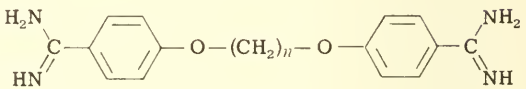
(A) Monamidines



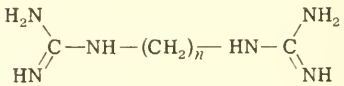
(B) Diamidines



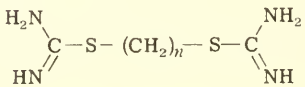
(C) Dibenzamidines



(D) Diguanidines



(E) Diisothioureas



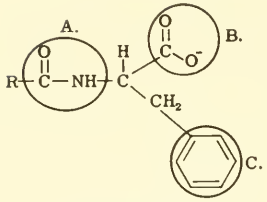
the substrate site entirely, or possibly to two substrate sites, or even to anionic groups outside the substrate site. The enzyme may have binding sites for the imidazole ring of histamine since imidazole inhibits 11%, imidazolelactate 20%, and histidine 4% at 6.7 m*M* when cadaverine is 3.3 m*M* (Zeller, 1941). Urate also competitively inhibits the oxidation of histamine, but rather weakly. An excellent review of the structure-action relationships among the amidine derivatives is by Fastier (1962).

CARBOXYPEPTIDASE, AMINOPEPTIDASES, AND DIPEPTIDASES

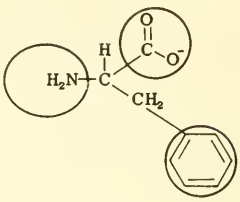
Certain aspects of the inhibition of carboxypeptidase by substrate analogs were discussed in Volume I (page 292) to illustrate how certain interaction contributions could be estimated. We shall now attempt to visualize more clearly the orientation of these analogs on the enzyme surface. The data indicate that a three-point attachment of the substrate is necessary for catalysis. The enzyme sites may be indicated as follows (see Fig. 2-9 for hypothetical orientation of substrate): (A) the *peptidatic site* contains the mechanism of the electron displacement necessary for hydrolysis and is probably positively charged, (B) the *cationic site* is a positively charged group that interacts electrostatically with the COO^- group, and (C) the *electrokinetic site* is perhaps a lipophilic region capable of reacting with alkyl or phenyl groups by dispersion forces. It is easy to see why D-substrates are not reacted since the peptide bonds would not be able to approach the peptidatic site. There is also an enzyme region near the projection direction of the fourth asymmetric carbon bond that sterically prevents attachment of groups larger than an amino group, and thus the D-isomers usually do not bind and are not inhibitors. Only two-point attachment is necessary for inhibitors, and most that have been studied bind at the cationic and electrokinetic sites.

The relative binding energies for inhibitors in Table I-6-26 were calculated from the data of Smith *et al.* (1951). Earlier studies by Elkins-Kaufman and Neurath (1949) provide additional information on the competitive inhibitors in the accompanying tabulation. It is interesting that D-phenylalanine is

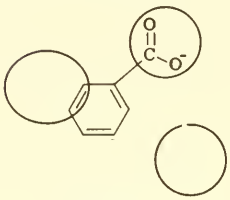
Inhibitor	K_i (m*M*)	Relative $-\Delta F$ of binding (kcal/mole)
β-Phenylpropionate	0.062	5.96
Phenylacetate	0.39	4.83
γ-Phenylbutyrate	1.13	4.17
D-Phenylalanine	2	3.82
p-Nitrophenylacetate	2.5	3.68



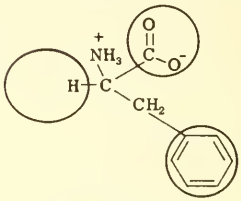
Acyl-L-phenyl-alanine (substrate)



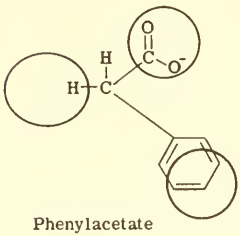
L-Phenylalanine



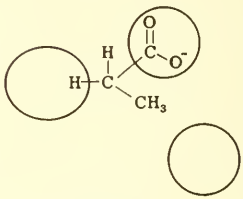
Benzoate



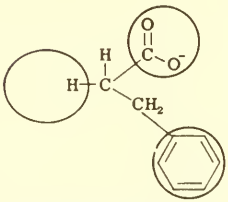
D-Phenylalanine



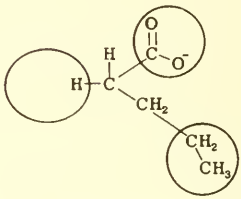
Phenylacetate



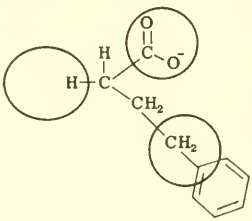
Propionate



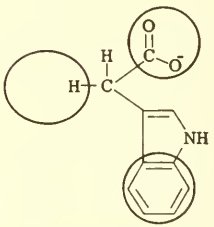
β-Phenylpropionate (hydrocinnamate)



Valerate



γ-Phenylbutyrate



3-Indoleacetate

FIG. 2-9. Possible orientations of substrate and inhibitors at the active center of carboxypeptidase. (A) is peptidatic site, (B) is cationic site, and (C) is electrokinetic site. The molecular configurations are only approximate.

a better inhibitor than L-phenylalanine under the usual conditions, since the NH_3^+ group of the latter is repelled by the positively charged peptidatic site while in the former it does not encounter serious steric interference (Fig. 2-9). However, β-phenylpropionate (hydrocinnamate) is bound more tightly than D-phenylalanine by 2.14 kcal/mole, suggesting that some steric repulsion of the latter analog occurs. *N*-Substitution increases the repulsion markedly and inhibitory activity is lost. A comparison of the possible orientations of some inhibitors in Fig. 2-9 with the relative binding energies may give some idea of the structural requirements for potent inhibition. It may be noted that benzylmalonate is an effective inhibitor but is somewhat less well bound than β-phenylpropionate; this is surprising because it might be thought that additional energy would be contributed by interaction of one of the COO^- groups with the peptidatic site. Neither *cis*- nor *trans*-cinnamate inhibits and it was suggested that the double bond restricts the orientation of the ring so that adequate binding cannot occur. The linearity of these molecules may also be a factor, since the active site is probably not flat as is implied by the two-dimensional representations in the figure.

Competitive inhibition by the following analogs has been demonstrated more recently: 3-indolepropionate, ε-aminocaproate, δ-amino-*n*-valerate (Folk, 1956; Greenbaum and Sherman, 1962), γ-aminobutyrate, δ-guanidinovalerate, argininate (Folk and Gladner, 1958), *N*-acetyl-L-tyrosine, D-leucinyl-L-tyrosine, glycyl-L-tyrosine, and other dipeptides (Yanari and Mitz, 1957). The inhibition apparently sometimes depends on the substrate used; for example, 3-indolepropionate inhibits the hydrolysis of carbobenzoxyglycyl-L-phenylalanine but not the hydrolysis of α-*N*-benzoylglycyl-L-lysine, where ε-aminocaproate exhibits just the opposite behavior.

Relatively little work has been done on analog inhibition of dipeptidases and aminopeptidases, and it will suffice to mention a few isolated observations. Yeast dipeptidase is inhibited by various amino acids; for

Amino acid	Concentration for 50% inhibition (m*M*)
L-Leucine	1.5
L-Isoleucine	1.8
L-Tryptophan	6.0
L-Histidine	8.0
L-Leucinamide	9.0
L-Arginine	18
DL-Valine	22
DL-Phenylalanine	22
DL-Serine	>100

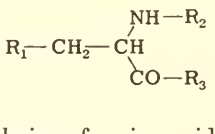
example, L-leucine, D-alanine, and glycine inhibit the hydrolysis of alanylglycine and glycylglycine (Grassmann *et al.*, 1935). This has been investigated more quantitatively by Nishi (1960) and his results are summarized in the accompanying tabulation (substrate is glycylglycine at 50 m*M*). These inhibitions are competitive with respect to substrate and uncompetitive with respect to Co^{++}. Some interesting inhibitions of pig kidney leucine aminopeptidase have been reported by Hill and Smith (1957). The hydrolysis of substrates of the type R—CH(NH_3^+)—CONH—R′ depends on a three-point attachment of the R group, the NH_3^+ group, and the amide N. The R groups interact by van der Waals' forces; further energy is contributed from the hydrogen bonding of water molecules displaced from the hydrophobic surfaces. The inhibitions given in the following tabulation are for L-leucinamide as substrate at 50 m*M* and at pH 8.50–

Inhibitor	Concentration (m*M*)	% Inhibition
L-Leucine	50	45
	100	61
L-Leucinol	50	38
	100	48
Isocaproamide	25	41
	50	56
Isocaproate	100	47
	200	65
n-Hexylamine	100	0
α-Ketoisocaproamide	25	21
L-*α*-Hydroxyisocaproamide	50	68

8.65. Every good inhibitor contains an R group that should give nearly optimal interaction with the electrokinetic site of the enzyme; in addition, at least one group that will react with the bound Mn^{++} is present.

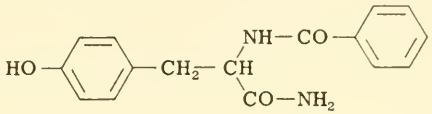
CHYMOTRYPSIN AND OTHER PROTEOLYTIC ENZYMES

Chymotrypsin hydrolyzes various amides and esters of the general type:



where R_1 represents the side chains of amino acids (phenylalanine, tyrosine, and tryptophan most commonly used), R_2 is an acyl group (acetyl, benzoyl,

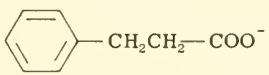
or nicotinyl), and R_3 is a group forming either an amide or ester bond. A typical substrate is benzoyl-L-tyrosinamide:



In addition, the NH—R_2 chain may be replaced by H, Cl, or OH groups. Only the derivatives of L-amino acids are hydrolyzed. The R_1 and R_2 groups are important in binding to the enzyme and thus, with the esteratic (paptidatic) site, one may again visualize a three-point attachment. Analogs either devoid of susceptible amide or ester bonds, or having in their place bonds resistent to hydrolysis, are often inhibitory. The R_1 group is the most important for binding, as is shown by the strong inhibitory activity of β-phenylpropionate (hydrocinnamate) (Kaufman and Neurath, 1949). The necessity for at least one aromatic ring in one of the side chains was pointed out by Neurath and Gladner (1951). Their data on the β-substituted propionates indicate the ring groups to have the following order of inhibitory activity:

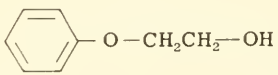
Indole > napthyl > phenyl > 2,4-dinitrophenyl > cyclohexyl

The distance between the COO^- group and the R_1 group is also of importance. The inhibitions are summarized in Table 2-19. The weaker binding of cyclohexyl derivatives compared to phenyl compounds (0.5–1 kcal/mole) could be explained by either the smaller polarizability of the cyclohexyl ring or the inability of the cyclohexyl ring to approach the enzyme surface as close as the phenyl ring. The strong binding of the indole compounds was explained on the basis of the enhancement of hydrogen bonding by the ring N. In fact, Neurath and Gladner interpreted the inhibitions by most of the analogs in terms of hydrogen bonding. Even the COO^- may not interact electrostatically with an enzyme cationic group since the binding energies are quite low; indeed, it may serve as a hydrogen acceptor. The equivalent bindings of



β-Phenylpropionate

and



2-Phenoxyethanol

would be difficult to explain otherwise; however, the latter compound can act as a hydrogen donor in forming a hydrogen bond. These two compounds have essentially the same molecular dimensions but the electronic configurations of the terminal groups differ.

TABLE 2-19

COMPETITIVE INHIBITION OF α-CHYMOTRYPSIN BY ANALOGS [a]

Inhibitor	Apparent K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
3-Indolepropionate	2.5	3.57
3-Indolebutyrate	3.6	3.35
α-Naphthylpropionate	4.0	3.28
2,4-Dinitro-β-phenylpropionate	5.3	3.08
β-Phenylpropionate	5.5	3.07
2-Phenoxyethanol	5.8	3.05
γ-Phenylbutyrate	14	2.53
Cyclohexylpropionate	30	2.08
Cyclohexylbutyrate	35	1.99
Benzoate	42	1.88
Phenylacetate	42	1.88
α-Naphthylmethylmalonate	55	1.72
α-Benzylmalondiamide	78.8	1.51
Cyclohexylacetate	86	1.46

[a] The substrate is acetyl-L-tyrosinamide ($K_m = 27$ mM). Experiments at pH 7.8 and 25°. (Data from Neurath and Gladner, 1951.)

For the past several years Niemann and his associates have conducted excellent quantitative investigations of the inhibition of chymotrypsin by a variety of analogs and their results, some of which are presented in Table 2-20, provide a basis for the interpretation of the binding mechanisms. Although final conclusions must await completion of their work, some tentative ideas may be expressed.

(*A*) Despite the fact that the derivatives of L-amino acids are hydrolyzed by chymotrypsin, the D-isomers of several inhibitors are bound on an average of 0.45 kcal/mole more tightly than the corresponding L-isomers (see also page 271). Although three-point attachment may be important for substrate binding, it is evident from this difference and other data that it is not for inhibitor binding.

(*B*) Comparing the derivatives of the three amino acids, it is seen that the phenylalanine and tyrosine analogs are equally bound, whereas the tryptophan analogs are bound some 1.19 kcal/mole more tightly, and this is probably to be attributed to the greater affinity of the enzyme for the

TABLE 2-20

COMPETITIVE INHIBITION OF α-CHYMOTRYPSIN BY ANALOGS [a]

Inhibitor	Apparent K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
Tryptophan series		
L-Tryptophanamide	8.5	2.93
D-Tryptophanamide	4.0	3.40
Acetyl-D-tryptophanamide	2.4	3.72
Trifluoroacetyl-D-tryptophanamide	4.0	3.40
Acetyl-L-tryptophanmethylamide	6.5	3.10
Acetyl-D-tryptophanmethylamide	1.8	3.89
Benzoyl-D-tryptophanamide	0.7	4.46
Nicotinyl-D-tryptophanamide	1.6	3.96
p-Methoxybenzoyl-D-tryptophanamide	0.6	4.55
Acetyl-L-tryptophanate	9.5	2.87
Acetyl-D-tryptophanate	7.5	3.01
Acetyl-D-tryptophan isopropylester	0.8	4.39
Tryptamine	2.3	3.74
Acetyltryptamine	1.8	3.89
Trifluoroacetyltryptamine	1.2	4.13
Indole	0.8	4.34
Indoleacetate	18	2.47
Indolepropionate	15	2.58
Indolebutyrate	23	2.32
Indolepropionamide	2.3	3.74
Tyrosine series		
Acetyl-D-tyrosinamide	12	2.72
Trifluoroacetyl-D-tyrosinamide	20	2.40
Chloroacetyl-D-tyrosinamide	6.5	3.10
Nicotinyl-D-tyrosinamide	9	2.90
Acetyl-L-tyrosinemethylamide	61	1.72
Formyl-L-tyrosinemethylamide	31	2.14
Nicotinyl-L-tyrosinemethylamide	8.8	2.92
Benzoyl-L-tyrosinemethylamide	6.4	3.12
Acetyl-L-tyrosinate	110	1.36
Fluoroacetyl-L-tyrosinate	120	1.30
Chloroacetyl-L-tyrosinate	150	1.16
Acetyl-D-tyrosine ethyl ester	4.7	3.30
Nicotinyl-D-tyrosine ethylester	0.8	4.39
D-Tyrosinehydroxamide	40	1.98
Acetyl-D-tyrosinehydroxamide	7.5	3.01

TABLE 2-20 (*continued*)

Inhibitor	Apparent K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
Phenylalanine series		
Acetyl-D-phenylalaninamide	12	2.72
Nicotinyl-D-phenylalaninamide	9	2.90
Acetyl-D-phenylalanine methylester	2.3	3.74
Benzoate	150	1.16
Benzamide	10	2.83
Phenylacetate	200	0.99
Phenylacetamide	15	2.58
Phenylpropionate	25	2.26
Phenylpropionamide	7.0	3.05
Phenylbutyrate	60	1.73
Phenylbutyramide	12	2.72

[a] The values of K_i are taken from work at pH 7.9. K_i varies with the pH and thus the degree of ionization may be of importance in some instances. The relative binding energies are therefore subject to some error but may provide an initial basis for discussion. Various substrates have been used in the different studies and this may introduce further uncertainties. (Data from Foster *et al.*, 1955; Foster and Niemann, 1955 a, b; Lands and Niemann, 1959.)

indole ring system. This is confirmed by comparing the phenyl and indole acids and amides, the indole derivatives being bound 0.77 kcal/mole more strongly on the average.

(*C*) Comparing the *N*-substituted R_2 groups one may calculate that the order of binding is:

$$\text{Benzoyl} \overset{\Delta = 0.35 \text{ (kcal/mole)}}{>} \text{nicotinyl} \overset{\Delta = 0.68 \text{ (kcal/mole)}}{>} \text{acetyl}$$

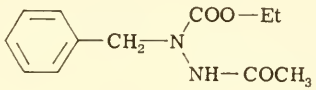
and it appears that the acetyl derivatives are better inhibitors than the analogs with a free NH_3^+ group. It is likely that these groups interact with the enzyme surface in a nonspecific fashion.

(*D*) Analogs with a $CONH_2$ group are bound around 1.10 kcal/mole more tightly than those with a free COO^- group. This might indicate that the peptidatic site is in an electric field arising from surrounding negative charges, but it could also mean that hydrogen bonds between the peptide linkage and the enzyme are important. The rather tight binding of

tryptamine indicates also a more favorable field for positive than negative ionic groups, but the positive charge is not important since acetyltryptamine is bound even more readily.

Foster and Niemann (1955 a) determined the values of K_i for several inhibitors at pH 6.9 and 7.9. The acetyltryptophanates and indolepropionate are bound 1.0–1.4 kcal/mole more tightly at pH 6.9 than at pH 7.9, whereas the binding of various amides is not significantly affected. This is interpreted as pointing to the development of a negative charge in the vicinity of the active site as the pH rises from 6.9 to 7.9; this charge would repel the negatively charged tryptophanates and other carboxylates. It is possible that the lack of effect of pH on amide binding is due to the simultaneous deprotonation of the $CONH_3^+$ with rise in pH ($pK_a = 7.5$). The concept of a negative charge on or near the active center was first postulated by Neurath and Schwert (1960) on the basis of the suppressing effect of a carboxylate group on the hydrolysis of an adjacent peptide bond. Whether this enzyme negative group participates in the hydrolysis along the lines suggested by Stearn (1935) and Vaslow and Doherty (1953) is not certain.

The nitrogen analogs of substrates are usually not hydrolyzed and can act as inhibitors (Kurtz and Niemann, 1961). In these compounds an α-methine group is replaced by a N atom, or an α-methylene group by an NH group. Thus ethyl 1-acetyl-2-benzylcarbazate is an analog of ethyl



acetyl-L-phenylalaninate and is an inhibitor, with $K_i = 20$ mM. Also φ—CH_2CH_2—COO—CH_3 and φ—NH—CH_2—COO—CH_3 are substrates of chymotrypsin whereas φ—CH_2—NH—COO—C_2H_5 is an inhibitor ($K_i =$ 6 mM). It is suggested that an α-N= or α-NH— group leads to a restriction of the rotation around the bond joining it to the CO group compared to that of a C—C bond, this constraint leading to loss of substrate activity or weakening of inhibitor binding.

Wallace *et al.* (1963) published the results of their studies of the interactions of 136 compounds with chymotrypsin, and it is seen that several substances not directly related to amino acids are fairly potent inhibitors; such are quinoline and the methylquinolines, hydroxyquinolines, and aminoquinolines, various acridines, α-naphthol, and the naphthylamines, all with K_i values less than 1 mM. They summarized their conclusions with respect to structure and inhibitory activity in ten postulates, from which the following comments are extracted. Aromatic compounds are more effective than the corresponding saturated derivatives, and monosubstituted benzene derivatives with polarizable uncharged groups are more inhibitory than the

parent compounds; both facts indicate the importance of polarization of the inhibitor molecule in the field of ionic groups on the enzyme. The active site has a locus for interaction with the aromatic nucleus, and vicinal to this there is at least one anionic group; the orientation of the inhibitor is determined by the interaction of the polarizable group with a sublocus. Molecules presenting a larger planar area are more inhibitory, and it seems that the site at which the aromatic compounds act is mainly flat, of greater length than breadth, and not straight but curved along the enzyme surface. The different loci involved in the interactions perhaps have different properties; e. g., it was postulated that the active site may be hermaphroditic* in that one locus may be electron-deficient and another electron-rich.

The chymotrypsin reaction proceeds in two steps, the acetylation of the enzyme:

$$EH + AcB \rightleftarrows (EH—AcB) \rightleftarrows (EAc \cdot HB) \rightleftarrows EAc + HB$$

and the solvolysis of the acetylchymotrypsin:

$$EAc + HOR \rightleftarrows (EAc \cdot HOR) \rightleftarrows (EH—AcOR) \rightleftarrows EH + AcOR$$

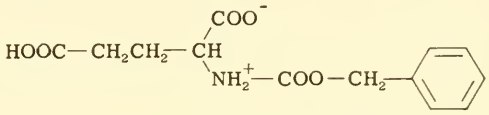
The competitive inhibitors tabulated here were shown by Foster (1961) to block the acetylation reaction and not the solvolysis. The inhibition by indole is not strictly competitive (Applewhite *et al.*, 1958).

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
Skatole	0.5	4.69
Indole	0.7	4.43
p-Nitrobenzoyl-D-tryptophan	1.5	4.01
Tryptamine	2.0	3.84
p-Nitrobenzoyl-L-tryptophan	2.3	3.74
Acetyl-D-tryptophan	4.0	3.41
Acetyl-L-tryptophan	6.0	3.16
D-Tryptophan	10	2.84
L-Tryptophan	20	2.41

The relation of chymotrypsin or a chymotrypsin-like enzyme to histamine release and the anaphylactic reaction was examined by Austen and Brocklehurst (1960) in guinea pig lung sensitized to ovalbumin. Inhibitors such as β-phenylpropionate, indoleacetate, and indolepropionate depress anaphylactic histamine release more than 50% at 2.5 mM, and indole and skatole are effective at 1 mM or below. Antigen apparently activates some proteolytic enzyme necessary for the release of histamine.

* This terminology connotes an entirely new way of looking at enzyme catalysis.

The hydrolysis of α-benzoyl-L-argininamide by papain is inhibited competitively by carbobenzoxy-L-glutamate (Kimmel and Smith, 1954; Stockell and Smith, 1957). The inhibition is very sensitive to pH between 3.9 and 5, the inhibitory activity almost disappearing at the upper end of this range (see Fig. I-14-6). The γ-carboxyl group has a pK_a of 4.4 so it is possible that the active form is un-ionized:



It is rather surprising that the ionization of this group should be so important in the binding, especially as it is at some distance from the other probable binding groups, and the enzyme as a whole is quite positively charged at these pH's (isoelectric point near 8.75). Perhaps a hydrogen-bonding function must be attributed to the COOH group instead. Benzoyl-L-arginine (K_i = 60 mM) and benzoyl-L-argininamide (K_i = 54 mM) also inhibit the hydrolysis of benzoyl-L-arginine ethyl ester (K_m =23 mM) by ficin at pH 5.5 (Bernhard and Gutfreund, 1956).

Beef spleen cathepsin C is competitively inhibited by various amides, esters, and dipeptides when glycyl-L-tyrosinamide is the substrate (Fruton and Mycek, 1956). L-Phenylalanine amides and esters are good inhibitors (as shown in the accompanying tabulation) but L-phenylalanine, acetyl-L-

Inhibitor	K_i (mM)
L-Phenylalaninamide	8.3
L-Phenylalanine ethyl ester	14
L-Phenylalanyl-L-phenylalanine	17
L-Tyrosinamide	22
DL-Phenylalanylglycine	25
D-Phenylalaninamide	68

phenylalanine, and glycyl-DL-phenylalanine are inactive. N-Acetylation or a free COO^- group seems to prevent binding. The interaction between the inhibitors and the enzyme may involve several groups with possible hydrogen bonding of the carbonyl group in a manner similar to that proposed for chymotrypsin. Decarboxylation and N-acylation of amino acids can lead to inhibitors, as in the competitive inhibition by tosylagmatine of thrombin (K_i = 13.2 mM) and trypsin (K_i = 3.45 mM) (Lorand and Rule, 1961). This inhibitor markedly slows clotting in a thrombin-fibrinogen system and in whole plasma.

HEXOKINASES

We shall now turn to various aspects of carbohydrate metabolism and begin with those enzymes responsible for the initial phosphorylation of sugars. Hexokinases catalyze the reaction between two types of substrate — hexoses and ATP — and analogs of either may inhibit. Discussion of certain particularly important glucose analogs (2-deoxy-D-glucose, 6-deoxy-6-fluoro-D-glucose, and related compounds), whose actions may involve not only hexokinases but other early steps in carbohydrate metabolism, will be postponed to the following section. The remaining analog inhibitors may be classed as (A) hexoses, (B) hexose phosphates, (C) glucosamine and derivatives, and (D) nucleotides and polyphosphates. It should be remembered that the values of K_m and K_i may often be composite in that the sugar or its derivative may occur in solution in a variety of forms (for example, α- or β-isomers, or pyranose or furanose rings). These forms may have quite different activities and different individual constants.

Inhibition by Hexoses

Most hexokinases are not specific for the phosphorylation of one sugar but act at varying rates with different hexoses. Thus yeast hexokinase phosphorylates glucose (K_m = 0.16 mM), fructose (K_m = 1.7 mM), and mannose (K_m = 0.1 mM); brain hexokinase is very similar (Slein *et al.*, 1950). If one active site on the enzyme is responsible, competition between the substrates should be observed. At equimolar concentrations, glucose and mannose almost completely inhibit the phosphorylation of fructose (88–98%), whereas fructose inhibits the phosphorylation of the former hexoses very little (15-18%), which would be expected on the basis of their relative K_m's. The calculated K_i's are essentially the same as the K_m's. The inhibition of *Schistosoma* fructokinase* by glucose and mannose is also quite marked, whereas galactose is much less potent (Bueding and MacKinnon, 1955). The fructokinase of rat intestinal mucosa shows similar specificity, the following inhibitions being observed with 6 mM inhibitor when fructose is 10 mM: glucose (90%), mannose (80%), mannoheptulose (65%), xylose (50%), allose (0%), and galactose (0%) (Sols, 1956). Ascites cell glucokinase is inhibited rather well by talose (K_i = 3.2 mM) and altrose (K_i = 6 mM), but not by allose, gulose, or idose (Lange and Kohn, 1961 b). Yeast glucokinase is competitively inhibited by mannose (K_i = 0.11 mM) (Fromm and Zewe, 1962). Mannoheptulose induces a temporary diabetic state by blocking the uptake of glucose into the tissues through inhibition of glucokinase (Coore and Randle, 1964).

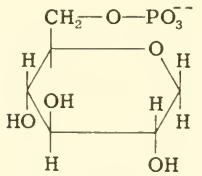
* The terms "glucokinase," "fructokinase," etc., will be used to designate kexokinases when the corresponding substrates are used without implying that the enzymes are specific for these substrates.

Such inhibitions may be of importance in the metabolism of mixtures of sugars. Fructose is usually phosphorylated more rapidly than glucose, but in mixtures of the two the phosphorylation of fructose is markedly suppressed and essentially only glucose is metabolized. Although little is known of the nature of the binding of these hexoses to the enzyme, it would appear that the configurations at C-3 and C-4 are particularly important; for example, allose differs from glucose only at C-3 and is not readily bound, and galactose differs from glucose only at C-4 and is much less bound.

Inhibition by Hexose Phosphates

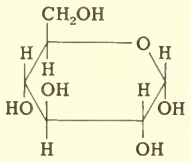
Three hexose phosphates have been found to be fairly specific and interesting inhibitors of hexokinases: these are α-D-glucose-6-P, α-L-sorbose-1-P, and 1,5-anhydro-D-glucitol-6-P. Inhibiting hexose phosphates should be visualized in their pyranose or furanose forms, since it is likely that interaction with the enzyme occurs with one side of these ring structures. (See formulas on page 378).

Glyceraldehyde has been known for many years to be an inhibitor of glycolysis and Lardy *et al.* (1950) in a study to determine the site of action found that L-glyceraldehyde prevents the phosphorylation of glucose or fructose in brain extracts, but yet has no direct effect on the hexokinase. This paradox was resolved by showing that aldolase catalyzes the condensation of L-glyceraldehyde with glyceraldehyde-3-P to give a mixture of D-fructose-1-P and L-sorbose-1-P. The latter compound was found to be a potent inhibitor of hexokinase (for example, 0.08 m*M* inhibits 67% the phosphorylation of glucose at 35 m*M*), whereas L-sorbose and L-sorbose-6-P are inactive. The conventionally written structure for L-sorbose-1-P (as above) bears little obvious resemblance to D-glucose-6-P, but if the former structure is inverted it is seen that the molecules are identical from one side and differ only by the transposition of a hydroxyl group, as illustrated by Lardy *et al.* with molecular models. D-Glucose-6-P is the product

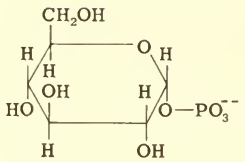


α-L-Sorbopyranose-1-P

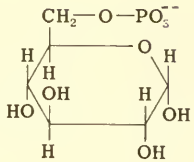
of the hexokinase phosphorylation of glucose and is an inhibitor of the reaction (see below). It is surprising that the inhibition by L-sorbose-1-P is not competitive with glucose; indeed, as the glucose concentration is increased, the inhibition becomes somewhat greater. It is possible that



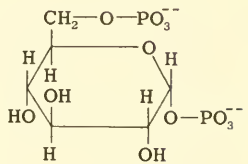
α-D-Glucose



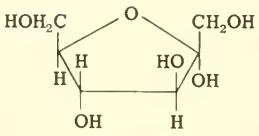
α-D-Glucose-1-P



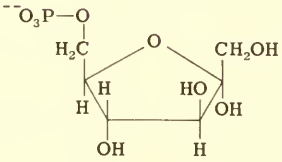
α-D-Glucose-6-P



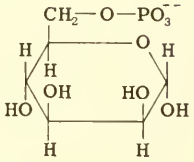
α-D-Glucose-1, 6-diP



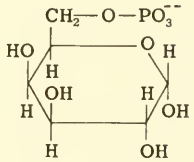
α-D-Fructose



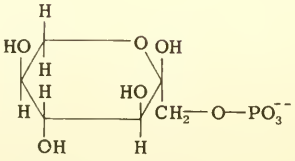
α-D-Fructose-6-P



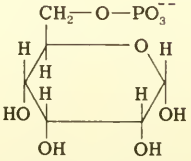
α-D-Mannose-6-P



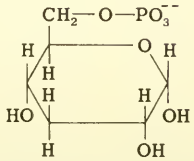
α-D-Galactose-6-P



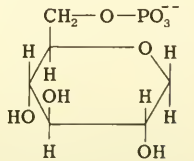
α-L-Sorbose-1-P



α-D-Allose-6-P



α-3-Deoxy-D-glucose-6-P



1, 5-Anhydro-D-glucitol-6-P

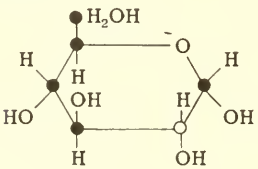
competition might be seen if rates of inhibition in the presence of various concentrations of glucose were determined, but once L-sorbose-1-P has combined with the enzyme the inhibition is essentially irreversible. It is possible that glucose forms an intermediary tightly bound complex with the enzyme-ATP during the reaction and that L-sorbose-1-P forms a similar complex that is stable. Actually the affinities of the brain enzyme for D-glucose-6-P (K_i = 0.4 mM) and L-sorbose-1-P (K_i = 0.7 mM) are close (Crane and Sols, 1954). Other hexokinases may not be so susceptible to L-sorbose-1-P, since Taylor (1960) found only slight inhibition by 0.5 mM of glucose uptake by *Scenedesmus*, the primary transfer site being hexokinase on the outside of the membrane.

A closely related inhibitor is 1,5-anhydro-D-glucitol-6-P (1,5-D-sorbitan-6-P), this lacking the 2-OH group in L-sorbose-1-P and binding somewhat less tightly (K_i = 1 mM) to the brain hexokinase (Crane and Sols, 1954). The nonphosphorylated compound is a very weak inhibitor (Sols, 1956). Since there are very few potent and specific hexokinase inhibitors, Ferrari *et al.* (1959) have recently investigated 1,5-anhydro-D-glucitol-6-P in some detail to see if it might be useful as a blocking agent of this enzyme in homogenates. It is stable to the enzymes attacking D-glucose-6-P, except for hydrolysis by liver glucose-6-phosphatase. No inhibition of glucose-6-P dehydrogenase is evident, but it inhibits phosphoglucomutase variably (depending on the concentrations of glucose-1-P, glucose-1,6-diP, and Mg^{++}) and phosphoglucose isomerase noncompetitively at higher concentrations. At 6.25 mM it blocks glucose respiration in heart homogenates but has no influence on the oxidation of glucose-6-P, indicating under these conditions a rather specific inhibition of hexokinase.

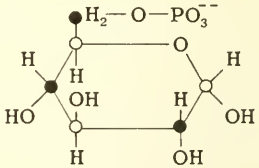
Brain hexokinase is inhibited by glucose-6-P whereas yeast hexokinase is not (L-sorbose-1-P also does not inhibit the yeast enzyme), and the inhibition has been found to be noncompetitive with respect to both glucose and ATP (Weil-Malherbe and Bone, 1951). Inhibitions by various hexose phosphates have been studied thorougly by Crane and Sols (1953, 1954); the accompanying tabulation summarizes their data. The following are noninhibitory: β-D-glucose-1,6-diP, D-mannose-6-P, D-fructose-6-P, D-fructose-1,6 diP, D-arabinose-5-P, D-ribose-5-P, D-galactose-6-P, α-glucose-1-P,

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
α-D-Glucose-6-P	0.4	4.80
α-D-Glucose-1,6-diP	0.7	4.46
α-L-Sorbose-1-P	0.7	4.46
1,5-Anhydro-D-glucitol-6-P	1.0	4.25
α-D-Allose-6-P	7.0	3.05

D-altrose-6-P, glucuronate, and glucuronate-6-P. It may be noted that glucose is the only hexokinase substrate that forms an inhibitory phosphate, indicating the importance of the configuration at C-2 for inhibition. Thus glucose phosphorylation in a closed system slows down progressively while that of mannose is linear, a phenomenon which may be significant in regulating the rate of sugar utilization. In a purer preparation of brain hexokinase, Crane and Sols confirmed that the inhibition is not formally competitive but that a reversible EI complex is formed. Phosphorylation at C-6 (or at C-1 in the sorbose structure) seems necessary for inhibition; e. g., glucose-1-P and 1,5-anhydro-D-glucitol lack inhibitory activity. It is interesting that the inversion of the phosphate-carrying group at C-1 to form β-glucose-1,6-diP abolishes the inhibition, possibly due to a static interference of the now closely apposed phosphate groups. Inversion of the groups on C-2 (mannose-6-P), C-3 (allose-6-P), or C-4 (galactose-6-P) reduces or abolishes the inhibition; it was felt by Crane and Sols that the configuration at C-3 influences the effect of an adjacent group and is not directly concerned in the binding. It is difficult in most cases to decide if the change in affinity on inversion of the groups is related to the hydroxyl group as a binding site or as producing steric hindrance; thus the lack of inhibition by galactose-6-P could be due either to the loss of hydrogen bonding through the OH group (occurring in glucose-6-P) or to a protrusion of the OH group preventing approach of the pyranose ring. Comparison with the corresponding deoxyglucose-6-P's might be informative. We cannot do this for C-4, but at C-2 removal of the OH group (2-deoxyglucose-6-P) abolishes inhibition, pointing to the OH group as a binding site. The weak inhibitory activity of 3-deoxyglucose-6-P substantiates the idea that the 3-OH group is not involved in binding. The retention of inhibition in 1,5-anhydro-D-glucitol-6-P likewise indicates that the 1-OH is not a binding site, but the loss of inhibition on C-1 methylation (α-methylglucoside-6-P) shows that steric repulsion occurs when the C-1 group becomes too large. One may conclude that binding sites are at the 2-OH and 6-phosphate groups, and possible at the 4-OH group.The inhibitors thus attach to a different set of enzyme sites than the substrates, only C-4 being common to both, and Crane and Sols visualized these differences in the following structures, where the solid circles indicate necessary binding positions:



Substrate



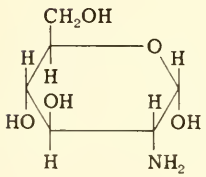
Inhibitor

Rat intestinal mucosa hexokinase is inhibited by glucose-6-P but only about one-tenth as readily as the brain enzyme (Sols, 1956). The hexokinase of *Schistosoma* is strongly inhibited by glucose-6-P when glucose or mannose is the substrate, but fructose phosphorylation is unaffected (Bueding and MacKinnon, 1955). Ascites tumor hexokinase behaves like the brain enzyme and the K_i for glucose-6-P is 0.4 mM (McComb and Yushok, 1959). Thus inhibition of various hexokinases by glucose-6-P has been observed, but the original observation that the yeast enzyme is resistant cannot as yet be explained.

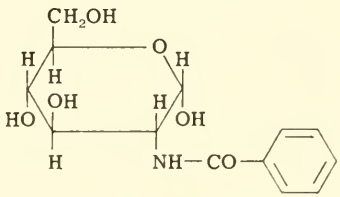
Inhibition by D-Glucosamine and Derivatives

Glucosamine (2-amino-D-glucose) is phosphorylated by brain hexokinase (Harpur and Quastel 1949) and it was postulated by Quastel and Cantero (1953) that it might be carcinostatic through ATP depletion. However, it also competitively inhibits glucose phosphorylation and any carcinostatic activity it possesses would be more likely related to this. Maley and Lardy (1955) thus attempted to find a more potent inhibitor among the *N*-acylated derivatives and were quite successful, as shown in the accompanying tabulation. Furthermore, these derivatives are not phosphorylated.

Glucosamine	K_i (mM)	
	Glucokinase	Fructokinase
N-(3,5-Dinitrobenzoyl)-	0.011	0.004
N-(*m*-Nitrobenzoyl)-	0.033	0.0084
N-(*p*-Nitrobenzoyl)-	0.04	0.05
N-Benzoyl-	—	0.036
N-(*m*-Aminobenzoyl)-	0.15	0.081
N-(*p*-Aminobenzoyl)-	0.2	0.11
N-Acetyl	—	0.46
N-Phenylacetyl-	—	0.86



α-D-Glucosamine



N-Benzoyl-α-D-glucosamine

Before considering the nature of this inhibition, let us examine other hexokinases to determine how widespread is the susceptibility. The fructo-

kinases of *Schistosoma* (Bueding and MacKinnon, 1955) and rat intestinal mucosa (Sols, 1956) and the glucokinase of *Spirochaeta recurrentis* (P. J. C. Smith, 1960 b) are moderately sensitive to glucosamine (50-65% inhibition by 6–10 mM) and more sensitive to N-acetylglucosamine (75% inhibition by 1–2 mM). The K_i for N-acetylglucosamine and the glucokinase of ascites tumor cells is 0.074 mM (McComb and Yushok, 1959), indicating a binding about 1 kcal/mole tighter than for glucose-6-P. Furthermore, both glucosamine and N-acetylglucosamine inhibit the metabolism of glucose-C^{14} and fructose-C^{14} to glycogen and CO_2 in rat liver slices (Spiro, 1958), and the N-(p-nitrobenzoyl) and N-(3,5-dinitrobenzoyl) derivatives inhibit glucose uptake by *Scenedesmus* (Taylor, 1960). The phosphorylation of glucosamine in liver extracts is competitively inhibited by glucose (K_i = 0.11 mM), fructose, N-acetylglucosamine, and hexose and glucosamine phosphates, illustrating mutual interference by these substrates and products (McGarrahan and Maley, 1962).

In all these instances the inhibitions are strictly competitive with glucose or fructose. The question arises as to why the N-acylated derivatives are not phosphorylated. Maley and Lardy (1955) showed by molecular models that the N-acyl groups do not overlap the 6-position so that some other explanation must be sought. It was suggested that the N-acyl groups might interfere with the binding of ATP to the enzyme, but it is also possible that they shift the position of the pyranose or furanose rings sufficiently so that the 6-position is not favorably oriented for phosphorylation. It may be mentioned that no carcinostatic activity was noted with any of these substances when tested in sarcoma-bearing mice, perhaps due to the hydrolysis of the N-acyl compounds by tissue cathepsins.

Kono and Quastel (1962) confirmed the glucosamine inhibition of glycogen formation in rat liver slices (50% inhibition by around 0.8 mM) and showed there to be no depression of the entry of glucose into the cells. Hexokinase, phosphoglucomutase, and UDP-glucose pyrophosphorylase are inhibited quite weakly by glucosamine, significant effects being exerted only at concentrations over 20 mM. UDP-glucose-glycogen glucosyltransferase in inhibited by glucosamine but not by N-acetylglucosamine, which inhibits glycogen synthesis as does glucosamine. The isolated phosphorylase is also resistant to glucosamine. Thus the enzymes involved in glycogen formation are not directly inhibited by glucosamine and N-acetylglucosamine. However, each of these substances stimulates phosphorylase activity in slices when added with glucose. Thus the explanation for the reduced glycogen formation may be an acceleration of glycogen breakdown and not a true inhibition. Silverman (1963) found a significant reduction of glucose oxidation by glucosamine in the rat epididymal fat pad in the presence of insulin and felt that an inhibition of glucokinase could not entirely account for the results, so that one must assume some action from nonmetabolized

products formed from glucosamine, possibly glucosamine-P. Glucosamine depresses the respiration and oxidation of pyruvate in ascites cells, as does glucose (Crabtree effect), and this is relieved by 2,4-dinitrophenol (Ram *et al.*, 1963). Since this is presumably related to the phosphorylation of glucosamine and the effects on ADP-ATP levels, it constitutes another mechanism whereby glucosamine can alter carbohydrate metabolism.

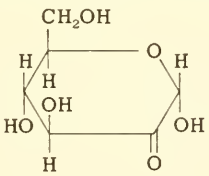
Inhibition by Adenine Nucleotides and Polyphosphates

The reaction rate of hexokinases falls with time due to the accumulation not only of glucose-6-P but also of ADP (Sols and Crane, 1954). Phosphate, pyrophosphate, and AMP do not inhibit. The nature of the ADP inhibition appears to vary with the source of the hexokinase. With yeast hexokinase the inhibition is noncompetitive with respect to ATP since around 50% inhibition is produced by 0.5 m*M* ADP at all levels of ATP used (Gamble and Najjar, 1955), and with *Schistosoma* glucokinase the inhibition actually increases slightly with ATP concentration (Bueding and MacKinnon, 1955). The inhibition of liver fructokinase by ADP is reduced slightly by increasing the ATP concentration from 5 to 10 m*M*, but not enough to indicate pure competitive inhibition; Parks *et al.* (1957) stated it is noncompetitive but it might better be designated as mixed. *Echinococcus* fructokinase, on the other hand, is inhibited competitively (Agosin and Aravena, 1959).

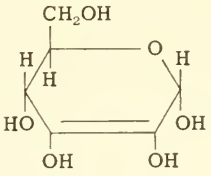
Tripolyphosphate ($P_3O_{10}^{5-}$) inhibits the fermentation of glucose by intact yeast cells, glycolysis in cell-free extracts, and pure hexokinase (Vishniac, 1950). The inhibition of hexokinase is quite potent when (ATP) = 3.75 m*M*: 13% at 0.47 m*M*, 31% at 1.4 m*M*, 74% at 4.7 m*M*, and 93% at 14 m*M* tripolyphosphate. The inhibition is reversed by both ATP and Mg^{++}. Wheat germ hexokinase appears to be more resistant to tripolyphosphate, only 8% inhibition being given by 5 m*M* (Saltman, 1953).

Inhibition by Glucosone

D-Glucosone may be formed from D-glucose by mild oxidation (e. g. with Cu^{++}) at C-2 but, although it has been known for over 75 years, its structure is not completely understood (Becker and May, 1949). The following forms are possible and it is difficult to choose between them:



(I)



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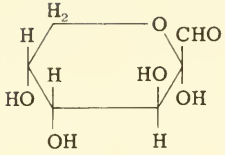

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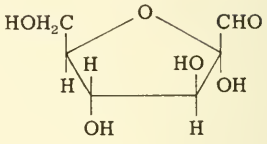


(IV)

There is some evidence that the enolic tautomer II is not important and the behavior with enzymes might favor structure I. Hynd (1927) at St. Andrews tested D-glucosone to determine if it could counteract insulin hypoglycemic convulsions, as glucose does, but found that, if anything, the effect of insulin is increased. Glucosone was then administered to normal mice giving toxic symptoms within 5 min and a well-developed insulin-like reaction in 20 min. The lethal dose range is very narrow, 2.4 mg/kg being nonlethal and 2.6 mg/kg generally lethal. Glucose injected before or with the glucosone reduces the effects somewhat. Moribund mice following lethal doses show an elevation in blood glucose from 0.161 to around 0.240 mg%; thus the symptoms are not due to a hypoglycemia. Although these results would point to glucosone interference with the utilization of glucose, Hynd unfortunately assumed that glucosone is formed from glucose by the action of insulin and that, indeed, it is responsible for the effects of insulin, the raised blood glucose levels being unexplained. Similar reactions to glucosone are seen in several species (Herring and Hynd, 1928). The theory that insulin induces glucosone formation was made untenable by Dixon and Harrison (1932), who found no glucosone in the blood during insulin convulsions.

The problem rested at this stage for 20 years and then was taken up at St. Andrews (Bayne, 1952; Mitchell and Bayne, 1952; Johnstone and Mitchell, 1953), but the results were published in a series of short and incomplete communications without adequate data. D-Glucosone effects in mice were not seen with up to 10 mg/kg of any other osone, including D-galactosone, D-arabinosone, D-xylosone, L-glucosone, and 3-methyl-D-glucosone. Turning to yeast glucose fermentation, they found no inhibition by 50 m*M* D-glucosone but marked inhibition at 200 m*M*, whereas L-glucosone has no effect at 200 m*M*. Becker (1954) reported almost complete inhibition of the aerobic and anaerobic utilization of glucose by yeast when (glucosone)/(glucose) = 5.

It was realized finally by Eeg-Larsen and Laland (1954) in Oslo that the structural similarity of glucosone to glucose might allow the former to interfere with the utilization of the latter by blocking its phosphorylation. This was demonstrated with ox brain hexokinase; glucosone is not phosphorylated but inhibits glucose phosphorylation 50% at 0.35 m*M* and 100% at 2.4 m*M* when glucose is 2.4 m*M*. They concluded that the inhibition is noncompetitive, but the small range of glucose concentrations used makes

it impossible to determine the type of inhibition; actually some decrease in the inhibition with increasing glucose was observed. As would be expected, glucosone does not produce a Crabtree effect but blocks it (Yushok and Batt, 1957). The inhibition of glucose fermentation in yeast depends on the pH and the buffer system present (Hudson and Woodward, 1958). At pH 6.5 in phosphate buffer an inhibition of 73% of the anaerobic fermentation of glucose was found at (glucosone)/(glucose) = 2, whereas no effect was found at pH 3.5–4.5. The fermentation and phosphorylation of fructose are inhibited more readily than with glucose, due to the higher K_m for fructose, and the inhibitions are completely competitive with K_i for glucosone around 0.061 mM. Marked inhibition of anaerobic glycolysis in rat tissues by glucosone was noted, brain being much more sensitive than tumor tissue; in brain complete inhibition occurs with (glucosone)/(glucose) = 0.0067. The susceptibility of brain glycolysis to glucosone is certainly much greater than of any hexokinase studied and possibly there is an additional site of action. In any event, these results provide sufficient explanation for the central toxic actions of glucosone. Despite the fact that glucosone can be formed in certain organisms (e. g., molluscan crystalline styles and red algae) and can be metabolized in streptococci and mammals, it would appear that it is not an important substance in intermediary metabolism and is not generally on the pathway for the synthesis of glucosamine (Becker and Day, 1953; Topper and Lipton, 1953; Dorfman *et al.*, 1955; Bean and Hassid, 1956). There is thus no evidence that glucosone can participate in the regulation of carbohydrate metabolism, but the high susceptibility of brain glycolysis suggests that one should withhold final judgment until the absence of glucosone in the body under various conditions has been demonstrated.

Inhibition of Phosphofructokinase by Cycle Intermediates

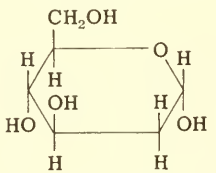
The phosphofructokinase from sheep brain is quite potently inhibited by certain cycle intermediates (see accompanying tabulation) (Passonneau and Lowry, 1963). Although this is not strictly analog inhibition, it is worth

Inhibitor	K_i (mM)
Citrate	0.03
cis-Aconitate	0.1
Isocitrate	0.2
Malate	0.6
Succinate	1.5
α-Ketoglutarate	2.5
Fumarate	>10

mentioning because of the implications such actions have for a feedback control of carbohydrate oxidation. A rise in the levels of the inhibitory intermediates would reduce the rate of formation of pyruvate. The steady-state concentrations of the cycle intermediates are certainly high enough to inhibit significantly, but the problem of compartmentalization arises since it is generally assumed that the tricarboxylates particularly are mostly confined to the mitochondria. Whatever the significance of this type of inhibition, it emphasizes the importance of compartmentalization in regulatory control of metabolism, a factor which has not always been taken into account.

EFFECTS OF 2-DEOXY-D-GLUCOSE ON CARBOHYDRATE METABOLISM

An inhibitor capable of specifically blocking the glycolytic pathway would not only be a valuable tool in biochemical investigation but might play a role in the chemotherapy of certain neoplasms. Glucose analogs, especially those entering the pathway and forming inhibitory intermediates, would be the most likely candidates, and 2-deoxy-D-glucose (2-DG) is the most interesting and best understood substance of this type. The volume of literature during the past 10 years on this analog precludes a complete discussion and emphasis will be placed on the sites and mechanisms of the inhibition in the glycolytic pathway. 2-DG was first examined by Cramer and Woodward (1952) at the Franklin Institute in the course of searching for carcinostatic glucose analogs, and they found that it does indeed produce some regression of Walker carcinoma and terminates embryonic development in rats. 2-DG differs from glucose in the substitution of the 2-OH group by a hydrogen atom and may be represented in the pyranose form as:



2-Deoxy-D-glucose

Absorption, Distribution, and Metabolism of 2-DG

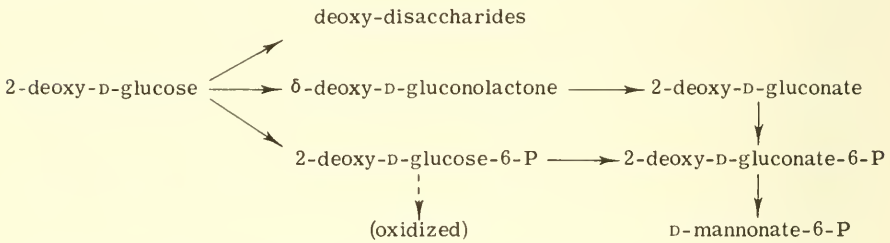
It will be well to discuss the uptake and fate of 2-DG in cells before turning to the metabolic disturbance produced. 2-DG enters most cells readily; this may involve a phosphorylation at the membrane in some cases, but in others it is phosphorylated only after entry. Whatever the transport mechanism there is usually competition between 2-DG and glucose, the

uptake of 2-DG being depressed progressively as the glucose concentration is increased in rat diaphragm (Nakada and Wick, 1956; Kipnis, 1958) and lymph node cells (Helmreich and Eisen, 1959). Nakada and Wick (1956) showed that insulin can double the rate of 2-DG uptake by diaphragm, and Kipnis and Cori (1959, 1960) studied this in greater detail. In normal diaphragm the 2-DG taken up appears as 2-deoxy-D-glucose-6-phosphate (2-DG-6-P), the rate of phosphorylation being apparently greater than the rate of penetration. Diabetic diaphragm takes up and phosphorylates 2-DG at a reduced rate but 2-DG does not accumulate in the cells, indicating the penetration is still rate-limiting. Addition of insulin accelerates the uptake and some free 2-DG appears in the cells so that the phosphorylation is not increased proportionately. Epinephrine does not influence the penetration but slows phosphorylation of 2-DG. Transport of 2-DG across the entire diaphragm is slow, being about one-fifth the rate for glucose and one-twenty-fifth the rate for galactose (Ungar and Psychoyos, 1963). It is possible that it is trapped in the muscle as 2-DG-6-P since insulin depresses the transfer. The uptake of 2-DG by yeast in glucose-phosphate medium is 5–10 times faster aerobically than anaerobically; when glucose is omitted the aerobic uptake of 2-DG is not altered, but anaerobically there is a loss of 2-DG from the cells, so that the uptake is dependent on aerobic processes and probably on the level of ATP since 2,4-dinitrophenol acts like anaerobiosis (Kiesow, 1959). Certain fungi, such as *Neurospora crassa* and *Aspergillus oryzae*, can grow with 2-DG as the sole source of carbon (Sols *et al.*, 1960 b) but not as rapidly as with glucose; indeed, growth with glucose or other sugars is inhibited by 2-DG. *E. coli* will not grow with 2-DG as the only carbon source and there is some evidence that it does not penetrate into the cells (Gershanovich, 1963). The evidence for the lack of entrance is that glycolysis is not inhibited in intact cells where it is in extracts. 2-DG diffuses across the intestinal wall but is not actively transported as are glucose, 1-DG, and 3-DG, indicating the importance of the 2-position in transport (Wilson and Landau, 1960), nor does 2-DG have an effect on the short-circuit current through the intestinal wall, such being associated with transport (Schultz and Zalusky, 1964; Barry *et al.*, 1964).

There is some information on the disposal of 2-DG in intact animals. Injected into rabbits, it is rapidly distributed in the extracellular space and some enters the tissues in eviscerated and nephrectomized animals, the uptake being markedly stimulated by insulin (Wick *et al.*, 1955). Since it produces a block of glucose uptake for at least 8 hr, it is evident that little 2-DG is metabolized beyond the 2-DG-6-P stage in extrahepatic tissues. This is confirmed by the finding that little or no $C^{14}O_2$ is expired following injection of 2-DG-C^{14} (Wick *et al.*, 1957). Blood levels of 2-DG are more consistent after subcutaneous injection than when it is given intraperito-

neally, due to erratic absorption from the peritoneum (Ball and Saunders, 1958). After subcutaneous administration there is a blood peak at 15 min, after which there is a gradual fall over 6 hr. In human subjects infused intravenously with 50–200 mg/kg of 2-DG over 30-min periods, approximately 30% is excreted in the urine (Landau *et al.*, 1958).

The pathways of 2-DG metabolism have not been completely worked out. The accompanying scheme shows some of the reactions encountered. The phosphorylation by hexokinase to 2-DG-6-P would seem to be the most important reaction, especially as 2-DG-6-P is not metabolized in most cells and tends to accumulate. HeLa cells can oxidize 2-DG-6-P but much more slowly than glucose-6-P (Barban and Schulze, 1961). Hexokinases for the



formation of 2-DG-6-P have been found in brain (Sols and Crane, 1953), kidney, intestine, liver (Lange and Kohn, 1961 a), skin (Brooks *et al.*, 1959), diaphragm(Kipnis and Cori, 1959), HeLa cells (Barban and Schulze, 1961), ascites carcinoma cells (McComb and Yushok, 1959; Lange and Kohn, 1961 b), and *Neurospora crassa* (Sols *et al.*, 1960 b). The Michaelis constants for 2-DG are usually higher than for glucose (see tabulation) but the rates of

Source	Hexokinase K_m (mM)	
	Glucose	2-DG
Brain	0.01	0.024
Ascites carcinoma	0.04	0.069
Intestine	0.065	0.09
Kidney	0.048	0.04
Liver	0.04	0.09

phosphorylation are often comparable. It is interesting that a strain of HeLa cells resistant to 2-DG has been obtained, and they are defective in hexokinase or contain a hexokinase inhibitor; the phosphorylation rates for 2-DG, glucose, fructose, and mannose are all lower than normal (Barban, 1961). Resistance is also associated with a 5- to 10-fold increase in alkaline phosphatase activity and this may partly account for the slower rate of

accumulation of 2-DG-6-P (Barban, 1962 a, b). How much of the 2-DG is oxidized directly is generally unknown, but the glucose oxidase from *Aspergillus niger* oxidizes it fairly well: the relative rates of oxidation are glucose (100), 2-DG (20), 3-DG (1), 4-DG (2), 5-DG (0.05), and 6-DG (10) (Pazur and Kleppe, 1964). The direct oxidation of 2-DG is apparently catalyzed by a variety of enzymes, some of the notatin type (Sols and de la Fuente, 1957) and some of the glucose dehydrogenase type (Williams and Eagon, 1959). The further metabolism of 2-deoxy-D-gluconate probably varies with the tissue and has been shown in the scheme above for skin (Brooks *et al.*, 1960). Other pathways for 2-DG metabolism may occur in plants, since Kocourek *et al.* (1963) have provided evidence for (1) β-glucosidation probably on C-6, (2) oxidation on C-1 to form 2-deoxyhexonate lactone, and (3) epimerization to 2-deoxygalactose in tobacco plants taking up 2-DG through the roots. The last reaction involves three enzymes and the epimerization occurs in a complex with UDP. The abnormal disaccharide, β-D-fructofuranosyl-2-deoxy-D-glucose, has been insolated from the excised leaves of several plants following incubation with 2-DG (Barber, 1959). It is possible that a number of abnormal polysaccharides containing 2-DG will eventually be found.

Effects of 2-DG and 2-DG-6-P on Glycolytic Enzymes

2-DG inhibits the utilization of glucose and other sugars in many organisms and tissues, and we shall now attempt to localize the site of this inhibition in the early phases of glycolysis. We must consider not only 2-DG but also its primary metabolic product, 2-DG-6-P, as inhibitors. The most likely sites for inhibition would be (1) 2-DG on hexokinases, or (2) 2-DG-6-P on phosphoglucose isomerase, 6-phosphofructokinase, or aldolase with respect to glucose metabolism. Since 2-DG is phosphorylated about as well as glucose by hexokinases it is clear that some competitive inhibition would be observed under certain circumstances, a suggestion first made by Cramer and Woodward (1952). However, this would appear to be generally an unimportant factor in the over-all glycolytic inhibition, since 2-DG equimolar with glucose does not inhibit the glucokinase of ascites cells (Nirenberg and Hogg, 1958) and at 10 times the glucose concentration does not inhibit HeLa cell glucokinase (Barban and Schulze, 1961). The situation may be somewhat more complex in certain tissues, however, inasmuch as rat liver contains two glucose-phosphorylating enzymes, called glucokinase and hexokinase (Walker and Rao, 1963). The sensitivities of these enzymes are quite different (see accompanying tabulation) and a kinetic analysis was made, the hexokinase being studied without interference by the glucokinase since fetal liver contains only the former. The effect of the various inhibitors, which are all competitive, varies in a complex fashion as the glucose concentration is changed because of the vary-

Inhibitor	K_i (mM)		
	Rat liver glucokinase	Rat liver hexokinase	Guinea pig hexokinase
2-DG	14	0.3	0.6
D-Glucosamine	0.8	0.3	0.2
N-Acetyl-D-glucosamine	0.5	0.2	0.3
Glucose (K_m)	10	0.04	0.03

ing importance of each enzyme, and it was pointed out that it is very difficult to assess the effects of such analogs in adult liver.

Most of the emphasis recently has been placed on the inhibition of phosphoglucose isomerase by 2-DG-6-P. This inhibition is competitive on the enzyme from rat kidney (Wick *et al.*, 1957), rat muscle (Ferrari *et al.*, 1959), and ascites cells (Nirenberg and Hogg, 1958). The inhibition is reasonably potent (see tabulation for kidney enzyme) and it would be quite

Glucose-6-P (mM)	2-DG-6-P (mM)	% Inhibition
1	0.5	9
1	1	24
0.5	1	79

easy for the 2-DG-6-P concentration to become greater than the glucose-6-P concentration in cells, especially as the former is usually not metabolized and accumulates. The inhibition of the skeletal muscle enzyme is very similar. Unfortunately the inhibitions of 6-phosphofructokinase and aldolase by 2-DG-6-P have not yet been adequately examined, so one is left with phosphoglucose isomerase as the site of the primary block, the conclusion of Wick *et al.* (1957). However, another possible site for inhibition is the membrane transport system for glucose, as suggested by the work of Kipnis and Cori (1959), and this might be by either 2-DG or 2-DG-6-P. Furthermore, Nirenberg and Hogg (1958) reported that the metabolism of fructose-1,6-diP is blocked by 2-DG-6-P and stated that some inhibition must occur after the phosphofructokinase step (which could be on aldolase). Other inhibitions on enzymes metabolizing glucose but not on the main glycolytic pathway may be mentioned. Rat liver microsomal glucose-6-phosphatase is inhibited weakly by 2-DG (Hass and Byrne, 1960), HeLa cell glucose-6-P

dehydrogenase is inhibited noncompetitively by 2-DG-6-P (Barban and Schulze, 1961), 2-DG-6-P competitively inhibits the activation of rat liver glycogen synthetase by glucose-6-P (Steiner *et al.*, 1961), and UDPG: α-1,4-glucan-α-4-glucosyltransferase from dog muscle is inhibited by 2-DG-6-P with $K_i = 1.3$ mM (Rosell-Perez and Larner, 1964). The importance of these inhibitions in the interference produced by 2-DG on glucose metabolism is not understood.

The block of fructose utilization by 2-DG may well present a different problem. Fructose phosphorylation is inhibited much more readily than glucose phosphorylation, presumably due to the lower affinity of the hexokinases for fructose (Sols, 1956; Nirenberg and Hogg, 1958; Barban and Schulze, 1961). The inhibition of phosphoglucose isomerase could not explain the suppression of fructose utilization inasmuch as the fructose pathway bypasses this step. In rat adipose tissue 2-DG has very little effect on the metabolism of fructose although glucose metabolism is quite strongly depressed (Fain, 1964). With glucose at 2.8 mM and 2-DG at 1.4 mM, the formation of CO_2 is reduced 70% and of fatty acids 89%. Nevertheless, the stimulatory effect of insulin on fructose utilization is blocked by 2-DG whereas the effects of insulin on glucose are unaltered. Certainly different tissued and organisms must have various transport and enzyme systems for the metabolism of fructose, so one should not expect a uniform action of 2-DG. The enzymes involved in the metabolism of fructose-1-P or fructose-6-P have not been studied with respect to 2-DG inhibition.

Effects of 2-DG on Carbohydrate Metabolism and Respiration

Investigations on isolated enzymes have indicated an important block of phosphoglucose isomerase by 2-DG-6-P and contributory inhibition of hexokinases under certain conditions. Let us now turn to studies on carbohydrate metabolism in intact cells and tissues in order to determine if the effects of 2-DG can be explained adequately on this basis, or to accumulate evidence of blocks elsewhere. Anaerobic glycolysis, aerobic glycolysis, and glucose respiration are inhibited by 2-DG but to very different degrees (Fig. 2-10). Indeed, respiration is inhibited only with high concentrations, usually 30–100 times that required to inhibit anaerobic glycolysis comparably (Woodward and Hudson, 1954; Tower, 1958), so that some workers have reported that respiration is not inhibited (Fridhandler, 1959; Taylor, 1960). In the case of sea urchin eggs, the inhibition can be almost completely counteracted by increasing glucose concentration (Bernstein and Black, 1959). However, glucose must be present when the 2-DG is added and is ineffective when the inhibition has developed. The respiration of guinea pig skin in the presence of various substrates is inhibited by 2-DG moderately and progressively (see accompanying tabulation) (Carney *et al.*, 1962). All substrates and 2-DG were 20 mM. There is no effect on the endogenous

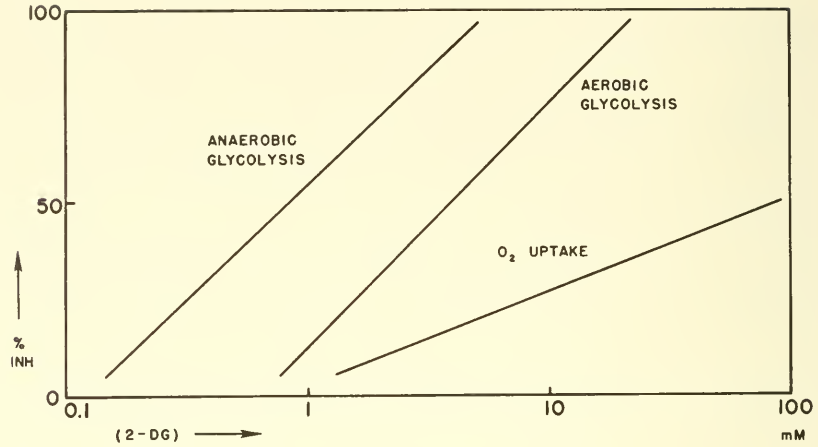


FIG. 2-10. Effects of 2-DG on the glucose metabolism in cat brain slices. (From Tower, 1958.)

respiration. The small effect on galactose respiration was felt to be due perhaps to a different hexokinase being used for the phosphorylation of galactose, since inhibition is exerted by 2- and 4-deoxygalactose.

Substrate	% Respiratory inhibition at:	
	0–2 hr	22–24 hr
Glucose	12	53
Mannose	10	43
Fructose	8	50
Galactose	3	21
Pyruvate	0	1

Glycolysis as measured by the formation of $C^{14}O_2$ from glucose-u-C^{14} is depressed by 2-DG in diaphragm (Nakada and Wick, 1956), kidney (Serif and Wick, 1958), lymph node cells (Helmreich and Eisen, 1959), and adipose tissue (Brooks *et al.*, 1961). The variation of inhibition with 2-DG concentration is shown in Fig. 2-11 for rat kidney slices. Glycolysis as measured by unlabeled CO_2 or lactate formation is also inhibited in yeast (Cramer and Woodward, 1952), ascites carcinoma and leukemic cells (Laszlo *et al.*, 1958), and cerebral cortex slices (Tower, 1958). The depression of aerobic and anaerobic glycolysis in tumor tissue is counteracted by increasing glucose concentrations (Woodward and Hudson, 1954). These results are quite

consistent in showing an inhibition of the glycolytic pathway by 2-DG. However, the formation of CO_2 from glucose is not always depressed. Fridhandler (1959) found that although 2-DG inhibits anaerobic glycolysis in rabbit blastocysts, the rates of respiration and CO_2 formation are not significantly affected. The formation of $C^{14}O_2$ from glucose-1-C^{14} in human fetal liver is actually increased by 2-DG, but this may be attributed to a partial inhibition of glycolysis (Villee and Loring, 1961). In ascites carcinoma cells 2-DG simultaneously inhibits the glycolysis of glucose and

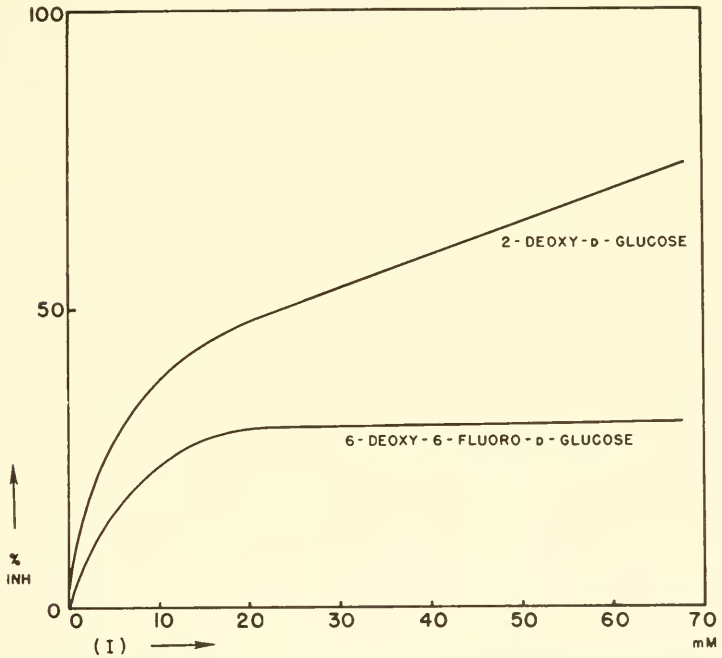


Fig. 2-11. Inhibition of the formation of $C^{14}O_2$ from glucose-u-C^{14} by glucose analogs in rat kidney slices. Glucose was 10 m*M*. (From Serif and Wick, 1958.)

increases its oxidation (Christensen *et al.*, 1961), while the disappearance of glucose from the medium is reduced (Fig. 2-12). The increased $C^{14}O_2$ formed from glucose coupled with the depressed glucose uptake must be taken to mean that the pathway of glucose utilization has been markedly altered, i. e., less glucose is going to lactate and more is being oxidized. One important factor in such tissues must be the activity of the pentose-P pathway, which is apparently not directly inhibited but is indirectly stimulated by 2-DG. The formation of $C^{14}O_2$ from glucose-6-C^{14} in calf thymus nuclei is inhibited essentially completely by 2-DG (McEwen *et al.*, 1963 b). However,

the C-1/C-6 ratio in brain slices remains the same when glucose uptake is reduced to one third by 2-DG (Tower, 1958).

Since hexose uptake into cells is often coupled with phosphorylation, one would expect 2-DG to inhibit this uptake by suppressing kinase activity directly or indirectly. Lymph node cells treated with 2-DG until 2-DG-6-P is formed and then washed free of 2-DG do not accumulate glucose, fructose, or mannose, and lactate formation is markedly reduced (Helmreich and Eisen, 1959). The uptake of glucose into chick embryo hearts is 35–45%

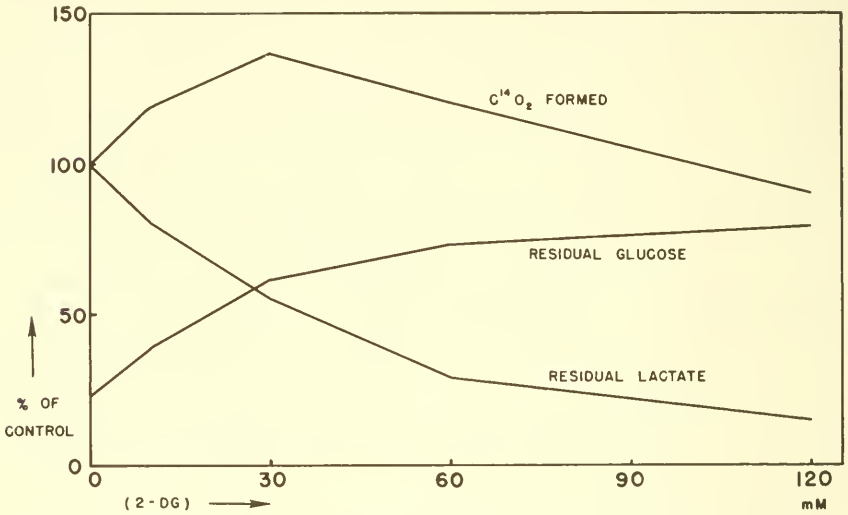


FIG. 2-12. Effects of 2-DG on the glucose metabolism in Ehrlich ascites carcinoma cells. Glucose-u-C^{14} was 10 mM. Control for residual glucose taken from nonincubated flasks with no 2-DG. Experiments 2 and 4 were averaged. (Data from Christensen *et al.*, 1961.)

reduced by 40 mM 2-DG (Modignani and Foà, 1963) and into carrot slices is reduced to about the same degree by 10 mM 2-DG, this inhibition not being overcome by increase in glucose concentration (Grant and Beevers, 1964). 2-DG interferes with galactose uptake into mouse strain L cells but not potently (K_i = 7.2 mM) (Maio and Rickenberg, 1962). It is quite likely that these inhibitions are exerted predominantly by 2-DG-6-P. On the other hand, the accumulation of α-methylglucoside, which is transported into *E. coli* by the glucose carrier, is well inhibited by 1-DG, poorly by 6-DG, and not at all by 3-DG (Hagihira *et al.*, 1963). Apparently 2-DG is not as potent an inhibitor here as 1-DG.

Another mechanism by which 2-DG could alter carbohydrate uptake and metabolism is by changing the levels of P_i, ADP, and ATP, since the rates

of hexose phosphorylation and glycolytic breakdown are controlled by these. In brain slices, 10 m*M* 2-DG causes a 50% fall in creatine-P and almost complete disappearance of the adenosine polyphosphates (Tower, 1958). There is also a diversion of phosphate to the stable 2-DG-6-P so that a certain amount of phosphate is removed from glycolytic participation, as also pointed out by Kiesow (1960 c). Furthermore, 2-DG has been shown to inhibit the incorporation of P_i^{32} into ADP and ATP in ascites carcinoma cells 70% at 10 m*M* (Creaser and Scholefield, 1960). El'tsina and Beresotskaya (1962) determined P_i and nucleotide levels in tumor cells exposed to 11 m*M* 2-DG and found marked decreases in ATP and ADP (see accompanying tabulation). On the other hand, rat liver and kidney slices show no

Tumor components	Control	2-DG
Zahdel hepatoma		
ATP	69.2	7.7
ADP	28.9	13.2
AMP	—	11.7
P_i	157	39
Sarcoma 37		
ATP	74.7	1.7
ADP	40.5	5.4
AMP	14.2	3.5
P_i	140	67

significant changes, a difference attributed to variations in the activity and cellular location of hexokinases. McComb and Yushok (1964 a) also reported marked falls in ATP in ascites cells within 12 min after exposure to 2-DG, and a 65% net loss of the cellular adenine nucleotides. The disappearance of nucleotides is at least partly accounted for by the phosphorylation of 2-DG by hexokinase, the formation of AMP mediated by adenylate kinase, the deamination of AMP to IMP, and the splitting of IMP to inosine by 5′-nucleotidase (McComb and Yushok, 1964 b). They observed a steady rise in inosine, correlated with a rise and subsequent fall in IMP. Changes in nucleotide levels should affect various phases of metabolism which involve these substances, and this is well seen in the effects of 2-DG on the oxidation of ethanol in yeast (Maitra and Estabrook, 1962). When 2-DG is added to previously starved yeast in the presence of ethanol, there is acceleration of respiration and the oxidation of NADPH, accompanied by a fall in ATP with an elevation of ADP. This stimulatory phase lasts less than a minute and is followed by a depressed phase characterized

by very low levels of P_i. During the oxidation of ethanol, the ATP/ADP and P_i/ADP ratios are high; when 2-DG is added it temporarily augments respiration by lowering these ratios, but within a minute enough phosphate has been trapped in 2-DG-6-P to cause a marked fall in the P_i/ADP ratio (perhaps from 17 to 1.5). The P_i may now be so low that it limits the respiration.

The respiration of certain tissues is diminished by the addition of glucose, a phenomenon often called the Crabtree, or reversed Pasteur, effect; it is particularly evident in Ehrlich ascites carcinoma cells and most of the studies of the mechanisms involved have been on these cells. It has been stated that an acceleration of glycolysis inhibits the oxidation of pyruvate, but there was no real evidence to link the entire EM pathway with respiratory control. The effects of 2-DG are thus of particular importance, since it is phosphorylated but not further metabolized to any extent. It was shown that 2-DG inhibits respiration to about the same degree as glucose (Ibsen *et al.*, 1958). Respiration and pyruvate decarboxylation are reduced 50% by 10 m*M* 2-DG (Ram *et al.*, 1963). There has been disagreement as to whether glucose and 2-DG act by the same mechanism or differently. Let us briefly compare the responses to these sugars. (1) 2-DG depresses the respiration more slowly than does glucose. Yushok (1964) has shown in a group of sugars that the rate of respiratory inhibition is correlated with the rate of phosphorylation. One would thus expect 2-DG to act more slowly than glucose, so this does not constitute a real difference in action. (2) The inhibition by glucose is released when it is all glycolyzed but the inhibition by 2-DG remains (Ibsen *et al.*, 1962; Hofmann *et al.*, 1962). This does not seem to me to be valid evidence for different mechanism of action. (3) The addition of glucose leads to the formation of lactate whereas 2-DG does not (Ram *et al.*, 1963). This is what would be expected, of course, but emphasizes that glycolysis, as defined classically, is not necessary for the effect. (4) Glucose at 10 m*M* inhibits the respiration 40%, 2-DG at 20 m*M* inhibits it 48%, and both together inhibit it only 23% (Wenner and Cereijo-Santalo, 1962). This was interpreted to mean that the inhibitory mechanisms are quite different. (5) It has been stated that amobarbital prevents the inhibition of respiration by 2-DG but not by glucose (Wenner and Cereijo-Santalo, 1962). This is true, however, only in the presence of succinate, since the endogenous respiration is not affected by either glucose or 2-DG in the presence of amobarbital (there is very little to be affected). (6) The respiratory inhibition by glucose is released by 2,4-dinitrophenol, but there is some disagreement as to the effect of the uncoupler with 2-DG, Ibsen *et al.* (1962) stating that it releases the inhibition and Ram *et al.* (1963) stating that it does not. The latter workers, however, did not feel that this is evidence for different mechanisms and were inclined to attribute the differences to the availability of glycolytic intermediates. (7) Both

glucose and 2-DG reduce the ATP level immediately and the ADP level very soon (Ibsen *et al.*, 1962). The most commonly accepted explanation of the Crabtree effect is a depletion of ADP, since the rate of mitochondrial oxidation in ascites cells depends on the level of ADP. (8) 2-DG causes the loss of enzymes from the ascites cells (measured with lactate dehydrogenase) whereas glucose does not (Hofmann *et al.*, 1962). Indeed, glucose seems to antagonize this action of 2-DG. Whether this observation has any bearing on the Crabtree effect is not known. (9) Both glucose and 2-DG still exert respiratory inhibition in the presence of sufficient iodoacetate to block almost completely the glycolytic pathway (Ibsen *et al.*, 1958; Wenner and Cereijo-Santalo, 1962). It seems that although the Crabtree effect is not abolished by iodoacetate, it may be diminished. If the initial phosphorylation of hexoses is responsible for the Crabtree effect, iodoacetate would not be expected to inhibit it, except as it might reduce ATP for the kinase reactions. (10) Glucosone inhibits the Crabtree effect produced by both glucose and 2-DG (Yushok, 1964), but this is probably the result of the inhibition of hexokinase by glucosone. Summarizing these results, it would appear that glucose and 2-DG inhibit respiration by basically the same mechanism and that this is related to their phosphorylation. It is difficult to understand the diminished Crabtree effect in the presence of glucose and 2-DG together, observed by Wenner and Cereijo-Santalo (1962), but this should be investigated further, inasmuch as Ram *et al.* (1963) stated that the respiratory inhibition by glucose is not enhanced by 2-DG, apparently no antagonism being noted.

Glucose and 2-DG not only depress the oxidation of pyruvate in ascites cells but even more strongly the oxidation and $C^{14}O_2$ formation from labeled palmitate (Sauermann, 1964). Inhibition of palmitate oxidation occurs to the extent of around 80% at the relatively low concentration of 1.8 m*M* 2-DG. The inhibitions are approximately 29% for acetate, 58% for pyruvate, and 92% for palmitate at 18 m*M* 2-DG. There would thus appear to be some effect on fatty acid oxidation which is exerted prior to the utilization of acetyl-CoA. This action is not related to the inhibition of glycolysis for several reasons, including the demonstration that it occurs in the presence of iodoacetate. Some effect on fatty acid oxidation might be expected from a lowering of the ATP level, but it was felt that this is not the entire explanation because of the relatively small effect on acetate oxidation. It is not necessary, however, that a fall in ATP should affect acetate and palmitate oxidations equally. One awaits with interest the elucidation of this interesting effect.

The Crabtree effect has been discussed in some detail because it illustrates one way in which 2-DG can alter carbohydrate metabolism through the alteration of levels of P_i and the adenine nucleotides. It is quite possible that part of the effect of 2-DG on tissues is the result of a lowering

of available ATP, this secondarily reducing hexokinase activity, and the uptake of sugars. The greater resistance of aerobic glycolysis and respiration to 2-DG, compared to anaerobic glycolysis, may be due in part to the higher ATP levels aerobically. The differential effects on the uptakes of different hexoses (Fig. 2-13) might be explained on the basis of whether a hexose is actively transported or not, and the steady-state rate of its phosphorylation (e. g., galactose metabolism is not depressed as much as that of glucose), and the different affinities of the hexoses for the hexokinases.

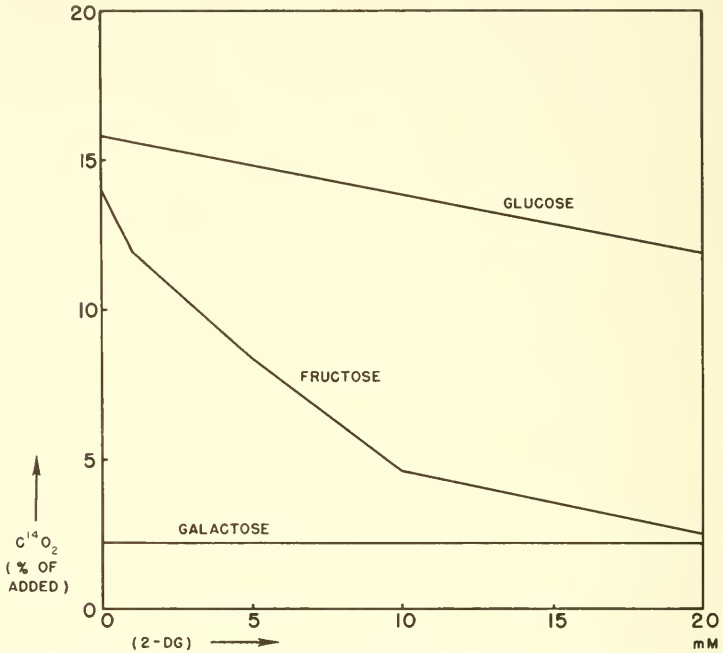


FIG. 2-13. Inhibition of hexose oxidation by 2-DG in isolated rat diaphragm. Hexoses were 10 m*M*. (From Nakada and Wick, 1956.)

A summary of the sites and mechanisms in the inhibition of carbohydrate utilization by 2-DG would then include: (1) primary competitive inhibition of certain hexokinases by 2-DG, (2) possible direct interference in the active transport of hexoses into the cell, (3) inhibition of phosphoglucose isomerase by 2-DG-6-P, (4) secondary reduction in transport and hexose phosphorylation through depletion of ATP, and (5) possible inhibitions by 2-DG-6-P of glycolytic enzymes not yet examined.

Effects of 2-DG on Various Metabolic Pathways

Infusion of acetate-$C^{14}OO^-$ into rabbits and determination of the respired $C^{14}O_2$ were done prior to and after 2-DG injections; no alteration of acetate oxidation was observed (Wick *et al.*, 1957). The total CO_2 produced decreased, partly due to the hypothermia brought about by 2-DG. This was taken as evidence that any block by 2-DG is previous to the tricarboxylate cycle. An effect of 2-DG on citrate levels in ascites carcinoma cells in the presence of pyruvate was reported by Letnansky and Seelich (1960). The citrate begins to rise 2 min after addition of 18.3 m*M* 2-DG and eventually reaches levels definitely higher than in untreated suspensions. The utilization of pyruvate is also depressed around 50%. These observations might be interpreted as originating from some action on the cycle, but more recently (Seelich and Letnansky, 1961) it was shown that methylene blue reduces the high citrate levels and promotes pyruvate utilization in the presence of 2-DG. It was postulated that this is caused by oxidation of NADPH to NADP, which is necessary for isocitrate oxidation. The rise in citrate may be due to a deficiency of NADP, possibly because lactate is not formed in the presence of 2-DG and NADPH is not oxidized, and also due to a drop in ATP. The results can thus be satisfactorily explained on the basis of the mechanisms previously discussed without assuming any direct action on the cycle.

Lipogenesis in human fetal liver from glucose is depressed by 2-DG, incorporation of 1-C^{14} being lowered 33% and of 6-C^{14} 18% at 6.1 m*M* (Villee and Loring, 1961). This can, of course, be attributed to an inhibition of glycolysis. Plasma fatty acids in man rise as much as 330% following intravenous infusion of 2-DG at 60 mg/kg, and this might be interpreted as a depression of fatty acid synthesis so that the normal equilibrium is disturbed and fatty acids are mobilized from the tissues (Laszlo *et al.*, 1961). On the other hand, there is evidence that 2-DG augments epinephrine release, so that this could be partly responsible.

The appearance of labeled amino acids from labeled glucose in brain slices is also depressed by 2-DG (Tower, 1958). 2-DG not only prevents the accumulation of glutamate but causes a profound fall in the intracellular level, glutamine decreasing less markedly. It is believed that glutamate accumulation is important in K^+ uptake by brain and it was found, as expected, that this is inhibited around 60% by 10 m*M* 2-DG. The role of such changes in the central actions of 2-DG in the whole animal is not as yet understood. The incorporation of L-valine-C^{14} into protein in ascites cells is almost abolished by 10 m*M* 2-DG; since this may be reversed by glucose, it is likely that the inhibition is related to glycolytic suppression (Riggs and Walker, 1963). The DNA content of a rat carcinoma is reduced by feeding 2–5% 2-DG in the diet and possibly this is related to the observed inhibition in growth (Sokoloff *et al.*, 1956).

Effects of 2-DG on Cell Division and Growth

The growth rate of *E. coli* is depressed at least 50% by 10 m*M* 2-DG but the differential rate of inducible β-galactosidase synthesis is not altered (Cohn and Horibata, 1959). Although *Neurospora* and *Aspergillus* can grow slowly on 2-DG alone, the growth is inhibited when the usual sugars are present (Sols *et al.*, 1960 b). The mutiplication of influenza virus in chick embryos is markedly inhibited by 2-DG but in preliminary studies there was no evidence that the development of lesions in mice is altered (Kilbourne, 1959). Glucose and, to some extent, pyruvate are able to counteract this depression. Sea-urchin egg cleavage is delayed by 2-DG and development is stopped at various stages, depending on the concentration: 1–10 m*M* prevents gastrulation and the eggs reach swimming blastulae, 100 m*M* delays first cleavage but the early blastula stage is eventually reached, 200 m*M* causes greater cleavage delay and development stops before the blastula stage (Bernstein and Black, 1959). Glucose can counteract the cleavage delay. The most sensitive tissue investigated appears to be chick embryo heart fibroblasts, since the mitotic index is reduced from 2.65 to 0.15 by 1.52 m*M* 2-DG (Ely *et al.*, 1952). Glucose at approximately equimolar concentration is not able to reverse this inhibition significantly. The migration of cells in the 2-DG-treated cultures is also limited and the cells become vacuolated. These observations all point to a general growth-inhibiting action of 2-DG, which is not unexpected, but there has been little study of differential growth effects.

The ability of tumors to derive a good part of their energy for growth from glycolysis prompted the original study of 2-DG as a possible carcinostatic agent, but surprisingly little work has been done on this aspect. It has been established that glycolytic inhibition of tumors *in vivo* is possible, in that patients with chronic myelogenous leukemia infused with 60 mg/kg 2-DG show 35–40% inhibition of glycolysis in the leukemic cells (Laszlo *et al.*, 1958). The growth of cultured HeLa cells from human carcinoma is inhibited readily by 2-DG, 5 m*M* producing essentially complete depression which is reversible up to 3 days but not afterward (Barban and Schulze, 1961). Glucose or mannose will counteract the growth inhibition. Cells in a fructose medium are inhibited more readily than when grown on glucose or mannose, which corresponds to the greater inhibition of fructose utilization discussed previously. When 2-DG is added at 2–5% to the diet of rats, the growth of carcinoma G-175 in reduced (Sokoloff *et al.*, 1956). Adult rats tolerate this dosage well but the growth of young rats is retarded. The 2-DG can also be injected subcutaneously at 2–4 mg/kg/day. Mouse sarcoma is similarly affected. The inhibition in either case is not very marked. Solid, transplanted, and ascitic tumors in mice grow more slowly when 2-DG is administered (Laszlo *et al.*, 1960). A modest prolongation of the survival time of the animals was noted. Definite carcinostatic

activity *in vivo* has thus been demonstrated but there should be much more work to establish if sufficient differential depression can be achieved, and other types of neoplasm should be studied.

Effects of 2-DG on Whole Animals

The intravenous infusion of 2-DG at doses of 50–200 mg/kg in cancer patients produces a feeling of warmth, flushing, diaphoresis, headache, drowsiness, tachycardia, a rise in blood glucose, and a fall in white cell count (Landau *et al.*, 1958). Hyperglycemia has been noted in all studies and might be attributed to a reduced utilization of glucose brought about by glycolytic inhibition. However, other factors must be considered. Brown and Bachrach (1959) showed that the rise in blood glucose from 2-DG can be partially prevented by demedullation of the adrenals, indicating that 2-DG may stimulate the release of epinephrine. Increases in urinary catecholamines during 2-DG infusion have also been noted (Laszlo *et al.*, 1961). Hökfelt and Bydgeman (1961) felt that this epinephrine release is the primary cause of the hyperglycemia and showed that 2-DG can deplete the adrenals of half their epinephrine. Spinal transection reduces the 2-DG effect, indicating epinephrine release to be mediated through the central nervous system. Pretreatment with dihydroergotamine, which blocks the effects of epinephrine, abolishes the 2-DG hyperglycemia (Altszuler *et al.*, 1963; Sakata *et al.*, 1963). It is interesting in connection with the possible effects of 2-DG on the central nervous system that 2-DG is transported from the blood into the cerebrospinal fluid faster than glucose; there is also competition between glucose and 2-DG for the carrier (Fishman, 1964). Landau *et al.* (1958), on the other hand, were inclined to discount the role of epinephrine since no rise in blood pressure is observed during maximal hyperglycemia. The hyperglycemia probably is responsible for the decreased stainability and degranulation of pancreatic islet cells produced by 2-DG, indicating increased activity of these cells, rather than a direct action (Hökfelt and Hultquist, 1961). The symptoms listed above must be due in part to the restricted glucose utilization caused by 2-DG. The rise in blood glucose must tend to counteract this inhibition, since glucose infusions reduce mortality from 2-DG, but not sufficiently. It is interesting that the anaphylactoid reaction to dextran and ovomucoid in rats is inhibited by 2-DG at 200 mg/kg intravenously, although the response to the histamine releasers is not affected, indicating an important role of glucose uptake and metabolism in certain inflammatory reactions (Goth, 1959). The LD_{50} in mice is around 2.5 g/kg for single intravenous injections and 5 g/kg subcutaneously or intraperitoneally (Laszlo *et al.*, 1960). The animals survive several hours to a day in most cases but may die within 10 min after intravenous injection. The major toxic effects are related to the central nervous system and are weakness, convulsions, and coma.

Effects of 2-DG on the Heart *

The contractile tension of rat atria is progressively depressed by 10 m*M* 2-DG when glucose is present at its usual concentration of 5.5 m*M*; the inhibition is 25% at 20 min, 50% at 40 min, and 70% at 90 min (A. Gimeno and M. Gimeno). If pyruvate is present, the rate of depression is slower and the inhibition is 30% at 90 min; if pyruvate is added at 30 min when the depression by 2-DG is around 40–45%, there is partial recovery to the − 30% level; if glucose and pyruvate are present and 2-DG is added at 30 min, there is a depression to the − 30% level at 90 min. The atria in the absence of glucose progressively fail so that at 30 min the contractile tension is 50% depressed (actually the course is quite similar to that when glucose and 2-DG are present), but with 2-DG the rate of fall is much faster, indicating that 2-DG can effect the endogenous metabolism. Addition of glucose at 30 min when the depression is 80% or more results in partial recovery, pyruvate is less effective, and both allow return to near the − 30% level. The rapid cessation of the 2-DG depression brought about by either glucose or pyruvate is noteworthy. The contractile levels reached in 90 min may be summarized in the following tabulation. The failure of

(I)	Glucose-free and glucose added at 30 min	− 0%
(II)	Glucose-free and pyruvate added at 30 min	−15%
(III)	Glucose + pyruvate + 2-DG (in any order)	−30%
(IV)	Glucose + 2-DG (in any order)	−70%
(V)	2-DG alone	−90% to −100%

pyruvate to maintain normal contractions might indicate that some 25–30% of the tension is dependent on glycolysis, but it is also possible that 2-DG is interfering in some way with the utilization of pyruvate. The ability of glucose to stimulate 30 min after depression by 2-DG at 10 m*M* shows that the block of glycolysis is only partial or that glucose is acting by some other mechanism. The atrial depression by anoxia (− 85% at 10 min) is accelerated by 2-DG (− 93% at 5 min and − 100% at 10 min), and recovery upon readmission of O_2 is much less when 2-DG is present (J. Lacuara). Rat ventricle strip contractility is not affected over 160 min by 0.5–2 m*M* 2-DG, but 4 m*M* causes a slow depression and partially prevents the positive inotropic effect of ouabain (E. Majeski).

* Inasmuch as so little is known of the effects of 2-DG on tissue functions and nothing has been reported relative to the heart, this section summarizes briefly some of the recent work done in our department and not yet published at the time of submission of the manuscript.

It is very interesting that the atrial depression is completely unassociated with demonstrable changes in the membrane electrical characteristics (E. Ruiz-Petrich). 2-DG at 10 m*M* depresses the contractile tension around 50% at 20–30 min under the conditions of these experiments, glucose being present, but there are no changes at all of the resting potential, the action potential magnitude, or the rates of depolarization and repolarization. The addition of 5 m*M* pyruvate rapidly stopped the progression of the contractile depression and allowed slight recovery, again without detectable alterations of the membrane characteristics. 2-DG is the only inhibitor with which we have worked that is able to affect the contractile processes so selectively, all other inhibitors decreasing the action potential duration to varying degrees and producing other correlated changes in the potentials. If 2-DG acts here by reducing the utilization of glucose or glycogen, these results would point to a close relation between the contractile process and some aspect of glycolysis other than the generation of ATP. It has also been shown that atrial K^+ influx and efflux are only very slightly altered by 11 m*M* 2-DG, and that intracellular K^+ is unchanged over a period during which the contractile activity is depressed 50% (Chin, 1963).

EFFECTS OF 6-DEOXY-6-FLUORO-D-GLUCOSE ON METABOLISM

Modification of hexoses at the 6-position should interfere with their phosphorylation by hexokinase, and hence any inhibition of glucose metabolism observed would probably not be exerted beyond the hexokinase step. Thus inhibition produced by 6-substituted sugars should be simpler than inhibition by 2-substituted sugars, which can be phosphorylated and may block at several sites. Brooks *et al.* (1961) compared 6-deoxy-D-glucose with 2-DG on the oxidation of glucose-u-C^{14} by various tissues and found it to be somewhat less potent. When glucose is 10 m*M* and 6-DG is 30 m*M*, the inhibitions of $C^{14}O_2$ formation are 43% for adipose tissue, 23% for kidney, and 19% for diaphragm. The inhibition in adipose tissue is reversed by increasing glucose concentration and appears to be competitive. 6-DG is not metabolized by adipose tissue and no $C^{14}O_2$ arises from 6-DG-u-C^{14}. The site of inhibition was considered to be either the membrane transport system or hexokinase. It has been found that galactose transport across the intestine is inhibited by 6-DG (47% at 5 m*M*) and that the transport of 6-DG is depressed by glucose (Wilson *et al.*, 1960).

The replacement of the 6-OH group with fluorine to give 6-deoxy-6-fluoro-D-glucose (6-DFG) leads, as expected, to an interesting inhibitor of glucose utilization. The original idea was apparently to produce a glycolytic inhibitor with fluorine analogous to the cycle inhibitor fluoroacetate. The initial work was done by Blakley and Boyer (1955) at Minnesota; they

showed that fermentation of glucose and fructose by yeast is competitively inhibited by 6-DFG. The apparent constants are shown in the following tabulation. 6-DFG is not fermented by intact cells or yeast extracts. In

Yeast	Glucose		Fructose	
	K_m (mM)	K_i (mM)	K_m (mM)	K_i (mM)
Baker's	1.8	7.3	5.0	3.3
Brewer's	6.9	2.7	27	2.3

extracts 6-DFG has essentially no effect even at 36 mM and hexokinase is very weakly inhibited. Glucose utilization by rat diaphragm is not inhibited as well as yeast fermentation, and 6-DFG is not taken up as readily as glucose by the muscle cells. Glucose oxidase (notatin) oxidizes 6-DFG at about 3% the rate of glucose oxidation so that direct oxidation in the tissues is probably negligible. It was concluded that the rate-limiting reaction for glucose utilization is different in intact cells and extracts, and that 6-DFG probably inhibits the membrane transport of normal hexoses.

The uptake and metabolism of 6-DFG may vary from tissue to tissue. For example, although 6-DFG is not actively transported into rat diaphragm and insulin has no effect on this transfer, it is well transported across the intestinal wall whereas 2-DG is not (Wick *et al.*, 1959; Wilson and Landau, 1960). And, although 6-DFG is not metabolized in most tissues, a particulate preparation from *Aerobacter aerogenes* is able to oxidize it to the corresponding gluconate (Blakley and Ciferri, 1961). It is fairly certain that the compound is quite stable and that release of fluoride does not occur sufficiently to inhibit glycolysis (Serif *et al.*, 1958).

6-DFG inhibits the formation of $C^{14}O_2$ from glucose-u-C^{14} in liver, kidney, and adipose tissue, without modifying the metabolism of acetate or lactate (Serif and Stewart, 1958; Serif and Wick, 1958; Serif *et al.*, 1958). In general 6-DFG is somewhat less potent than 2-DG (Fig. 2-11), but the relative sensitivities depend on the tissue studied. Nevertheless, in the eviscerated rat 6-DFG is able to inhibit the intracellular transport of glucose quite appreciably, e. g., 42% from 200 mg/kg (Wick *et al.*, 1959). There is no direct evidence that 6-DFG acts elsewhere than on membrane transport and the comparable inhibitions produced on the metabolism of glucose-1-C^{14}, glucose-6-C^{14}, and glucose-u-C^{14} would point to a block prior to the glycolytic–pentose phosphate shunt division (Serif and Wick, 1958). However, hexokinases from other than yeast have not been adequately tested. Although the toxicities of 2-DG and 6-DFG have not been directly compared, Blakley and Boyer (1955) found that 250 mg/kg 6-DFG intraperitoneally

in rats produces toxic symptoms with recovery. Since the LD_{50} for 2-DG is probably around 10 times this, it would indicate that 6-DFG must affect the central nervous system more than the other tissues which have been studied.

VARIOUS ANALOG INHIBITORS OF CARBOHYDRATE METABOLISM

Numerous inhibitions of enzymes in the glycolytic and pentose phosphate pathways by analogs have been reported. Some of these may be important in attempts to block carbohydrate metabolism specifically and some are undoubtedly significant in mechanisms regulating the rates in these pathways. We shall discuss a few of the more important enzymes and inhibitions, no effort being made to include all of the observations.

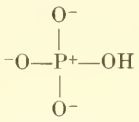
Phosphorylases

The enzymes involved in the synthesis and phosphorolysis of polysaccharides, such as starch or glycogen, are very stereospecific with respect to substrates and inhibitors. The fairly potent inhibition of glycogen phosphorolysis by the product glucose-1-P in preparations from rabbit muscle was reported by Cori *et al.* (1939), 7 m*M* inhibiting 93%, whereas glucose-6-P at the same concentration inhibits only 17%. The reverse reaction of glycogen synthesis from glucose-1-P is inhibited competitively by glucose, and to a lesser extent by mannose, galactose, and maltose, but the affinity of the enzyme for these sugars is low since 30% inhibition occurs when glucose and substrate are approximately equimolar at 17 m*M* (Cori and Cori, 1940). However, the lobster muscle phosphorylase is inhibited only 25% by 250 m*M* glucose when glucose-1-P is 100 m*M* (Cowgill, 1959).

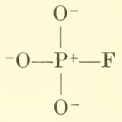
The only known substrate for rabbit muscle phosphorylase is α-D-glucose-1-P with respect to glycogen synthesis, and Cori and Cori (1940) had found that only α-D-glucose inhibits, β-D-glucose having little if any effect, so the question of α, β-specificity was studied in detail on the crystalline enzyme by Campbell *et al.* (1952). It was found that β-D-glucose-1-P is neither a substrate nor an inhibitor, and that whereas α-methyl glucoside inhibits, β-methylglucoside is without activity. The β-anomers appear to have no affinity for the enzyme. Furthermore, a large number of sugars and derivatives at 50 m*M* were found to be inactive. The configurations of the hydroxyl groups on all the positions of glucose seem to be necessary for combination with the enzyme. A pyranose structure seems also to be a requirement for inhibition, since sorbitol and inositol are without effect, and a primary alcohol group on C-5 is necessary since D-xylose is inactive. The failure of fructose to inhibit might be explained in several ways: (1) the more planar furanose form is dominant, (2) the configurations on the

C-1 of D-glucopyranose and C-2 of D-fructopyranose are different, (3) there is a primary alcohol group on the C-5 of glucose but not on the C-6 of fructose, and (4) the β-anomer of fructose may predominate.

An interesting type of inhibition on the phosphorolysis of starch by monofluorophosphate was observed by Rapp and Sliwinski (1956). The



Orthophosphate

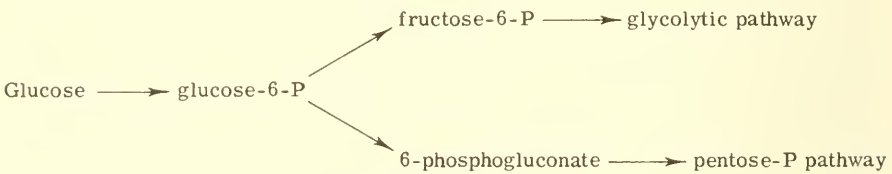


Monofluorophosphate

sizes and electronic configurations of orthophosphate and monofluorophosphate are quite similar so that interference with many reactions involving phosphate might be anticipated. The inhibition of potato phosphorylase is completely competitive (K_m = 3.2 mM and K_i = 2.8 mM calculated from their i/v-i/(S) plot) and the affinity of the enzyme for the two substances corresponds to this tectonic resemblance. The inhibition is not due to the release of fluoride since 20 mM fluoride inhibits only 6.4% and 2.1 mM monofluorophosphate inhibits 50%.

Phosphoglucose Isomerase

This enzyme is important in regulating carbohydrate metabolism since its activity, along with other factors, determines how much glucose-6-P enters the glycolytic pathway; in other words, this enzyme represents a branching point of metabolism in the terminology of Krebs. We have seen that inhibition by 2-DG-6-P is probably an important component of the mechanism of action of 2-DG. The potent inhibition by 6-phosphogluconate is particularly interesting because this substance is formed from glucose-6-P in the pentose phosphate pathway and could determine to some extent the diversion at the branching point. Parr (1956, 1957) reported inhibition of the enzymes from blood, liver, muscle, and potato and found it to be competitive; in the reaction glucose-6-P → fructose-6-P, 1 mM inhibits 75% and



2 mM 95% (glucose-6-P 2 mM). Similar competitive inhibitions of the enzymes from yeast (Noltmann and Bruns, 1959) and *Trichinella spiralis* (Mancilla and Agosin, 1960) have been noted. Rabbit muscle phosphoglucose isomerase may be even more sensitive to 6-phosphogluconate, since the

K_i is around 0.005 mM (Kahana *et al.*, 1960). Another potent inhibitor that is an intermediate in the pentose-P pathway is erythrose-4-P, for which the K_i is 0.0007– 0.001 mM (K_m for fructose-6-P is 0.08 mM) (Grazi *et al.*, 1960). Under conditions that would lead to an accumulation of erythrose-4-P, more glucose-6-P might be diverted into the pentose-P pathway or, if fructose-6-P is being metabolized, a negative feedback effect would be exerted. A third potent inhibitor is glucosamine-6-P (Wolfe and Nakada, 1956), which on the *T. spiralis* enzyme is around 40 times more inhibitory than 6-phosphogluconate, 0.06 mM inhibiting 64% when glucose-6-P is 5 mM (Mancilla and Agosin, 1960). The deamination of glucosamine-6-P in *E. coli*:

$$\text{Glucosamine-6-P} \rightarrow \text{fructose-6-P} \rightleftarrows \text{glucose-6-P}$$

thus terminates at fructose-6-P initially because of the inhibition of the second reaction, but eventually disappearance of glucosamine-6-P relieves the inhibition and glucose-6-P is formed, another example of metabolic regulation through inhibition.

Aldolase

This enzyme splitting fructose-1,6-diP to glyceraldehyde-3-P and dihydroxyacetone-P has unfortunately been studied very little from the standpoint of inhibition by hexose or triose phosphates. Yeast aldolase binds several hexose phosphates quite tightly but splits them very slowly (Richards and Rutter, 1961), as may be seen from the K_i's and the relative reaction rates (rate with fructose-1,6-diP as 1) in the accompanying tabulation.

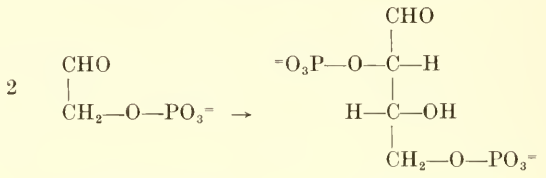
Inhibitor	K_i (mM)	Relative rate
L-Sorbose-1,6-diP	0.13	0.0014
L-Sorbose-1-P	0.2	0.0002
D-Fructose-1-P	1.0	0.0004
D-Fructose-6-P	3.8	0.0001

Muscle aldolase splits these analogs much more readily. The aldolase from rabbit muscle was found by Herbert *et al.* (1940) to be competitively inhibited by fructose-6-P (31%), fructose (16.3%), and glucose (5.4%), the percentages being for 20 mM inhibitor and fructose-1,6-diP. The difficulty in interpreting these results lies in our ignorance of the preferred form of the substrate (i. e., α or β, pyranose, furanose, or linear) for the enzyme, and the distribution of the substrates and inhibitors among these forms under the experimental conditions. The values of K_i may be quite misleading

because the inhibiting form could represent only a small fraction of the total concentration of the inhibitor.

Glyceraldehyde-3-Phosphate Dehydrogenase

A new, potent, and apparently specific inhibitor of this enzyme has been found and is likely to be useful as a blocking agent of this step in glycolysis. Glycolaldehyde-2-P freshly prepared does not inhibit glyceraldehyde-3-P dehydrogenase, but either aging the preparation or allowing it to react in 1 *N* NaOH for a short time leads to the formation of a potent inhibitor, termed tetrose-diP by Racker *et al.*, (1959) and isolated as D-threose-2,4-diP by Fluharty and Ballou (1959). Both the D- and L-isomers



of the inhibitor were synthesized by the latter workers and only the D-isomer was found to inhibit strongly. The inhibition is reversible but noncompetitive with respect to glyceraldehyde-3-P; the inhibition may actually increase with NAD concentration. The value of K_i for rabbit muscle enzyme is 0.0001-0.0002 m*M*. D-Threose-2,4-diP is oxidized by the enzyme but only as much as the NAD present. Fluharty and Ballou postulated that it might react with a site other than the normal catalytic site for oxidation of glyceraldehyde-3-P but Racker *et al.*, showed spectroscopically that a stable acyl-enzyme complex is formed, probably with the SH group known to be involved in the catalysis and the binding of NAD. The substrate reactions might be written as:

$$\text{Enzyme-NAD} + \text{Glyceraldehyde-3-P} \rightleftarrows \text{phosphoglyceryl-enzyme-NADH}$$

$$\text{Phosphoglyceryl-enzyme-NADH} + \text{P} \rightleftarrows \text{glycerate-1,3-diP} + \text{enzyme-NADH}$$

whereas the reaction with the inhibitor is:

$$\text{Enzyme-NAD} + \text{threose-2,4-diP} \rightleftarrows \text{diphosphothreonyl-enzyme-NADH}$$

Normally an inorganic phosphate is transferred from a second site to the phosphoglyceryl-enzyme to form glycerate-1,3-diP, but the location of the 2-phosphate group on the inhibitor is such as to occupy this second site so that no phosphate can enter the reaction. This is thus an example of an inhibition in which a substance enters the reaction sequence in the same

manner as the substrate, forming a stable complex with the enzyme, but is unable to complete the sequence.

The use of D-threose-2,4-diP to block glycolysis in intact cells or tissues will probably not meet with general success due to the poor penetration. Extracts of ascites tumor cells are inhibited readily, but glycolysis in intact ascites or HeLa cells is unaffected (Racker *et al.*, 1959). Preparations of glycolaldehyde-2-P. containing the tetrose-diP, inhibit the growth of some bacteria and not others, probably depending on the degree of penetration. The possibility was considered that the inhibitor, can be formed intracellularly but so far appropriate precursors have not been found.

If photosynthesis involves the reversal of the glycolytic sequence, D-threose-2,4-diP should inhibit, and it has been found that the total $C^{14}O_2$ fixation in sonically ruptured spinach chloroplasts is reduced 57% by this analog at 0.1 m*M* (Park *et al.*, 1960). The photoreduction of 3-phosphoglycerate is inhibited and this substance accumulates in the presence of the inhibitor. This inhibition is due primarily to an action on glyceraldehyde-3-P dehydrogenase leading to a deficiency of ribulose-1,5-diP, the CO_2 acceptor. Carboxy dismutase is inhibited only slightly by 0.1 m*M* D-threose-2,4-diP but higher concentrations inhibit appreciably. This inhibitor may thus play a role in photosynthetic studies.

Keleti and Telegdi (1959) examined various glyceraldehyde-3-P dehydrogenases and found inorganic phosphate to stimulate the activity at low concentrations but to inhibit progressively above 20–30 m*M*. The inhibition of the yeast enzyme is competitive with both glyceraldehyde-3-P and NAD. Taylor *et al.*, (1963) also found phosphate inhibition of the hydrolysis of *p*-nitrophenylacetate by glyceraldehyde-3-P dehydrogenase, the muscle enzyme being much more sensitive than the yeast enzyme. Indeed, the muscle enzyme is 56% inhibited already at 10 m*M* phosphate. Phosphate inhibitions of the glycolytic enzymes have seldom been considered as playing a role in the regulation of carbohydrate metabolism.

Enolase

Enolase catalyzes the reaction

$$\text{D-2-phosphoglycerate} \rightleftarrows \text{phosphoenolpyruvate}$$

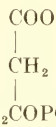
and various analogs of these substances inhibit competitively (see tabulation) (Wold and Ballou, 1957). The following substances do not inhibit: D-lactate, D-glyceraldehyde-3-P, dihydroxyacetone-P, and glycerol-2-P. An inhibitor must have a carboxyl and a phosphate group, and the distance between them is of some importance, although not critical. The 3-methylation of 2-phosphoglycerate does not lead to much reduction in binding, but 3-methylation of the 3-phosphoglycerate reduces the affinity by approxi-

Inhibitor	K_i (mM)
D-Phospholactate	0.35
β-Hydroxypropionate-P	0.45
D-3-Phosphoglycerate	0.45
D-*erythro*-2,3-Dihydroxybutyrate-2-P	0.60
D-*erythro*-2,3-Dihydroxybutyrate-3-P	3.3

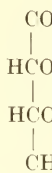
D-2-Phosphoglycerate

D-3-Phosphoglycerate

D-Phospholactate



β-Hydroxypropionate-P



D-*erythro*-2,3-Dihydroxybutyrate-2-P

D-*erythro*-2,3-Dihydroxybutyrate-3-P

mately 1.2 kcal/mole. This would indicate that 2- and 3-phosphates fit comparably as long as there is no bulky group on the 3-position, but when there is it sterically interferes with the bending of the 3-phosphate to fit the active site.

Glucose and Glucose-6-P Dehydrogenases

Beef liver glucose dehydrogenase is inhibited strongly and competitively by glucose-6-P (Strecker and Korkes, 1952) and fructose-1,6-diP (Brink, 1953 a), the K_i's being 0.0025 mM and 0.062 mM, respectively. The K_m for glucose is around 31 mM at pH 7, so that if this represents a dissociation constant the phosphorylated compounds are bound much more tightly (around 5.8 kcal/mole). The rat liver enzyme is similarly inhibited: glucose-6-P at 0.015 mM inhibits 78% when glucose is 200 mM (Metzger *et al.*, 1964). Glucose-1-P also inhibits but is about one-tenth as effective as glucose-6-P. Ribose-5-P and fructose-6-P are also less inhibitory (Brink, 1953 a). The potency of the glucose-6-P inhibition is surprising and it is quite likely that it may be important in metabolic regulation or conserving glucose for phosphorylation. Not enough is known about this enzyme or the reaction mechanism to speculate on the nature of the interaction of these inhibitors.

Yeast glucose-6-P dehydrogenase is inhibited competitively by glucosamine-6-P but in this case the affinity for the inhibitor is not as great as for the substrate (K_m = 0.058 mM, and K_i = 0.72 mM) (Glaser and Brown, 1955). Neither mannose-6-P nor N-acetyl-D-glucosamine-6-P inhibits. Phosphate inhibits the yeast enzyme rather weakly but it is competitive with respect to NADP. The enzyme from *Prototheca zopfii*, however, is inhibited 13% by 0.07 mM phosphate, 43% by 0.14 mM, and 70% by 0.7 mM (glucose-6-P = 3 mM and NADP = 0.67 mM) (Ciferri, 1962). There needs to be much more study of the inhibition of such enzymes if we are to understand the controlling factors of carbohydrate metabolism.

Phosphopentose Isomerases

The phosphoribose isomerase of alfalfa is potently inhibited by 5-phosphoribonate and much less so by a variety of related substances (as shown in the accompanying tabulation) (Axelrod and Jang, 1954). Since ribose-5-P

Inhibitor	Concentration (mM)	% Inhibition
5-Phosphoribonate	0.13	50
Glucose-6-P	11.3	32
Phosphate	25	49
Ribose	11.5	0
Ribose-3-P	12.5	0

was 2.5 mM, the 5-phosphoribonate is bound more tightly. However, even 19 mM 5-phosphoribonate does not inhibit the growth of *Leuconostoc mesenteroides* or *Lactobacillus arabinosus* in glucose medium; this could mean that the pentose phosphate pathway is not necessary for growth of these organisms or that the inhibitor does not penetrate readily. The *Ophiodon elongatus* muscle enzyme is also inhibited by 5-phosphoribonate but less potently (28% inhibition at 0.5 mM and 45% at 5 mM) (Tarr, 1959).

The phosphoarabinose isomerase of *Propionibacterium pentosaceum* is not inhibited by D-ribose, D-xylose, D-arabinose, L-arabinose, ribose-5-P, and glucose-6-P at 10 times the concentration of arabinose-5-P (Volk, 1960), and the phosphopentose isomerase of *Echinococcus granulosus* hydatid cysts is not inhibited by glucose, fructose, glucosamine, glucose-6-P, fructose-6-P, and mannose-6-P at 4 mM (Agosin and Aravena, 1960). However, the latter enzyme is inhibited 51% by 1.2 mM dihydroxyacetone-P and 54% by 4 mM dihydroxyacetone, so that conditions favoring accumulation of these substances might suppress the alternate pentose-P pathway, which could be important in the results obtained with iodoacetate or D-threose-2,4-diP.

Glucose-6-Phosphatase

The hydrolysis of glucose-6-P in rat liver preparations (probably microsomal) is inhibited by glucose with a K_i around 29 mM (Langdon and Weakley, 1957). This inhibition is noncompetitive with respect to glucose-6-P, and it was postulated by Segal (1959) that the glucose competes with the second substrate, water, for its site; the transfer of phosphate could occur either to water or another molecule of glucose. If this is true, incorporation

(1) Enzyme + glucose-6-P $\rightleftarrows$ enzyme-glucose-6-P

(2) Enzyme-glucose-6-P $\rightleftarrows$ enzyme-P + glucose

(3) Enzyme-P + water $\rightleftarrows$ enzyme + P_i

(4) Enzyme-P + glucose $\rightleftarrows$ enzyme-glucose-6-P

of C^{14} from glucose-C^{14} into glucose-C^{14}-6-P should occur and this was demonstrated. It was shown that with the appropriate rate constants the reactions (1)–(4) (reaction (4) is of course the reverse of (2) and is included to visualize competition between water and glucose) lead to noncompetitive kinetics. A study of the inhibition of this incorporation by Hass and Byrne (1960) showed that glucose is the most potent inhibitor of various sugars tested.

Miscellaneous Inhibitions

Numerous other analog inhibitions of enzymes involved in carbohydrate utilization have been reported, some of which have been summarized in Table 2-21. These inhibitions along with those previously discussed point to many mechanisms for the control of glucose metabolism. The complex interplay between all the sugars and their phosphorylated derivatives with respect to the inhibition of various enzymes in the different available pathways must always be borne in mind in work on intact cells. Many of these enzymes are also inhibited to varying degrees by inorganic phosphate; hence the level of phosphate can also be a regulating factor. Enzymes inhibited by phosphate include phosphodeoxyribomutase, phosphoribose isomerase, phosphoglucose isomerase, triosephosphate isomerase, glucose-6-P dehydrogenase, enolase, glyceraldehyde-3-P dehydrogenase, phosphorylase, transaldolase, transketolase, glucose-6-phosphatase, and ribulose-P carboxylase. In many instances appreciable inhibition is exerted by 5–20 mM phosphate. An interesting study of phosphate inhibition of transaldolase was made by Bonsignore *et al.* (1960) in which the following reactions were examined:

Fructose-6-P + glyceraldehyde $\rightarrow$ glyceraldehyde-3-P + fructose

Sedoheptulose-7-P + glyceraldehyde-3-P $\rightarrow$ fructose-6-P + erythrose-4-P

Phosphate inhibits the first reaction competitively with respect to fruc-

TABLE 2-21

MISCELLANEOUS COMPETITIVE INHIBITORS OF ENZYMES INVOLVED IN CARBOHYDRATE METABOLISM

Enzyme	Source	Inhibitor	Reference
Phosphoglucomutase	Rabbit muscle	Glucose-6-P	Bodansky (1961)
Phosphoribomutase	Bovine uterus	2,3-Diphosphoglycerate	Guarino and Sable (1956)
Phosphodeoxyribomutase	*Sarcina lutea*	Fructose-6-P Galactose-6-P Glucose-6-P Ribose-6-P	Smith and Bernstein (1961)
Phosphoribulokinase	Spinach	5-Phosphoribonate	Hurwitz *et al.* (1956)
2-Keto-3-deoxy-D-*arabo*-heptonate-7-P synthetase	*E. coli*	Sedoheptulose-1,7-diP Sedoheptulose-7-P Fructose-1,6-diP 3-Deoxy-D-*arabo*-heptonate-7-P	Srinivasan and Sprinson (1959)
Glycerate-2,3-diphosphatase	Chicken muscle	3-Phosphoglycerate	Joyce and Grisolia (1958)
	Yeast	2-Phosphoglycerate	Joyce and Grisolia (1958)
Mutarotase	Rabbit lens	Galactose Xylose Xylitol Sorbitol	Keston (1963)

tose-6-P but inhibits the second reaction noncompetitively with respect to sedoheptulose-7-P or glyceraldehyde-3-P. Since the K_i's are roughly the same for all inhibitions (around 60 mM), a single binding site for phosphate was postulated. Phosphate prevents the formation of the transaldolase-dihydroxyacetone complex but does not interfere with the transfer of the dihydroxyacetone to its acceptor.

Multivalent anions in general are inhibitors of glycolysis. The anaerobic formation of lactate from glucose in pigeon hemolysates is depressed 84% by 40 mM sulfate, 64% by 20 mM phosphate, 100% by 4.2 mM oxalate, and 48% by 0.1% ribonucleate (Dische and Ashwell, 1955). The reactions from glucose → lactate are inhibited more strongly than from fructose-1,6-diP → lactate; therefore there is inhibition previous to fructose-1,6-diP. The sequence from glucose → glyceraldehyde-3-P is inhibited strongly by sulfate and ribonucleate. Aldolase is inhibited about 20% by 40 mM sulfate but is not affected by oxalate. It was concluded that there are three sites of action, the strongest on hexokinase, next on glyceraldehyde-3-P dehydrogenase, and the last possibly on pyruvate kinase, the inhibitions being competitive with ATP or NAD. Actually little positive evidence was provided for these sites and other possibilities are just as likely; furthermore, competition with hexose phosphate and glycerol phosphate is also possible. In addition the complexing of Mg^{++} by these anions must be considered since several glycolytic enzymes are activated by this ion.

It will suffice to mention four additional interesting examples of analog inhibition on this phase of metabolism. Cataractogenic sugars and polyols inhibit lens mutarotase while noncataractogenic sugars do not; the K_i's are 4 mM for galactose, 15 mM for xylose, and 100 mM for sorbitol (Keston, 1963). Despite the weak inhibitory activity of sorbitol, there is a large amount in the lens in certain conditions, such as diabetes (perhaps around 30 mM). Mannose is quite toxic to honeybees; of bees offered 1 M mannose solution, 50% were dead in 90 min and over 90% in 3 hr (Sols *et al.*, 1960 a). It was found that bees have a hexokinase very active toward mannose coupled with a negligible amount of phosphomannose isomerase, so that mannose not only may interfere with phosphorylation of glucose and fructose, but many accumulate as mannose-6-P, which could disturb glycolysis in a number of ways. Xylose appears to be in some manner a specific inhibitor of photosynthesis, since *Chlorella* propagation is not inhibited by 0.5–1.5% xylose when glucose, fructose, or mannose is present (thus it is not inherently toxic), but under photosynthetic conditions the cells rapidly lose their color and ability to divide, an effect that can be reversed by glucose (Hassall, 1958). It was postulated that xylose may compete with xylulose-5-P for an enzyme in the transketolase pathway and block photosynthesis; on the other hand, a phosphorylated product may be the active inhibitor. Analogs without the usual hexose structure may also inhibit glycolysis and

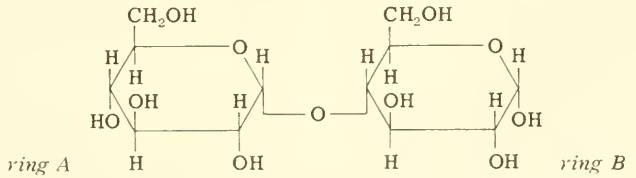
the pentose-P pathway. Sahasrabudhe *et al.* (1960) in looking for carcinostatic analogs found that thiophene-2,5-dicarboxylate, which might be considered as an analog of substances such as ribose-5-P, inhibits the formation of $C^{14}O_2$ from glucose-1-C^{14} and glucose-6-C^{14} around 43% (concentration unspecified) in tumor tissue, and suppresses the growth *in vivo* of rat sarcoma. No evidence was given as to the site of action and so the assumption is tenuous, but the concept of using heterocyclic substances analogous to the furanose and pyranose structures may be important.

GLYCOSIDASES

This large group of enzymes hydrolyzing the glycosidic bonds of simple glycosides, oligosaccharides, and polysaccharides has been studied for many years and it is not surprising that numerous instances of analog inhibition have been observed. Most of the reports, although important in themselves, do not lend themselves to interpretations on the molecular level; some of the data have been briefly summarized in Table 2-22. Most of the inhibitions are relatively weak but a few could definitely be significant in metabolic regulation. The α, β configuration of the inhibitor is seen to be important in some instances. However, the enzymes are seldom completely specific for the α- or β-forms and the affinities often differ very little, as with maltose transglucosylase (amylomaltase) from *E. coli* where (see accompanying tabulation) the binding difference between the α- and β-glu-

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
β-Methylmaltoside	2.5	3.70
α-Methylglucoside	8	2.98
β-Methylglucoside	10	2.84
α-Phenylglucoside	10	2.84
β-Phenylglucoside	30	2.16

cosides is 0.14–0.68 kcal/mole (Wiesmeyer and Cohn, 1960). It appears that the hydroxyl groups on C-2, C-4, and C-6 of ring A are involved in the



Maltose

TABLE 2-22

ANALOG INHIBITIONS OF GLYCOSIDASES[a]

Enzyme	Source	Substrate	Analog	Type of inhibition	(I) (mM)	% Inh.	K_i (mM)	Reference
α-Glucosidase	Yeast	α-Me-glucoside(50)	Glucose	C	20	49		Michaelis and Rona (1914)
		(250)	Glucose	C	20	29		
			Galactose		—	(i)		
			Mannose		—	(i)		
			β-Me-glucoside		—	(ni)		
			Fructose		—	(ni)		
	E. coli	Maltose	β-Me-maltoside	C	—	—	2.5	Wiesmeyer and Cohn (1957)
			α-Me-glucoside	C	—	—	10	
			β-Me-mannoside		—	(ni)		
			Sucrose		—	(ni)		
			Lactose		—	(ni)		
	Pig intestine	Maltose	Turanose	C	—	—	3.3	Dahlqvist (1959)
			Phenyl-α-gluco-pyranoside	C	—	—	15	
			Isomaltose	C	—	—	20	
			Melezitose	C	—	—	500	
	Aspergillus	Maltose	Trehalose	C	—	—	23	Kato *et al.* (1960)
			α-Me-glucoside	C	—	—	39	
			Raffinose	C	—	—	150	

		α-φ-Glucoside	Glucose	C	—	—	4.7	Kato *et al.* (1960)
			Xylose	C	—	—	140	
			Sucrose	C	—	—	180	
			Mannose		—	(ni)		
			Galactose		—	(ni)		
β-Glucosidase	*Aspergillus*	*p*-Nitrophenol-β-glucoside(0.5)	Maltose		5	40		Ezaki (1940)
			Cellobiose		5	55		
			Gluconate		5	65		
			Gluconate ethyl ester		5	76		
			Gluconic-γ-lactone		5	92		
			Phenol-α-glucoside		5	43		
	Sheep rumen bacteria	*o*-Nitrophenol-β-glucoside	Cellobiose	C	—	(i)		Festenstein (1958)
			Glucose	C	—	(i)		
			Gluconolactone	C	—	(i)		
α-1,6-Dextran-glucosidase	Rabbit liver	Dextran	Glucose		—	(i)		Lukomskaia and Rozenfel'd (1958)
			Isomaltose		—	(i)		
			Maltose		—	(i)		
			Sucrose		—	(ni)		
α-Galactosidase	*Trichomonas foetus*	*o*-Nitrophenyl-α-galactoside(10)	Glucose		37	22		Watkins (1959)
			Galactose		37	22		
			N-Acetylglucosamine		28	20		
			N-Acetylgalactosamine		28	0		
			Fucose		41	0		

TABLE 2-22 (*continued*)

Enzyme	Source	Substrate	Analog	Type of inhibition	(I) (m*M*)	% Inh.	K_i (m*M*)	Reference
	E. coli	*o*-Nitrophenyl-*α*-galactoside(1.6)	Raffinose		10	78		Sheinin and Crocker (1961)
			α-Me-galactoside		10	52		
			Galactose		10	44		
			Melibiose		10	41		
			β-Me-galactoside		10	20		
			Fructose		2	6		
β-Galactosidase	*E. coli*	*o*-Nitrophenyl-*β* galactoside	Lactose		—	58		Lester and Bonner (1952)
			Lyxose		—	47		
			Galactose		—	34		
			Melibiose		—	33		
			Glucose		—	28		
			Maltose		—	15		
			Arabinose		—	13		
			Xylose		—	12		
			Raffinose		—	8		
	Trichomonas foetus	*o*-Nitrophenyl-*β*-galactoside(10)	Galactose		37	2		Watkins (1959)
			Glucose		37	0		
			N-Acetylglucosamine		28	0		

			N-Acetylgalactosamine		28	0		
N-Acetyl-β-glucosaminidase	*Trichomonas foetus*	*p*-Nitrophenyl-*N*-acetyl-β-glucosaminide(10)	*N*-Acetylglucosamine		28	36		Watkins (1959)
			N-Acetylgalactosamine		28	70		
N-Acetyl-β-galactosaminidase	*Trichomonas foetus*	*p*-Nitrophenyl-*N*-acetyl-β-galactosaminide(10)	*N*-Acetylglucosamine		28	37		Watkins (1959)
			N-Acetylgalactosamine		28	68		
N-Acetyl-β-glucosaminidase	Rat kidney	Phenyl-*N*-acetyl-β-glucosaminide	*N*-Acetylglucosamine	C	—	—	4.4	Pugh *et al.* (1957)
			Acetamide	C	—	—	10.2	
			Acetate	C	—	—	17.0	
			p-Nitrophenyl-*N*-acetylgalactosaminide	C	—	—	0.07	Walker *et al.* (1961)
			N-Acetylglucosamine	C	—	—	3.1	
			N-Acetylgalactosamine	C	—	—	0.4	
			2-Acetamido-2-deoxygluconolactone	C	—	—	0.0007	
			2-Acetamido-2-deoxygalactonolactone	C	—	—	0.0005	

TABLE 2-22 (*continued*)

Enzyme	Source	Substrate	Analog	Type of inhibition	(I) (m*M*)	% Inh.	K_i (m*M*)	Reference
N-Acetyl-β-galactosaminidase	Rat kidney	*p*-Nitrophenyl-*N*-acetyl-β-galactosaminide	Phenyl-*N*-acetylglucosaminide	C	—	—	3.5	Walker *et al.* (1961)
			N-Acetylglucosamine	C	—	—	7.6	
			N-Acetylgalactosamine	C	—	—	1.6	
			2-Acetamido-2-deoxygluconolactone	C	—	—	0.0049	
			2-Acetamido-2-deoxygalactonolactone	C	—	—	0.0018	
α-Amylase	*Candida tropicalis*	Starch	Maltose	C	—	—	0.5	Sawai (1960)
			α-Phenylglucoside	C	—	—	1.4	
			Glucose	~C	—	—	2.1	
			β-Phenylglucoside	NC	—	—	17	
			α-Me-glucoside	~C	—	—	20	
			Sucrose	NC	—	—	43	
			Mannose		—	(i)		
			Xylose		—	(i)		

β-Amylase	Malt	Starch	Maltose		56	30		Wohl and Glimm (1910)
					139	42		
					195	59		
			Glucose		278	49		
			Galactose		—	(i)		
			Fructose		—	(ni)		
			Sucrose		—	(ni)		
	Sweet potato	Starch	Maltose	C	—	—	6	Misra and French (1960)
Sucrose trans-fructosylase	*Aerobacter levanicum*	Sucrose	β-Me-fructofuranoside	C	—	—	85	Hestrin and Avigad (1958)
			α-Me-glucopyranoside	C	—	—	180	
Polygalacturonase	Yeast	Pectate	Galacturonate		50	13		Luh and Phaff (1954)
β-Fructofuranosidase	Yeast	Sucrose(250)	α-Me-glucoside		50	75		Michaelis and Pechstein (1914)
			β-Me-glucoside		—	(ni)		
			Fructose		—	(i)		
	Yeast	Sucrose(100)	α-Glucose		110	0		Kuhn (1923)
			β-Glucose		110	15		
			α, β-Fructose		110	17		
			β-Fructose		110	15		

[a] The type of inhibition is given as C = competitive or NC = noncompetitive. Under % inhibition (i) indicates that inhibition was found but no data are provided and (ni) indicates no inhibition. The substrate concentration (m*M*) where it is important is given in parentheses after the substrate.

binding since alteration of the positions, as in α-methylmannoside or α-methylgalactoside, abolishes the inhibition, and omission, as in α-methylxyloside, also prevents binding. The substitution of a methyl group on C-1 of ring B (β-methylmaltoside) does not interfere with the binding, since this analog has approximately the same affinity for the enzyme as the substrate maltose. Alteration of the glycosidic link from the α-1,4 in maltose to the β-1,4 in cellobiose does not result in an inhibitor, and other changes (as in trehalose, sucrose, dextran, or lactose) likewise reduce the affinity. The requirements for binding may be summarized as (1) a glucose-like configuration of hydroxyl groups in ring A, (2) a glycosidic link of the α-1,4 type, and (3) a widely variable glycosidic group.

An α-mannosidase of *Streptomyces griseus* with α-phenylmannoside as the substrate is inhibited competitively (probably) by several sugars and glycosides (Hockenhull *et al.*, 1954 c) and the relative binding energies have been estimated on this basis (see tabulation), although the reliability of

Inhibitor	% Inhibition at 50 m*M*	Relative $-\Delta F$ of binding (kcal/mole)
α-Methylmannoside	96	5.23
Mannose	83	4.25
Cellobiose	82	4.20
Maltose	73	3.88
α-Methylglucoside	14	2.15
Xylose	11	1.98
Arabinose	10	1.91
Sucrose	7.5	1.72
Glucose	5	1.45
Fructose	5	1.45
Ribose	4	1.31
Mannitol	2	0.87
Rhamnose	0	—

these figures is quite low due to variations between experiments. The mannose configuration seems to confer strong binding, as expected, but the high inhibitory activities of maltose and cellobiose (α and β glucosides, respectively), especially in view of the weak inhibitions produced by α-methylglucoside and glucose, are unexpected and perhaps indicate that these substances are oriented on the enzyme surface in a different way than the substrate, although there is no necessity to postulate an irreversible complex as did Hockenhull and his co-workers. The active centers for such enzymes probably possess several binding groups for the hydroxyls arranged in a certain pattern; glycosides other than the substrate could conceivably

interact satisfactorily with this pattern by appropriate translation or rotation of the molecules (indeed, the other sides of these roughly planar molecules could possibly fit the pattern in some cases).

One of the most interesting studies of analog inhibition was reported by Halvorson and Ellias (1958) for the α-glucosidase of *Saccharomyces italicus*, the data from which are given in the accompanying tabulation.

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
α-Phenylglucopyranoside*	0.3	5.00
Glucose	1.2	4.15
α-Butylglucopyranoside	1.9	3.86
α-Ethylthioglucopyranoside	4	3.40
Turanose*	9.5	2.87
Isomaltose	11	2.78
Xylose	12	2.72
Sucrose*	23	2.32
β-Methylmaltoside*	37	2.04
α-Ethylglucopyranoside	45	1.91
Maltose*	81	1.55
α-Ethylthioglucofuranoside	86	1.51
Arabinose	110	1.36
α-Methylglucopyranoside	400	0.56

Several of the substances (marked by *) are also substrates; maltose, in fact, is probably the natural substrate. Inversion of hydroxyl groups on C-2 and C-4, and substitution or oxidation at C-6, abolish the affinity. Increasing the size of the aglycone groups increases the affinity. The rather potent inhibition by glucose is surprising and indicates that the alkyl aglycone groups actually reduce the affinity. This might mean that the C-1 hydroxyl group can be involved in the binding; a small substituent prevents this but the binding increases as the alkyl group is lengthened.

Inhibition of β-Glucuronidases by D-Glucaro-1,4-lactone and Related Compounds

These widely distributed enzymes catalyze the hydrolysis of β-glucuronides occurring naturally in plants and animals, and in addition may play a role in mucopolysaccharide metabolism. They are not involved in the glucuronide syntheses of detoxification, these reactions being catalyzed by glucuronyl transferases. There is evidence that β-glucuronidases are related in some manner to growth and the activities of certain tissues, and it was

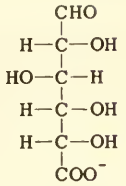
to establish their role in metabolism that Karunairatnam and Levvy (1949) searched for specific inhibitors. The most potent of the analogs tested is glucarate ($K_i = 0.06$ mM, and $K_m = 3.5$ for phenyl-β-glucuronide) but subsequent studies in different laboratories showed great variability in activity. The problem was solved by Levvy (1952), who found that the active inhibitor is glucaro-1,4-lactone,* which is formed from glucarate and occurs to varying extents in different preparations. The tabulation shows

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
Glucaro-1,4-lactone	0.00054	8.87
3-Methylglucaro-1,4-lactone	0.13	5.50
Glucarate	0.17	5.33
Glucaro-3,6-lactone	0.48	4.70
Glucuronate	1.6	3.96
Galacturonate	6.0	3.14
Galactarate (mucate)	6.0	3.14

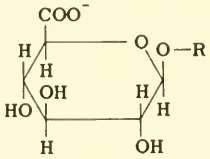
the inhibitor constants obtained on mouse liver β-glucuronidase hydrolyzing phenolphthalein-β-glucuronide, and the particular potency of the glucaro-1,4-lactone is evident.† No inhibition at 1 mM is observed with mannarate, mannaro-1,4-3,6-dilactone, 4-methylglucuronate, and ascorbate, or at 10 mM with 3-methylglucarate, glucurone, 3-methylglucuronate, mannuronate, mannurone, and 2-keto-L-gulonate. Heat and acid treatments of glucarate and galactarate increase the inhibitory activity greatly and it is possible that the dicarboxylates are completely inactive. The inhibition by glucaro-1,4-lactone is competitive and the high potency probably due to the structural similarity between the inhibitor and the β-glucofuranuronide form of the substrate. The hydroxyl configuration in the furan ring is very important since the glucaro-3,6-lactone is bound much less tightly to the enzyme, and the 3-OH must also be involved since methylation reduces the binding by over 3 kcal/mole. These rather large energy differences argue

* This substance has also been called saccharo-1,4-lactone, 1,4-saccharolactone, glucosaccharo-1,4-lactone, and other names. The generic name for the dicarboxylic acids derived from sugars is saccharic acid and that specifically from glucose is glucosaccharic acid. However, it seems that glucaric acid is used most commonly today and this terminology is more consistent with the naming of the monocarboxylic acids, so that the lactones will here be named accordingly.

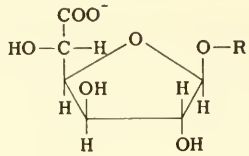
† The figures in this and other reports for inhibitors such as glucarate, galactarate, glucuronate, and related acids may reflect to varying degrees the presence of lactones, since, due to the high potency of the lactones, only small amounts need be present.



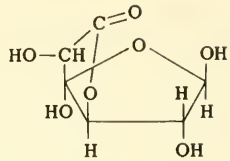
β-Glucuronate
(free acid)



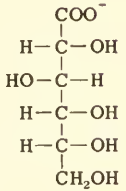
β-R-glucopyran-
uronide



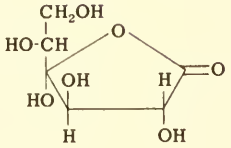
β-R-glucofuran-
uronide



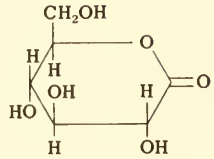
β-Glucofuranurono-
3, 6-lactone
(glucurone)



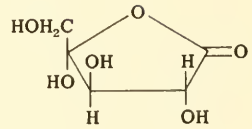
Gluconate
(free acid)



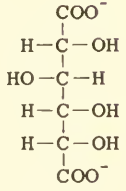
Glucono-1, 4-
lactone



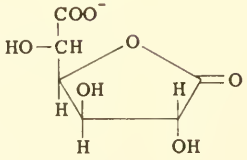
Glucono-1, 5-
lactone



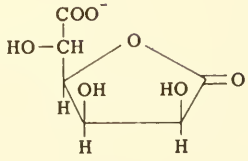
Xylono-1, 4-
lactone



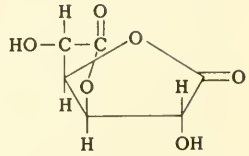
Glucarate
(free acid)



Glucaro-1, 4-
lactone



Glucaro-3, 6-
lactone



Glucaro-1, 4-3, 6-
dilactone

against van der Waals' forces being a major factor in the hydroxyl interactions, and suggest hydrogen bonding. The terminal carboxylate group also participates, since glucaro-3,6-lactone is much more inhibitory than glucurone. (See formulas on page 425).

Some inhibitor constants for various β-glucuronidases are shown in Table 2-23. Not enough β-glucuronidases have been tested with glucaro-1,4-lactone to determine the variations in susceptibility, but from the limited data it appears that the animal enzymes are quite sensitive whereas the plant enzyme baicalinase is much less readily inhibited. It is clear that glucaro-1,4-lactone is one of the most potent inhibitors known and that none of the other analogs so far examined on β-glucuronidase is comparable, although galactaro-1,4-lactone is undoubtedly a very effective inhibitor. It is interesting that the β-galacturonidases of limpet and preputial gland are approximately as susceptible to these lactones as are the β-glucuronidases (Marsh and Levvy, 1958).

Limpet α-glucuronidase shows quite a different pattern of inhibition (see accompanying tabulation for inhibitions at 5 mM with phenyl-α-glucur-

Inhibitor	% Inhibition	Relative $-\Delta F$ of binding (kcal/mole)
Glucuronate	68	3.73
Mannuronate	43	3.09
Galacturonate	28	2.68
Glucurone	9	1.84
Xylono-1,4-lactone	22	2.48
Glucono-1,5-lactone	7	1.67
Arabono-1,4-lactone	4	1.31
Glucono-1,4-lactone	3	1.13
α-Glucuronate-1-phosphate	55	3.39
β-Glucuronate-1-phosphate	22	2.48
Menthyl-α-glucuronide	55	3.39
Menthyl-β-glucuronide	6	1.57
Borneol-α-glucuronide	41	3.04
Veratroyl-β-glucuronide	9	1.84

onide at 1 mM with relative binding energies calculated on the basis of competitive inhibition). α-Glucuronides are more inhibitory than β-glucuronides, as expected, but no particularly potent inhibitors were found (K_i for glucuronate is 0.54 mM and for galacturonate 4.7 mM). The following do not inhibit: mannurone, glucaro-1,4-lactone, mannono-1,4-lactone, and galactarate. The difference between the α and β enzymes with respect to glucaro-1,4-lactone is especially striking.

TABLE 2-23

COMPETITIVE INHIBITORS OF VARIOUS β-GLUCORONIDASES

Source	Substrate	Inhibitor	K_i (mM)	Reference
Beef spleen	*p*-Chlorophenyl-β-glucuronide	Glucarate[a]	0.88	Spencer and Williams (1951)
		Glucuronate	11.1	
		Galactarate[a]	36.2	
		Glucurone	(ni)[b]	
	Phenyl-β-glucuronide (pH 3.4 enzyme)	Glucarate[a]	0.057	Mills *et al.* (1953)
		Galactarate[a]	0.41	
		Galacturonate	(ni)	
Mouse liver	Phenolphthalein-β-glucuronide	Veratroyl-β-glucuronide	0.035	Levvy and Worgan (1955)
		α-Ethylhexanoyl-β-glucuronide	0.22	
		α-Ethylbutyryl-β-glucuronide	0.79	
Scutellaria baicalensis	Phenolphthalein-β-glucuronide	Glucaro-1,4-lactone	0.19	Levvy (1954)
		Veratroyl-β-glucuronide	6.6	
		Methyl-β-glucuronide	14	
		Glucaro-3,6-lactone	(ni)	
		Menthyl-α-glucuronide	(ni)	
		Methyl-α-glucuronide	(ni)	
		Methyl-β-glucuronide	(ni)	
Patella vulgata	Phenolphthalein-β-glucuronide	Glucaro-1,4-lactone	0.000089	Levvy *et al.* (1957)
		Galactarate (boiled)[a]	0.0098	
Rat preputial gland	Phenolphthalein-β-glucuronide	Glucarate (boiled)[a]	0.00031	Marsh and Levvy (1958)
		Galactarate (boiled)[a]	0.022	
		Glucuronate	1.5	
		Galacturonate	4.3	

[a] Preparations of glucarate and galactarate contain varying amounts of lactones and thus K_i's are not necessarily accurate; in boiled preparations perhaps one third is in the lactone form. [b] The designation (ni) indicates no inhibition noted.

It was previously stated that β-glucuronidases do not seem to participate in glucuronide synthesis in tissues and the evidence is mainly the lack of inhibition by glucaro-1,4-lactone usually observed (Levvy and Marsh, 1960). For example, Lathe and Walker (1958) found no effects of 2 m*M* glucaro-1,4-lactone on the formation of *o*-aminophenylglucuronide or bilirubin-glucuronide in liver suspensions. However, Fishman and Green (1957) reported that glucarate potently inhibits glucuronyl transfer (50% inhibition by 0.05 m*M*), and Sie and Fishman (1954) found glucarate and glucurone to inhibit glucuronide synthesis in liver slices. It is not definitely known if these inhibitions relate to a β-glucuronidase or a transferase, but the question of the role of the β-glucuronidases in glucuronide synthesis should probably be left open.

The urinary bladder cancer that occurs in people employed in certain industries may be related to the formation of glucuronides of aromatic amines in the body and their subsequent hydrolysis by urinary β-glucuronidase. The oral administration of inhibitory analogs to patients is a possible approach to the prevention of such cancers. Boyland *et al.* (1957) found that the urinary enzyme can be inhibited by administration of gluconate and glucarate at a dose of 10 g/day, but the most potent inhibition is produced by glucaro-1,4-lactone, 73% inhibition occurring from 1.5–2 g/day and 90% inhibition from 4 g/day. Nevertheless, these inhibitions are less than expected and the urinary pH was reported later to be an important factor, the inhibition decreasing as the pH rises above 6 (Boyland *et al.*, 1959). Inhibition of liver β-glucuronidase in mice by the administration of glucaro-1,4-lactone at doses from 50 to 800 mg/kg (17% to 76%) was found by Akamatsu *et al.* (1961). The maximal inhibition occurs at 30–60 min and after 2–4 hr most of the activity has returned. Similar results were found in rat liver and kidney. Since β-glucuronidase and β-*N*-acetylglucosaminidase hydrolyze products from chondroitin and hyaluronate and may participate in the metabolism of connective tissue mucoproteins, and since certain tumors have relatively large amounts of these enzymes, Carr (1963) administered the two inhibitors — glucaro-1,4-lactone and 2-acetamido-2-deoxygluconolactone — to mice bearing Tumor 2146. At 150 mg/kg these substances are nontoxic and cause regression of the tumors. It was suggested that the inhibitors prevent the penetration of the tumor cells through the intercellular cement substance, but this is admittedly only a tenuous hypothesis. Whatever the explanation, it represents an interesting approach to tumor chemotherapy.

Inhibition of Various Glycosidases by Glucono- and Glucaro Lactones

The inhibition of the β-glucuronidases by the saccharo-1,4-lactones suggested that the β-glycosidases might be inhibited by the aldonolactones, and this was found to be so by Conchie (1953, 1954). A sheep rumen β-

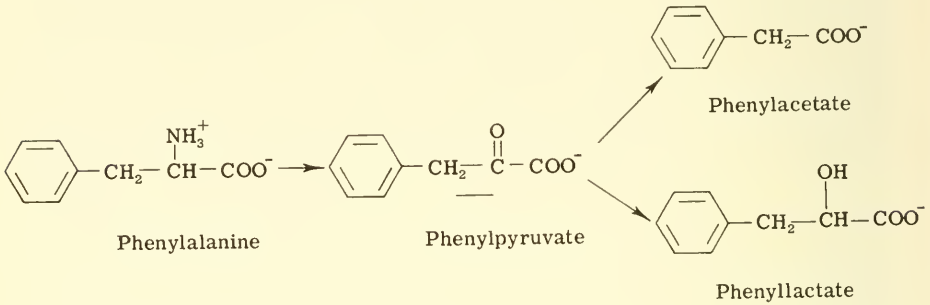
glucosidase, possibly involved in the digestion of cellulose, is strongly inhibited by glucono-1,4-lactone ($K_i = 0.094$ mM) and glucono-1,5-lactone ($K_i = 0.091$ mM) in a competitive manner, the affinity of the enzyme for these analogs being about 10 times that for the substrate *o*-nitrophenyl-β-glucoside, whereas gluconate itself has no action. Ox liver β-galactosidase is inhibited much more potently by the galactono- and fucono-1,5-lactones than the corresponding 1,4-lactones, and this can be readily explained on the basis of the relationship to substrate configuration (Levvy *et al.*, 1962). A remarkable degree of specificity is exhibited by the α and β glycosidases, inhibition usually resulting only from the corresponding aldonolactone in a number of enzymes from different sources (Conchie and Levvy, 1957). For example, limpet α-mannosidase is inhibited markedly by mannono-1,4-lactone but not by the glucono-, galactono-, arabono-, or xylonolactones. On the other hand, galactono-1,4-lactone is specific for β-galactosidase (Conchie and Hay, 1959). Cellulytic rumen enzymes are inhibited to varying degrees by glucono-1,4-lactone, depending on the substrate chain length; hydrolysis of cellobiose is inhibited 99%, of cellotriose 90%, and of cellotetraose 75% by 0.5 mM (Festenstein, 1959). Glucono-1,4-lactone also inhibits a yeast debranching isoamylase (Gunja *et al.*, 1961) and a mammalian thioglycosidase (Goodman *et al.*, 1959), so that this type of inhibition appears to be widespread.

Very potent and specific inhibitions are exerted on N-acetyl-β-glucosaminidase and N-acetyl-β-galactosaminidase by the corresponding lactones (Marsh and Levvy, 1957; Findlay *et al.*, 1958). The former enzyme from epididymis is inhibited by N-acetylglucosaminolactone competitively, with $K_i = 0.000072$ mM, but the limpet enzyme is less sensitive, the K_i being 0.027 mM. The N-acetyl-α-glucosaminidase, on the other hand, is inhibited much less. The acetyl group is essential for the inhibition and cannot be replaced by other acyl groups. Certainly the use of the corresponding lactones for specific and potent inhibition of the glycosidases has been one of the most successful endeavors in the application of analogs.

PYRUVATE METABOLISM

The usefulness of a direct and specific inhibitor of pyruvate utilization is obvious and the natural occurrence of certain pyruvate analogs makes this field of inhibitor study an important one, but relatively little work has been done. Fluoropyruvate will be taken up with other fluorinated compounds, such as fluoroacetate, in a separate chapter. Phenylpyruvate is the best studied of the other pyruvate analogs. It is readily formed from phenylalanine and is usually decarboxylated to phenylacetate, although some may be reduced to phenyllactate. In phenylketonuria (phenylpyruvic oligophrenia), the accumulation of phenylpyruvate could be responsible for

some of the disturbances by interfering with pyruvate metabolism, but no work has been done on the metabolic changes in tissues resulting from such levels of the analog. Hydroxyphenylpyruvate is similarly formed from tyrosine.

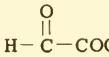
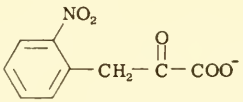
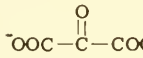
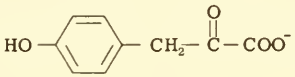
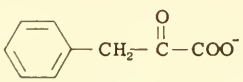
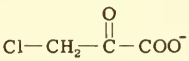
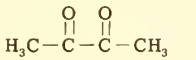
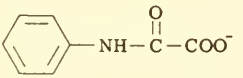
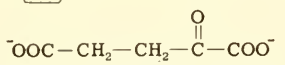


The formation of acetoin from pyruvate in *Streptococcus fecalis* is inhibited 13% by 1 m*M* and 75% by 10 m*M* phenylpyruvate, whereas the formation of acetoin from acetoacetate is unaffected by 10 m*M* (Dolin and Gunsalus, 1951). Pyruvate oxidase is inhibited to about the same degree. Several anaerobic pyruvate pathways are inhibited by phenylpyruvate in several bacteria and yeast, including the formation of acetoin, the phosphoroclastic reaction, and decarboxylation, whereas the oxidative metabolism of pyruvate is not so readily affected (Watt and Werkman, 1954). The concentrations of pyruvate and phenylpyruvate used (120 m*M*) are unfortunately too high to be physiologically significant, but further study on extracts of *Aerobacter aerogenes* showed competitive inhibition of acetoin formation with $K_m = 123$–197 m*M*, and $K_i = 0.59$–1.1 m*M*, so that in this case phenylpyruvate is bound to the enzyme much more tightly than pyruvate. The reduction of hydroxypyruvate to glycerate by glycerate dehydrogenase from spinach is inhibited by phenylpyruvate (5%), pyruvate (34%), and bromopyruvate (52%) at 10 m*M* (Holzer and Holldorf, 1957). These inhibitions are competitive but rather weak.

The most pertinent study with respect to blocking an important pyruvate pathway is that of Gale (1961) on yeast pyruvate decarboxylase, which reports the inhibitions given in Table 2-24. The following compounds are inactive: pyruvic ethyl ester, oxalacetate, propionate, phenyllactate, phenylalanine, acetamide, oxamate, and oxalate. One might infer that (1) the C=O group is necessary for inhibition (reduction or substitution abolishes activity), and (2) the COO^- group is necessary for strong inhibition (amides and esters inactive). The nature of the R group in R—CO—COO^- can vary quite widely and it is difficult to correlate structure with activity; for example, it is surprising that ketomalonate is bound so well and chloropyruvate relatively poorly, and that oxanilate is bound so very weakly.

TABLE 2-24

INHIBITION OF YEAST PYRUVATE DECARBOXYLASE BY ANALOGS[a]

Inhibitor	Structure	Concentration (m*M*)	% Inhibition	Relative activity
Glyoxylate		0.023 0.45	33 95	32
o-Nitrophenylpyruvate		0.09 0.14	61 70	17
Ketomalonate		0.23 0.34	71 82	12
p-Hydroxyphenylpyruvate		0.45 1.1	62 71	2.9
Phenylpyruvate		1.1 2.3	68 80	1.8
Chloropyruvate		0.45 2.3	27 73	1.0
2,3-Butanedione		9	56	0.14
Oxanilate		9	31	0.05
α-Ketoglutarate		27	24	0.012

[a] Pyruvate concentration 4.5 m*M* and preincubation with inhibitor 15 min. Relative inhibitory activity calculated from the formula $i/(1 - i)(I)$ and roughly would be inversely proportional to K_i for noncompetitive inhibition. (From Gale, 1961.)

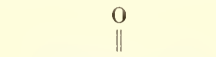
The inhibition by ketomalonate is more surprising when one considers that oxalate and oxalacetate are inactive. The kinetics of the inhibitions produced by the five most potent substances were studied in greater detail and a typical noncompetitive mechanism was established. However, presence of the substrate prevents development of the inhibitions, suggesting that the inhibitors combine at the substrate site. These results indicate an irreversible or pseudoirreversible type of inhibition, and it was indeed demonstrated that the inhibitions are all progressive with time. Phenylpyruvate is the only inhibitor whose effects are even partially reversible by

dialysis. It was considered that the true inhibitors are the aldehydes corresponding to the keto acids, and it was shown that CO_2 is evolved from phenylpyruvate and *p*-hydroxyphenylpyruvate but not from the other three inhibitors. Although this does not completely invalidate the aldehyde proposal, it makes it unlikely. On the other hand, it is possible that the tight complex is formed with some intermediate prior to decarboxylation.

Hydroxypyruvate is decarboxylated by yeast decarboxylase at a rate 1/76 that of pyruvate and strongly inhibits the pyruvate reaction (Holzer *et al.*, 1955 d). It was thought that hydroxypyruvate might exert some regulatory function on pyruvate metabolism in yeast. Formaldehyde and acetaldehyde inhibit pyruvate decarboxylase but the mechanism may not be competitive with substrate (Bauchop and Dawes, 1959). The oxidation of pyruvate in rat kidney slices is inhibited around 70% by 20 m*M* *meso*-tartrate, but not at all by D- and DL-tartrates (after correction for the inhibition of endogenous respiration) (Quastel and Scholefield, 1955). This inhibition is completely reversed by fumarate and malate. However, when mitochondria were examined it was found that there is little direct effect on pyruvate oxidation but a rather strong inhibition of α-ketoglutarate oxidation, which is progressive with time. In the presence of bicarbonate, pyruvate is oxidized and this is inhibited by *meso*-tartrate. It was assumed that the inhibition is upon the incorporation of CO_2, and it was stated that *meso*-tartrate is a specific inhibitor of pyruvate oxidation in slices of rat kidney cortex, a conclusion that would seem to be unjustified since so few systems were tested.

LACTATE METABOLISM

A specific inhibitor of lactate dehydrogenase would be useful in studying the effects of a block of glycolytic lactate formation and for determining the role of this enzyme in the functioning of tissues. One of the most interesting lactate dehydrogenase inhibitors is oxamate, first reported by Hakala *et al.* (1953), who stated that it is the most potent inhibitor of many lactate and pyruvate analogs examined. The inhibition is competitive with



Oxamate

respect to pyruvate,* noncompetitive with respect to lactate and NAD, and uncompetitive with respect to NADH (Papaconstantinou and Colo-

* Examination of the double reciprocal plot reveals that the situation is not purely competitive but mainly so.

wick, 1957; Novoa *et al.*, 1959). The substrate and inhibitor constants have been summarized by Papaconstantinou and Colowick (1961 a), as shown in the accompanying tabulation; if the K_m's are dissociation constants,

Source	K_m (pyruvate) (mM)	K_i (oxamate) (mM)
Beef heart	0.137	0.0374
Ascites carcinoma	0.212	0.0563
Rabbit muscle	0.302	0.10

oxamate is bound around 0.77 kcal/mole more tightly than pyruvate. The difficulty in interpreting K_i is that oxamate complexes with the apodehydrogenase, E, with E-NAD, and with E-NADH, the dissociation constants being different for each (Novoa *et al.*, 1959). The values for three different pH's are shown in the accompanying tabulation. Such ternary

Complex	K_i (mM) at:		
	pH 6.40	pH 8.45	pH 9.70
E-oxamate	0.10	0.78	20
E-NAD-oxamate	0.069	0.57	11
E-NADH-oxamate	0.026	0.17	1.6

complexes have recently been demonstrated by ultracentrifugal separation, 1 mole of oxamate being bound for each mole of NAD or NADH (Novoa and Schwert, 1961). The dissociation constant for the E-NADH-oxamate complex determined ultracentrifugally is 0.011 mM at pH 7.4. No evidence could be found for a complex with the apoenzyme alone; whether such a complex occurs or not, the inhibition is mainly due to the ternary complexes. The lactate dehydrogenase from human liver and heart is also inhibited by oxamate (Plummer and Wilkinson, 1963). The reduction of 2-ketobutyrate is inhibited more strongly than pyruvate reduction, presumably because 2-ketobutyrate is bound to the enzyme less tightly, but the reduction of succinic semialdehyde by a rat brain lactate dehydrogenase is inhibited to the same degree as the reduction of pyruvate, namely, 88% by 0.1 mM (Fishbein and Bessman, 1964). Not all lactate dehydrogenases are sensitive to oxamate; that from *L. mesenteroides* is inhibited only 50% by 7 mM (Papaconstantinou and Colowick, 1961 a). Oxamate specifically inhibits the L(+)-lactate dehydrogenase and does not affect D(−)-lactate dehydroge-

nase up to 8 m*M* (Dennis and Kaplan, 1960). The active site of the L(+)-lactate dehydrogenase was represented as containing a cationic group for electrostatic interaction with the COO^- group, and a hydrogen bonding group which interacts with the OH group of lactate and the NH_2 group of oxamate (it might also bond to the CO or enolized COH group of oxamate). The role of the amino group of oxamate on the binding is not known, nor can the importance of the enolic tautomer of oxamate be evaluated. The marked decrease in inhibition between pH 8.45 and 9.70 (see tabulation above) could indicate the deprotonation of an amino group, but it could be on the enzyme as well as the inhibitor.

The aerobic lactate production in human leucocytes is inhibited less than 25% by 10 m*M* oxamate, even in broken cell suspensions, suggesting that lactate dehydrogenase is not solely responsible for lactate formation, although the sensitivity of the leucocytic enzyme to oxamate has not been examined (McKinney *et al.*, 1955). The effects of oxamate on tumor cell metabolism and growth have been studied thoroughly by Papaconstantinou and Colowick (1957, 1961 a, b). Anaerobic glycolysis in ascites carcinoma cells is inhibited 50% by 8 m*M* oxamate and aerobic glycolysis is similarly depressed. This may indicate that oxamate does not penetrate into cells readily, since the K_i of 0.0563 m*M* for ascites cell lactate dehydrogenase would lead one to expect a greater effect at this concentration. The inhibition of anaerobic glycolysis decreases with time due to the accumulation of pyruvate, whereas no accumulation of pyruvate occurs aerobically, indicating that oxamate has little effect on pyruvate oxidase (an 18% inhibition of pyruvate oxidation by 10 m*M* oxamate was observed). A decrease in the inhibition anaerobically with time was also noted in *Tetrahymena pyriformis* (Warnock and van Eys, 1963). The growth of HeLa cells is completely inhibited by 40–80 m*M* oxamate and this is paralleled by decreases in glucose uptake and lactate formation, so that lactate dehydrogenase appears in some manner to be essential for the growth of these cells (assuming that oxamate acts specifically on lactate dehydrogenase). It was proposed that oxamate might be a useful inhibitor for selectively blocking glycolysis in mammalian cells. Mice can tolerate quite large doses (1 g/kg), however. A block of glycolysis, of course, refers here only to an inhibition of lactate formation, and the formation or utilization of pyruvate should not be significantly affected, so it would seem that aerobic glucose metabolism, at least with respect to the generation of energy, would be resistant to oxamate.

A number of disturbing observations have appeared which cast some doubt on the simple concept that oxamate specifically inhibits lactate dehydrogenase. Leached HeLa cells restored to normal conditions actively extrude Na^+ and accumulate K^+; these processes are inhibited 50% and 77%, respectively, by 38 m*M* oxamate (Wickson-Ginzburg and Solomon,

1963). Inasmuch as the conditions were aerobic here, it is difficult to understand how an inhibition of lactate dehydrogenase would account for the marked effects observed, unless there is an elevation of the NADH/NAD ratio due to the prevention of pyruvate reduction, this slowing the oxidation of glyceraldehyde-3-P and reducing the generation of ATP. This does not seem to occur in Ehrlich ascites carcinoma cells inasmuch as oxamate stimulates the formation of $C^{14}O_2$ from glucose-6-C^{14} without affecting that from glucose-1-C^{14} (Christensen and Wick, 1963). The stimulation is thus associated only with oxidation through the cycle and there is no effect on the fraction going through the pentose-P pathway. More pyruvate enters the cycle since less goes to lactate. The effects of oxamate on glucose utilization will depend for one thing on how rapidly pyruvate can be oxidized. The Crabtree effect is abolished almost completely by 40 m*M* oxamate, i.e., in the presence of glucose, oxamate stimulates the respiration in ascites cells (Papaconstantinou and Colowick, 1961 a). Simultaneously, glucose uptake is depressed 40% and lactate formation 70%. In view of the conclusion about the nature of the Crabtree effect in the section on 2-DG, it would seem that inhibition of lactate dehydrogenase could not be responsible for this effect of oxamate. It is possible that oxamate diverts more pyruvate into the cycle and hence stimulates respiration under these conditions, but this would not be a true abolition of the Crabtree effect. One would not expect glucose respiration to be depressed by oxamate in any case if the only action is on lactate dehydrogenase, but 31% respiratory depression is produced by 10 m*M* oxamate in guinea pig alveolar macrophages (Oren *et al.*, 1963). Despite the statements relative to the specificity of oxamate, it must be admitted that very few enzymes or metabolic pathways have been studied. It is quite likely that certain phases of amino acid metabolism might also be inhibited, since oxamate could be considered as an amino acid analog. It will also be noted that all the effects discussed in this paragraph were produced by oxamate at the high concentration of 10 m*M* or above.

Oxalate often inhibits lactate-metabolizing enzymes as potently as does oxamate and similarly forms ternary complexes with lactate dehydrogenase and NAD. However, it differs from oxamate in being competitive with lactate instead of pyruvate; this has been shown on beef heart lactate dehydrogenase ($K_i = 0.015$ m*M* at pH 6.7) (Novoa *et al.*, 1959), yeast D-lactate dehydrogenase ($K_i = 0.007$) (Labeyrie and Stachiewicz, 1961), yeast D-lactate cytochrome c reductase ($K_i = 0.0016$ m*M*) (Nygaard, 1961 b), and yeast D-hydroxy acid dehydrogenase ($K_i = 0.0025$ m*M*) (Boeri *et al.*, 1960), although the inhibition seems to be uncompetitive on the lactate dehydrogenase of *Propionibacterium pentosaceum* (Molinari and Lara, 1960). The complex kinetics have been treated in detail by Novoa *et al.* (1959), but it is still rather puzzling that oxalate inhibits all these enzymes so

potently and competes with lactate rather than pyruvate. Tartronate and malonate also inhibit competitively with lactate and noncompetitively with respect to pyruvate, although by no means as strongly as oxalate, and Ottolenghi and Denstedt (1958) concluded that pyruvate and lactate react with different sites on the enzyme surface, but this is not necessary, as Novoa *et al.* (1959) have shown, since the configurations of the active sites on E-NAD and E-NADH are different. The five lactate dehydrogenase (LD) isoenzymes from human tissues are inhibited to different degrees by oxalate at 0.02 m*M* (see accompanying tabulation) (Emerson *et al.*, 1964).

Isoenzyme	% Inhibition
LD_1	70
LD_2	64
LD_3	56
LD_4	46
LD_5	32

There are A and B monomers, the B monomer being more sensitive to oxalate. LD_1 is a pure B tetramer, LD_5 is a pure A tetramer, and the others are intermediate in the proportion of A to B. This is an illuminating example of how enzymes may respond differently to inhibitors by reason of varied composition.

There are several interesting studies on lactate dehydrogenases from which deductions on the nature of the active site and certain interaction energies may be derived. Dikstein (1959) found that yeast lactate dehydrogenase is not inhibited potently by monocarboxylates: the concentrations for 50% inhibition are 6000 m*M* for formate, 2300 m*M* for acetate, 800 m*M* for propionate, 300 m*M* for butyrate, 120 m*M* for valerate, and 10 m*M* for caprylate. By plotting log $(I)_{50}$ against the number of carbon atoms in the aliphatic chains he obtained a straight line, from the slope of which it was possible to calculate that the transfer of a methylene group from the solvent to the enzyme surface involves an over-all energy change of 0.5 kcal/mole. It was assumed that the interaction energy between the COO^- group and an enzyme cationic group could be calculated from the intercept of this line, but it is doubtful that at the high concentrations used the residual energy can be attributed with certainty to ion-ion interactions, since nonspecific effects cannot be excluded.

The D-α-hydroxy acid dehydrogenase of yeast is competitively inhibited by several dicarboxylates (see accompanying tabulation) (Cremona, 1964). Monocarboxylates inhibit much more weakly and the inhibitions are usually not purely competitive. Oxalate is bound approximately 3.7 kcal/mole more

tightly than malonate, suggesting that there are two cationic groups quite close on the enzyme.

Inhibitor	K_i (mM)
Oxalate	0.0025
Tartronate	0.84
Malonate	0.95
L-Malate	1.05
α-Ketoglutarate	1.4

The D-lactate dehydrogenase of yeast studied by Boeri *et al.* (1960) is inhibited potently by oxalate ($K_i = 0.0025$ mM), moderately by malonate ($K_i = 0.9$ mM), and not at all by 16 mM succinate or fumarate, indicating the importance of the position of the second COO^- group. The enzyme is inactivated gradually by EDTA and reactivated with Zn^{++}. Although this does not prove that Zn^{++} is the normal metal ion involved, it points to the possibility of chelation between α-hydroxy carboxylates, or dicarboxylates, with an enzyme-bound metal ion. The configuration around the α-carbon atom is important since L-lactate binds very weakly to the enzyme ($K_i =$ $= 62$ mM).

The lactate cytochrome c reductases of yeast are flavoproteins and are competitively inhibited by a variety of lactate analogs (Nygaard, 1961 a, b, c). Fatty acid inhibitions lead to the calculation of 0.37 kcal/mole for the interaction of methylene groups with the enzyme and 2.80 kcal/mole for the COO^- group in the case of the D-lactate cytochrome c reductase, and of 0.37 kcal/mole and 0.88 kcal/mole, respectively, for the L-lactate cytochrome c reductase. Neither enzyme is inhibited by dicarboxylates, with the exception of oxalate, and from the differences in inhibition patterns it is probably safe to assume that the active sites of the two enzymes are quite different.

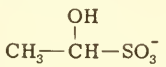
Some analog inhibitors of lactate dehydrogenases need only be listed for reference: pyruvate (Green and Brosteaux, 1936; Das, 1937 b; Neilands, 1952; Labeyrie and Stachiewicz, 1961), bromopyruvate, chloropyruvate, hydroxypyruvate (Busch and Nair, 1957), phenylpyruvate (Dikstein, 1959), α-ketoglutarate (Boeri *et al.*, 1960), malate (Boeri *et al.*, 1960; Busch and Nair, 1957), tartronate (Green and Brosteaux, 1936; Lehmann, 1938), tartrate (Labeyrie and Stachiewicz, 1961), mandelate (Lehmann, 1938), α, γ-diketovalerate (Meister, 1950), benzenesulfonate (Baptist and Vestling, 1957), mercaptoacetate, mercaptosuccinate, α-mercaptopropionate, α-mercaptobutyrate, and α-mercaptovalerate (Chaffee and Bartlett, 1960). The benzenesulfonates, where the COO^- group is replaced by a SO_3^- group,

and the mercapto fatty acids, where the α-OH is replaced by an α-SH group, are particularly interesting and deserve further study to determine their specificity on lactate metabolism.

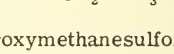
The oxidation of glycolate is catalyzed by glycolate oxidase and the

$$\underset{\text{Glyoxylate}}{CHO\text{-}CHO^-} + NADH + H^+ \rightleftarrows \underset{\text{Glycolate}}{CH_2OH\text{-}COO^-} + NAD^+$$

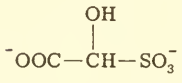
reverse reaction by glyoxylate reductase, both enzymes being found in plants. This reaction is similar to the pyruvate $\leftrightarrows$ lactate interconversion and, indeed, L-lactate is slowly oxidized by the oxidase. Since these enzymes bear some relationship to those involved in lactate metabolism due to this similarity, it is not inappropriate to discuss them at this point, particularly as Zelitch at the Connecticut Agricultural Experiment Station has reported some very interesting inhibitions by analogs. Glyoxylate reductase is apparently not especially susceptible to analogs: the following inhibitions were observed with 16.5 mM glyoxylate and 10 mM analogs — phenylglyoxylate 21%, oxalacetate 22%, oxamate 31%, and pyruvate 56% (Zelitch, 1955). Glycolate oxidase, on the other hand, is well inhibited by α-hydroxysulfonates. Competitive inhibition was found with hydroxymethanesulfonate ($K_i = 0.0018$ mM), α-hydroxyethanesulfonate ($K_i = 0.0023$ mM), and sulfoglycolate ($K_i = 0.0021$ mM); since $K_m = 0.38$ mM, these analogs are reasonably potent (Zelitch, 1957). Rabbit muscle lactate dehydrogenase is inhibited comparably, D-glycerate dehydrogenase less strongly, and malate dehydrogenase not at all, so that some specificity toward enzymes oxidizing α-hydroxy acids is evident. The inhibition of lactate oxidation by the glycolate oxidase is inhibited very strongly because lactate is bound less tightly to the enzyme. Another competitive inhibitor of comparable potency is α-hydroxy-2-pyridinemethanesulfonate (Zelitch, 1959) which has been used in most of the *in vivo* work apparently because it is more effective in cells, although sulfoglycolate would seem to act very similarly (Zelitch, 1958).



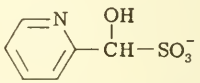
α-Hydroxyethanesulfonate



Hydroxymethanesulfonate



Sulfoglycolate



α-Hydroxy-2-pyridinemethanesulfonate

Normal tobacco leaves contain around 0.5–1 μmole/g wet weight glycolate and this level can be increased as much as 10-fold by placing them in solutions containing the α-hydroxysulfonates (Zelitch, 1959). Since glycolate is formed photosynthetically, marked accumulation occurs only in the light. The concentration of α-hydroxy-2-pyridinemethanesulfonate for maximal inhibition is near 10 mM, indicating that penetration into the cells is quite poor. At higher concentrations of inhibitor the glycolate level falls, due presumably to inhibition of glycolate formation; in fact, even at 10 mM there must be some inhibition since photosynthetic incorporation of $C^{14}O_2$ is inhibited about 33%. The pattern of C^{14} distribution is, however, more markedly altered; in controls, glycolate-C^{14} accounts for 5.5% of the labeling but in inhibited leaves it is almost 50%. This is a good example of a specific analog inhibitor useful in studying the importance of an intermediate in a complex metabolic pathway, and valuable information may result from more detailed studies on photosynthesis.

PHOSPHATASES

These enzymes are commonly inhibited by the products of the hydrolysis. We have already noted the inhibitions of phospho-L-histidinol phosphatase, *O*-phosphoserine phosphatase (page 270), and glucose-6-phosphatase (page 442) by dephosphorylated products, and there are other examples related to analog inhibition. Orthophosphate also frequently inhibits: The following may be cited as instances of well-marked inhibition of different types of enzyme — calf intestinal phosphatase (Schmidt and Thannhauser, 1943), mouse liver acid phosphatase (Macdonald, 1961), mouse liver pyrophosphatase (Rafter, 1958), calf brain carbamyl and acyl phosphatases (Grisolia *et al.*, 1958), sweet potato phosphatase (Ito *et al.*, 1955), and *E. coli* alkaline phosphatase (Garen and Levinthal, 1960). Another class of inhibitor comprises the phosphates that are substrates. Thus sweet potato phosphatase with phenyl phosphate as substrate is competitively inhibited by β-glycerophosphate (K_i = 2 mM), pyrophosphate (K_i = = 0.33 mM), metaphosphate (K_i = 3.2 mM), and ATP (K_i = 0.67 mM), all of which are also substrates (Ito *et al.*, 1955). Likewise the *E. coli* phosphatase with *p*-nitrophenyl phosphate as substrate is competitively inhibited by uridine phosphate (K_i = 0.044 mM), guanosine phosphate (K_i = 0.046 mM), β-glycerophosphate (K_i = 0.05 mM), glucose-1-phosphate (K_i = 0.063 mM), and adenosine-5′-phosphate (K_i = 0.093 mM) (Garen and Levinthal, 1960).

More interesting are the inhibitions by various anions that may be considered as analogs of either phosphate or the substrate phosphates. Thus arsenate (Garen and Levinthal, 1960; Ito *et al.*, 1955; Macdonald, 1961), borate (Ito *et al.*, 1955), and silicate (Umemura *et al.*, 1961) inhibit various

phosphatases about as potently as phosphate and probably combine with the enzymes in a similar manner. Certain carboxylates (oxalate, malonate, malate, citrate, glucarate, gluconate, lactate, and others) have been found to be inhibitory, but most of these are not remarkably effective, probably binding to enzyme cationic groups to various degrees or chelating with metal ions. However, (+)-tartrate* is a very potent inhibitor of certain phosphatases, as first shown by Abul-Fadl and King (1949) and confirmed by Anagnostopoulos (1953), and is so much more active than other anions that the mechanism has been investigated in several excellent studies.

The phylogenetic relationships of (+)-tartrate inhibition and the variable susceptibilities of phosphatases from different tissues are quite interesting. Abul-Fadl and King (1949) found that although prostatic acid phosphatase is very sensitive, no effects are exerted on the acid phosphatases of erythrocytes or plasma, and Anagnostopoulos (1953) noted no inhibition with the phosphatases of mustard, wheat germ, or *Aspergillus*. Kilsheimer and Axelrod (1958) investigated the effects of (+)-tartrate on the phosphatases from many sources and found that at 20 mM the inhibitions vary from 0 to 93%. They concluded that animal phosphatases are more susceptible than plant phosphatases, and that bacterial phosphatases are generally resistant. It was suggested that (+)-tartrate may be of taxonomic use in those more primitive organisms where it is difficult to decide whether they are plants or animals.

The earliest work demonstrated that the inhibition is stereospecific, neither (−)-tartrate nor *meso*-tartrate exerting appreciable effects on prostatic phosphatase, and this has been confirmed in all the recent studies. In the series of phosphatases tested by Kilsheimer and Axelrod (1958) it was observed that very few of the enzymes are affected by (−)-tartrate and these are inhibited only slightly. The K_i's were determined by London *et al.* (1958) as 0.13 mM for (+)-tartrate and 93 mM for (−)-tartrate on prostatic acid phosphatase. It is strange that they found *meso*-tartrate to be an effective inhibitor ($K_i = 0.4$ mM) in contrast to all other work. The acid phosphatase from *Neurospora crassa* is inhibited completely by 12 mM (+)-tartrate but is unaffected by 50 mM (−)-tartrate or *meso*-tartrate (Kuo and Blumenthal, 1961). In all cases the inhibition by (+)-tartrate is competitive with respect to substrate. The inhibition is thus more marked with β-glycerophosphate as the substrate than with phenyl phosphate or *p*-nitrophenyl phosphate, since the former substrate is bound less tightly (Nigam *at al.*, 1959; Kuo and Blumenthal, 1961).

Tartrate and related inhibitors have been used to map the active site of prostatic phosphatase (London *et al.*, 1958). The K_i's and estimated

* There has been confusion in the nomenclature of the tartrates and the dextrorotatory (+)-tartrate has been designated as L- or D-, depending on the system used. To avoid ambiguity I shall indicate the isomers by the signs of their rotation.

relative binding energies are shown in Table 2-25. It was assumed that (+)-tartrate is bound to the enzyme at four points; the two carboxylate groups interact with two enzyme cationic groups and the two hydroxyl groups

TABLE 2-25

COMPETITIVE ANIONIC INHIBITORS OF PROSTATIC PHOSPHATASE[a]

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
Fluoride dimer (HF_2^-)	<0.1	>5.68
(+)-Tartrate	0.13	5.51
DL-Glycerate	0.2	5.25
meso-Tartrate	0.4	4.82
Arsenate	1	4.25
D-Malate	1.8[b]	3.90
Sulfamate	8	2.98
Nitrate	16	2.55
D-Alanine	27	2.23
L-Leucine	30	2.16
Diphenylphosphate	32	2.12
D-Lactate	50	1.85
L-Serine	50	1.85
L-Glutamate	60	1.74
L-Aspartate	64	1.69
(−)-Tartrate	93	1.47
L-Lactate	110	1.36
L-Malate	280	0.79
L-Alanine	470	0.47
Glycine	470	0.47

[a] The substrate is β-glycerophosphate (K_m = 16 mM). Experiments with amino acids at pH 7.2 and with the rest at pH 5. (From London *et al.*, 1958.)

[b] K_i for D-malate estimated from value for DL-malate since L-malate is a weak inhibitor.

form hydrogen bonds with the enzyme (Fig. 2-14). On the basis of this model it is possible to explain most of the variations in inhibitor binding. (−)-Tartrate can make contact at only two points while *meso*-tartrate can make a three-point attachment in two ways. Only D-malate can attach at three points like (+)-tartrate but the second hydroxyl group is missing. The most important requirement for binding is a negative group separated from the nucleophilic group by around 2.9 Å. (+)-Tartrate has two such units. In the substrates the oxygen atom of phosphate is the nucleophilic atom. The amino acids are rather poor inhibitors, probably because at

pH 7.2 most of the amino groups are protonated and interfere with the binding. HF_2^- may bind across two attachment points and its potency may be related to the strong hydrogen bonds formed by fluorine. The nitrate

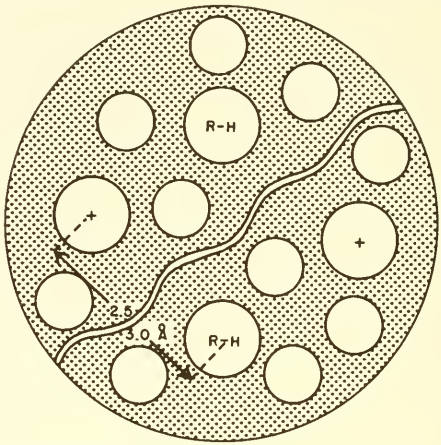


FIG. 2-14. Scheme for the active site of prostatic acid phosphatase showing two cationic groups and two hydrogen-bonding groups separated by a seam in the enzyme. The unmarked circles represent various enzyme groups and the stippled area between them a lipophilic region. (Modified from London *et al.*, 1958.)

ion also can bridge two of the attachment points, one oxygen atom being negatively charged and the other nucleophilic.

Some of the results of Nigam *et al.* (1959), shown in the accompanying tabulation, differ from those of London *et al.* (1958). These inhibitions were

Inhibitor (10 m*M*)	% Inhibition
(+)-Tartrate	100
Glucarate	91
Pyruvate	80
Oxalate	72
Malonate	66
Maleate	45
Glutamate	42
Glucuronate	40
Lactate	0

observed with 3 mM β-glycerophosphate as substrate. The potency of (+)-tartrate is not evident in the tabulation since 50% inhibition is produced by 0.07 mM. The failure of lactate to inhibit is surprising, especially as pyruvate is fairly potent. The susceptibility to glucarate observed here has not been observed with *Neurospora* phosphatase, 12.5 mM having no effect (Kuo and Blumenthal, 1961), and Jeffree (1957) found only a moderate inhibition ($K_i = 10$ mM) on prostatic phosphatase, it being definitely less potent than oxalate in contrast to the results above. Whether the lactone plays a role in this inhibition is not known. Two anionic polymers have been found to be more potent inhibitors than (+)-tartrate: polyxenyl phosphate inhibits the *Neurospora* phosphatase completely at 0.16 mM (Kuo and Blumenthal, 1961), and alginate (556 residues) inhibits the prostatic enzyme with a K_i of 0.0054 mM (Jeffree, 1957). These polymer inhibitions are only partially competitive.

SULFATASES

Arylsulfatases of type II (liver enzymes A and B and molluscan enzymes) are generally inhibited by sulfate and phosphate, but type I arylsulfatases (liver enzyme C and bacterial enzymes) are resistant. The product inhibition by sulfate was first reported by Tanaka (1938) and shown to be competitive with the substrate nitrocatechol sulfate by Roy (1953). The susceptibilities of ox liver arylsulfatases to sulfate vary markedly: The K_i for sulfatase A is 0.75 mM, for sulfatase B is 70 mM, and sulfatase C is not affected (Roy, 1953, 1954 b, 1956). The sulfatase A is inhibited also by several organic sulfates, although not very potently (see accompanying tabulation). The following do not inhibit at 25–50 mM: methyl sulfate, glucose-3-sulfate, glucose-6-sulfate, and phenyl sulfite. The fact that methyl sulfate does not inhibit and the affinity for the enzyme increases with size of the ring system esterified might indicate that the sulfate group is not

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
Sulfite	0.002	8.07
Phosphate	<0.21	>5.21
Sulfate	0.75	4.42
2-Phenanthryl sulfate	4	3.40
Phenyl phosphate	5.3	3.23
2-Naphthyl sulfate	30	2.15
1-Naphthyl sulfate	40	1.98
m-Tolyl sulfate	100	1.42
Phenyl sulfate	300	0.74
Benzyl sulfate	300	0.74

involved in the binding of these substances. The inhibition by these organic sulfates was stated to be noncompetitive but since some are slowly hydrolyzed substrates, the inhibition may be mixed. Phosphate seems generally to be a more potent inhibitor than sulfate; this has been observed on the arylsulfatases of rabbit liver (Maengwyn-Davies and Friedenwald, 1954), ox liver (Webb and Morrow, 1959), *Helix pomatia* (Dodgson and Powell, 1959), *Charonia lampas* (Takahashi, 1960 a), and beef and rabbit cornea (Wortman, 1962). The inhibitions, where examined, are competitive. Weak inhibitions by xylenesulfonate (Maengwyn-Davies and Friedenwald, 1954) and benzenesulfonate (Dodgson *et al.*, 1955; Dodgson and Powell, 1959) have been observed.

The potent inhibition by sulfite is interesting but the mechanism is not yet understood, although it would appear to be competitive (Roy, 1953). For all the ox liver arylsulfatases, sulfite is bound 3–4 kcal/mole more tightly than is sulfate; indeed, sulfatase C, although resistant to sulfate, is inhibited 55% by 0.1 m*M* sulfite (Roy, 1956). Sulfite is also more inhibitory than sulfate on sulfatases from bacteria and molluscs (Dodgson *et al.*, 1955; Dodgson and Powell, 1959; Dodgson, 1959), although in most cases the concentrations used were too high to evaluate the potency readily.

Choline sulfatase of *Pseudomonas nitroreducences* is inhibited very strongly by sulfite (33% by 0.01 m*M* and 83% by 0.1 m*M* when choline sulfate is 10 m*M*), but is not affected or somewhat stimulated by sulfate and phosphate (Takebe, 1961). The steroid sulfatase and glucosulfatase of *Patella vulgata* are inhibited by sulfate and even more potently by phosphate (Roy, 1954 a). Since the sulfatases comprise so heterogeneous a group of enzymes and relatively few have been adequately studied, it is difficult to draw general conclusions or make valid correlations, but it is at least evident that analogs are occasionally effective inhibitors. It is also likely that phosphate must exert a regulatory action on sulfatase activity *in vivo*.

ADENOSINETRIPHOSPHATASES AND TRANSPHOSPHORYLASES

Enzymes hydrolyzing ATP or transferring its terminal phosphate to various acceptors are frequently inhibited by other nucleotides. Competitive product inhibition by ADP has been noted for ATPases from several sources; the inhibition is never marked, since ADP is usually bound somewhat less tightly than ATP to the enzyme, but is sufficient to slow progressively the rate of ATPase reactions. The ATP-P_i and ATP-ADP exchange reactions catalyzed by mitochondria and digitonin particles are also inhibited by ADP (Löw *et al.*, 1958; Cooper and Kulka, 1961). Some results with ADP and other related substances are shown in Table 2-26. The inhibitions however, depend on the pH and the concentrations of Ca^{++} and Mg^{++}, as

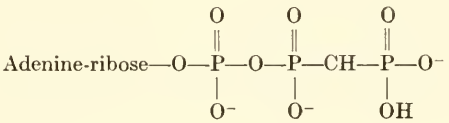
Table 2-26

Inhibition of ATPases by Analogs

Source	ATP concentration (mM)	Analog	Analog concentration (mM)	% Inhibition	Reference
Liver mitochondria	4	ADP	2	23	Kielley and Kielley (1953)
			4	42	
		IDP	2.2	0	
			4	16	
		ITP	4	13	
	15	AMP	15	22	Löw *et al.* (1958)
		ADP	15	45	
	3.5	ADP	3.5	50	Cooper and Kulka (1961)
Muscle myosin	1.07	ADP	0.4	7	Blum (1955)
			2.4	12	
	0.21	ADP	2.4	29	
Spinach chloroplasts	4	AMP	4	37	Wessels and Baltscheffsky (1960)
		ADP	4	29	
Brain	—	Adenine	5	23	Gore (1951)
		Adenosine	20	16	
		Guanine	5	2	

shown by Green and Mommaerts (1954). Addition of Ca^{++} decreases ADP binding at pH 6.4 and increases it at pH 9, whereas Mg^{++} has no effect at the lower pH but decreases affinity at the higher pH. The K_i for ADP is around 0.13 mM in the absence of Ca^{++} and Mg^{++}, but around 0.5 mM at pH 6.4 and 40 mM Ca^{++}. Kielley and Kielley (1953) had shown with liver mitochondrial ATPase that ADP does not alter the optimal Mg^{++} concentration for ATPase activity, indicating that the inhibition is not by the binding of Mg^{++}, but Nanninga (1958) reported that part of the inhibition of myosin ATPase by ADP is due to chelation of Ca^{++}. In the presence of excess Ca^{++} this chelation can be neglected and the true K_i for the enzyme-ADP complex is found to be 4.6 mM at pH 7.

An interesting phosphonic analog of ATP, adenylmethylenediphosphonate:



was found by Moos *et al.* (1960) to be unable to replace ATP in contracting glycerinated muscle and not to be hydrolyzed by myosin ATPase. However, some inhibition on ATPase is exerted (the affinities of the enzyme for ATP and its analog appear to be roughly the same), although this is not competitive. Mg^{++} is able to overcome the inhibition at a concentration lower than that of the analog, indicating that Mg^{++} is not simply complexing with and removing free analog. The mechanism of the inhibition was represented by the following reactions:

$$\begin{array}{ccc} Mg & + & E \rightarrow EMg \\ + & & + \\ I & & I \\ \downuparrows & & \downuparrows \\ MgI & & EI \end{array}$$

where E is low-activity enzyme and EMg is high-activity enzyme. The inhibition has a dual basis: (1) removal of Mg^{++}, thus decreasing the fraction of the enzyme in the high-activity form, and (2) reaction of the low-activity enzyme directly with the analog. This situation may be fairly common in inhibitions on enzymes with activating metal ions.

A few other nucleotidase inhibitions may be mentioned. ITPase is inhibited by IDP and ADP (Blum, 1955; Kielley and Kielley, 1953). Indeed, ADP inhibits ITPase more strongly than ATPase. The ITPase of fly muscle is strongly inhibited by ADP ($K_i = 0.0165$ mM) and much less readily by IDP ($K_i = 1.59$ mM), the inhibition being competitive at low but noncompetitive at higher concentrations (Sacktor and Cochran, 1957). GTPase is likewise inhibited but UTPase is unaffected by either ADP or IDP. In phage-infected *E. coli* the hydrolysis of deoxycytidine diphosphate (deoxyCDP) is inhibited by deoxyCMP and deoxyCTP, and the hydrolysis of deoxyCTP is inhibited by deoxyCMP and deoxyCDP, in both cases the deoxyCMP being relatively less active (Zimmerman and Kornberg, 1961).

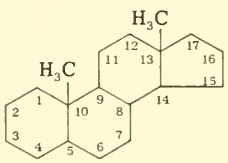
AMP-ATP transphosphorylase (myokinase) from rabbit muscle is inhibited by ADP ($K_i = 0.33$ mM) and this is competitive with respect to both AMP and ATP (Noda, 1958). The reverse reaction from 2ADP→AMP + ATP is inhibited by AMP ($K_i = 0.5$ mM) and ATP ($K_i = 0.32$ mM), the K_i's being the same as the K_m's for these substances (Callaghan and Weber, 1959). A much more effective analog is adenosine monosulfate ($K_i = 0.0186$ mM). Creatine kinase is inhibited competitively by ADP ($K_i = 0.27$ mM), AMP ($K_i = 7$ mM), adenosine ($K_i = 7$ mM), tripolyphosphate ($K_i = 8$ mM), orthophosphate ($K_i = 13$ mM), sulfate ($K_i = 6$ mM), and nitrate ($K_i = 22$ mM) (Noda *et al.*, 1960). The substrate here is $MgATP^{=}$ and it is possible that the most effective inhibitors form Mg complexes. Most of the anions inhibit the forward reaction competitively with respect to $MgATP^{=}$ and the reverse reaction competitively with re-

spect to creatine phosphate (Nihei *et al.*, 1961). However, ADP competes with $MgADP^-$ in the reverse reaction.

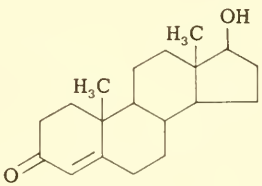
Since oxidative phosphorylation may in some ways be related to ATPase activity and transphosphorylations, it is not out of place to discuss the effects of various inorganic phosphorus compounds on this process. The phosphorylation in a rat liver mitochondrial suspension oxidizing fumarate, and with glucose and hexokinase to trap the phosphate, was studied by Thomson and Sato (1960) (Table 2-27). Some of the analogs investigated reduce the P : O ratio by depressing phosphate uptake more than oxygen uptake, but the only compound that can be considered as a true uncoupler is thiophosphate. In such a complex system a number of sites for inhibition are evident. It is known that anions can inhibit fumarase and it is possible that other enzymes attacking dicarboxylates might be inhibited. Hexokinase is inhibited at concentrations interfering with oxidative phosphorylation only by triphosphate. Some of these compounds might deplete Mg^{++} but it was shown that thiophosphate does not. It is not known if any of these substances can enter into the phosphorylative reaction but fail to form ATP. Further study of thiophosphate would seem warranted by these preliminary results.

HYDROXYSTEROID DEHYDROGENASES

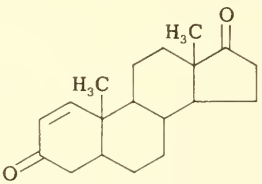
The β-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* catalyzes the oxidations of 3β- and 17β-hydroxysteroids to their respective ketones with NAD as acceptor. The oxidations of testosterone and 17β-estradiol are competitively inhibited by 17α-estradiol (Talalay and Dobson, 1953).



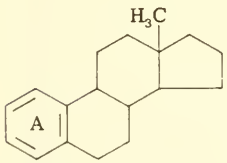
Androstane



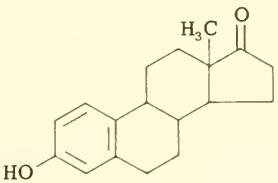
Testosterone



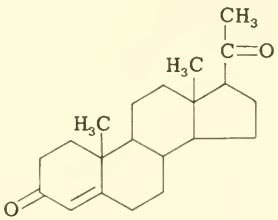
Androst-1-ene-3, 17-dione



Estra-1, 3, 5-triene



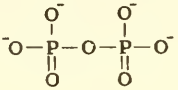
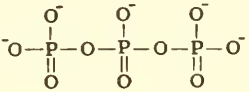
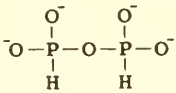
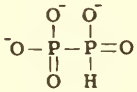
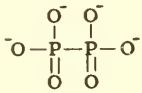
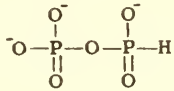
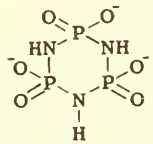
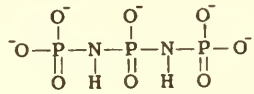
Estrone



Progesterone

TABLE 2-27

EFFECTS OF INORGANIC PHOSPHORUS COMPOUNDS ON OXIDATIVE PHOSPHORYLATION[a]

Inhibitor	Structure	Concentration (m*M*)	% Change		
			O_2 uptake	P_i uptake	P:O
Pyrophosphate		1.5	- 22	- 36	- 16
		3	- 20	- 51	- 38
Triphosphate		0.6	+ 8	- 11	- 17
		3	- 30	- 48	- 25
Pyrophosphite		1	- 4	- 4	0
Diphosphite		3	- 12	- 4	+ 10
		15	- 11	- 18	- 16
Hypophosphate		1	- 25	- 38	- 20
Isohypophosphate		3	+ 10	- 4	- 15
		15	- 25	—	—
Trimetaphosphimate		3	+ 12	+ 8	- 4
Diimidotriphosphate		3	- 9	- 24	- 19
Thiophosphate	PSO_3^{3-}	0.6	- 4	- 2	+ 2
		3	+ 18	0	- 15
		5	+ 26	- 28	- 42
		15	- 59	- 91	- 78

[a] Rat liver mitochondria oxidizing fumarate with phosphate at 15 m*M*. (From Thomson and Sato, 1960.)

This observation was extended to a number of estra-1,3,5-trienes, most of which are inhibitory to the oxidation of testosterone and are not themselves attacked (Marcus and Talalay, 1955). The most potent inhibitors are the following derivatives of estratriene: -3,17α-diol (α-estradiol), -3,16α-diol; -3,16α,17β-triol (estriol); -3-ol; -3,17β-diol-16-one; and -3,16β-diol. It appears that the aromaticity of ring A combined with the 3-OH group results in strong binding. The 17-ols are not inhibitory although there are enzyme regions for the oxidation of either 3-OH or 17-OH groups in other ring systems. The total ring system is not necessary since diethylstilbestrol and hexestrol are potent inhibitors. The K_i's for most of the effective inhibitors are around 0.001–0.01 mM corresponding to over-all interaction energies of 7–8.5 kcal/mole, implying a rather close fit over the surface of the molecules and a summation of dispersion and polarization forces. It is possible that the high polarizability of the aromatic ring A is important in augmenting binding, this ring overlying some ionic group on the enzyme, and the 3-OH interacts to form a hydrogen bond. The α,β specificity indicates that the steroids attach to the enzyme by their "rear" surfaces. The α-hydroxysteroid dehydrogenase is not inhibited so readily by the estratrienes as is the β enzyme (Talalay and Marcus, 1956). It was stated that the inhibitions are neither exactly competitive nor noncompetitive, but no data or plots were given, nor was the exact experimental procedure described, so that it is impossible evaluate the nature of the inhibitions.

The most potent inhibitor of the *Pseudomonas* β-hydroxysteroid dehydrogenase yet found is the noncompetitive 2-hydroxymethylene-17α-methylandrostan-17β-ol-3-one ($K_i = 0.0003$ mM), although the competitive 4,4-dimethyl-17β-hydroxyandrost-5-eno(3,2-c)pyrazole ($K_i = 0.0005$ mM) is almost as active (Ferrari and Arnold, 1963 a, b). The inhibitions by these and simpler steroids are dependent on the pH; e.g., diethylstilbestrol is approximately 20 times as effective at pH 8.5 than at pH 5.5. Since it is unlikely that the phenolic groups would ionize in this range (pK_a for diethylstilbestrol is 12.2), this implies the ionization of enzyme groups at or near the active site. This emphasizes the importance of polarization of the aromatic rings by anionic groups on the enzyme in determining the tightness of binding.

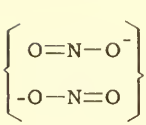
The Δ^1- and Δ^4-dehydrogenases which introduce unsaturation into ring A of the 3-ketosteroids at the 1- and 4-positions, respectively, are inducible enzymes in *P. testosteroni*, and both are inhibited quite potently by estrone (Levy and Talalay, 1959). The Δ^4-3-ketosteroid reductase (5α) of rat liver microsomes, which catalyzes the hydrogenation of the 4–5 double bond, is a NADPH-requiring enzyme acting on cortisone, cortisol, desoxycorticosterone, and related steroids (McGuire *et al.*, 1960). It is competitively inhibited by a variety of less substituted steroids, such as androst-1-ene-3,17-dione and 5α-androstane-3,17-dione; the 5β-androstane-3,17-dione is, how-

ever, inactive, indicating very specific attachment to the enzyme. The K_i for 17β-hydroxyandrosta-1,4-diene-3-one is 0.146 mM when the substrate is cortisone (K_m = 0.14 mM), and the other inhibitors presumably have similar values for K_i. It may also be noted that 6α- and 6β-methyl-11-keto-progesterone are inhibitors.

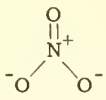
The normal roles of these enzymes in bacteria or mammals are not as yet well understood, although the steroid dehydrogenases in *Pseudomonas* enable this organism to grow with the appropriate steroids as the sole source of carbon. There are many other enzymes involved in steroid synthesis and catabolism which have not been studied with respect to analog inhibition. When such studies are made it may well be found that this is an important regulating mechanism in controlling the levels of the steroid hormones.

NITRITE AND SULFITE METABOLISM

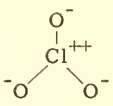
Nitrobacter is a soil autotroph that can obtain its energy from the oxidation of nitrite to nitrate. Chlorate at low concentrations (0.01–0.1 mM) inhibits growth without affecting nitrite oxidation, whereas at higher concentrations (above 1 mM) nitrite oxidation is progressively depressed (Lees and Simpson, 1955). The inhibition is completely reversible and it was postulated that chlorate combines with a nitrite-oxidizing enzyme at a stage when it is bound to nitrate; however, the inhibition seems to be unrelated to the concentrations of either nitrite or nitrate, and is not reversed by increasing the nitrite concentration (Lees and Simpson, 1957). It was then postulated that chlorate does not inhibit directly but is first converted to some substance, perhaps chlorite, which inhibits rapidly and irreversibly.



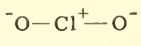
Nitrite



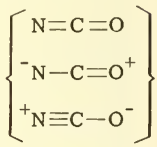
Nitrate



Chlorate



Chlorite



Cyanate

Inhibitor (2 mM)	% Inhibition
ClO_3^-	80
BrO_3^-	8
IO_3^-	1
PO_3^-	0

Related anions do not inhibit comparably with chlorate (see tabulation, where nitrite is 8 mM), but cyanate is apparently even more inhibitory

(Butt and Lees, 1960). However, the effect of cyanate is markedly dependent on the oxygen concentration (see following tabulation), so that at low oxygen levels inhibition is reversed to stimulation. It was suggested

Cyanate (mM)	% Change	
	$O_2 = 2.5\%$	$O_2 = 20\%$
0.2	+30	−50
0.3	+45	−66
0.5	+18	−76
0.7	− 3	−82
1.0	−46	−92

that a membrane transport system brings nitrite into the cells, and at moderate nitrite concentrations and normal oxygen pressures the rates of transport and oxidation are comparable. At low oxygen concentrations there is accumulation of nitrite in the cells with consequent inhibition of the nitrite-oxidizing enzyme (since substrate inhibition occurs when nitrite is above 1 mM). Cyanate may interfere with the transport so that at normal oxygen concentrations the accumulation of nitrite is suppressed and the substrate inhibition released. It would be interesting to know what effect, if any, chlorate has on the nitrite transport.

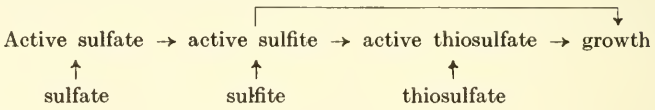
The oxidation of sulfite by the liver is inhibited by thiosulfate and this action is probably exerted on sulfite oxidase (Fridovich and Handler, 1954, 1956). Thiosulfate inhibits both aerobically and anaerobically (methylene blue reduction). Although it was originally stated that the inhibition is competitive, it has been found more recently (MacLeod *et al.*, 1961) that it is not, at least with respect to sulfite. The following reaction scheme was suggested:

$$\left|\begin{array}{l}\text{—S}\\ \quad|\\ \text{—S}\end{array}\right. + HSO_3^- \longrightarrow \left|\begin{array}{l}\text{—S—H}\\ \text{—S—}SO_3^-\end{array}\right. \xrightarrow{(+\,H_2O)} \left|\begin{array}{l}\text{—S—H}\\ \text{—S—H}\end{array}\right. + SO_4^{=} + H^+$$

and it was proposed that thiosulfate competes with the active thiosulfonate in the hydrolytic step of the sequence. Methanesulfonate, ethanesulfonate, benzenesulfonate, and pyridine-3-sulfonate are noninhibitory.

Wild-type *Neurospora crassa* can utilize sulfate as the sole source of sulfur but certain mutants require more reduced forms for growth (Ragland and Liverman, 1958). Some strains can use sulfite and others only thiosulfate. Since sulfate inhibits competitively the utilization of thiosulfate but does not interfere with the utilization of sulfite, and inasmuch as previously it

had been postulated that sulfite must pass through thiosulfate to be utilized, a shunt around thiosulfate was proposed:



Sulfate would thus block the formation of active thiosulfate from exogenous thiosulfate, or it could block the utilization of active thiosulfate. Another possibility is that thiosulfate transport into the cell is depressed by sulfate, in which case there is no necessity to assume a shunt as above.

SIMPLE ION ANTAGONISMS

Many examples of the inhibition of biological, metabolic, or enzymic processes by simple inorganic ions are known and in certain instances it is likely that the mechanism involves a competition between the inhibiting ion and a necessary ionic cofactor. The inhibiting ions may be considered as analogs of the normally functional ions. It is impossible here to treat this subject completely but it may be worthwhile to mention briefly some specific examples of competitive enzyme inhibition to illustrate the phenomenon. MacLeod and Snell (1950) emphasized the possible importance of such competitions in the effects of certain ions on the growth of bacteria. The growth of *Lactobacillus arabinosus* is suppressed by various ions and it was concluded that some of these effects are due to the fact that the inhibitory ions are structural analogs of the metal ions which are cofactors in metabolism. The following antagonisms, among others, were demonstrated: K^+ reverses the inhibitory effects of Na^+ and NH_4^+; K^+ reverses the inhibitory effects of Rb^+; Zn^{++} reverses the inhibitory effects of Mn^{++}; and Mg^{++}, Ca^{++}, and Sr^{++} reverse the inhibitory effects of Zn^{++}. Actually it is not certain that these antagonisms relate to metabolic events, since it is possible that physiological ions play nonmetabolic roles in bacteria, and it would be interesting to investigate these antagonisms on a metabolic level. Inasmuch as so many enzymes either involve ionic cofactors or are affected by physiological ions, it is likely that such competitions are very common and important in metabolic regulation.

The phosphotransacetylase of *Clostridium kluyveri* is active only in the presence of K^+ or NH_4^+ ions. Inhibition is exerted by Na^+ and Li^+ ions, and this can be overcome by increasing the K^+ or NH_4^+ concentration, so that competition for an enzyme site would seem likely (Stadtman, 1955). Yeast acid phosphatase requires Mg^{++} and is inhibited by Ca^{++}, the inhibition being strictly competitive, as shown by double reciprocal plots (Tsuboi and Hudson, 1956). Ba^{++} does not inhibit at all, but Mn^{++} and Zn^{++} depress

the activity, although less than Ca^{++}, and this may be competitive also. Ca^{++} is a competitive inhibitor of rabbit muscle phosphorylase *b* kinase with respect to the activator Mg^{++}, but is noncompetitive with respect to ATP ($K_m = 1.9$ mM for Mg^{++}, and $K_i = 0.3$ mM for Ca^{++}) (Krebs *et al.*, 1959). Most ATPase are activated by Mg^{++} and some are inhibited by Ca^{++}, but L-myosin ATPase is activated by Ca^{++} and inhibited by Mg^{++}; in both cases competition may occur. One might speculate that the uncoupling of oxidative phosphorylation by Ca^{++} may involve displacement of other ions such as Mg^{++} or Mn^{++}. The question of metal ion competition will be taken up in greater detail in the chapters on inhibitions produced by Zn^{++}, Pb^{++}, Cd^{++}, and other metal cations. Some new ideas on the regulatory role of simple cations in metabolism, especially glycolysis, may be found in the interesting discussion of Wyatt (1964). One example of ion inhibition in an enzyme system in which an ion is the substrate will be mentioned. This is the formation of δ-chlorolevulinate from β-ketoadipate by an enzyme, β-ketoadipate chlorinase, from *Caldariomyces fumago* (Shaw and Hager, 1959). F^-, Br^-, and I^- inhibit this reaction around 85% when they are present in equimolar concentration with Cl^- (10 mM). The nature of the inhibition is not known but it is rather surprising that the three inhibiting ions are of the same degree of potency.

INHIBITION BY MACROIONS

The inhibition by high molecular weight substances of enzymes attacking related high molecular weight substrates may be thought of as analog inhibition in certain instances, especially where competitive kinetics has been demonstrated (the type of inhibition has seldom been studied in work on polymers). On the other hand, these inhibitions are probably often nonspecific in the sense that the polymers interact with any or all regions of the enzyme surface rather than just the active center. Spensley and Rogers (1954) reviewed inhibitions of this type and suggested the terms *macrocationic* and *macroanionic* as applicable to effects exerted by positively charged and negatively charged polymers, respectively. Early work was mainly with naturally occurring macroions, such as heparin or protamine, but the preparation and use of more homogeneous and physically characterized synthetic polymers and copolymers have enabled the nature of the interactions to be better understood. Some of these inhibitions may well be physiologically important, for example, the intracellular effects of the various types of nucleic acids on enzymes involved in nucleic acid metabolism or their role in protein synthesis. The suggestion by Jones and Gutfreund (1964), that certain enzymes participating in metabolic sequences may interact specifically with each other to form complexes facilitating flow along the pathway, brings up the possibility that macroions can interfere in this interaction

and thereby depress metabolism by a mechanism unrelated to direct action on the individual enzymes. General macroion inhibition will be briefly discussed in this section, whether specific or nonspecific, and that these are all instances of analog inhibition is not implied. Bernfeld (1963) has recently reviewed some aspects of macroanionic inhibition.

General Nature of the Interactions between Macroions

Inasmuch as enzymes are macroions, the interactions involved, whether specific or nonspecific, will be mainly electrostatic and will depend primarily on the total charges and the distributions of charges on the enzymes and the inhibitory polymers. It is likely that enzymes at pH's removed from their isoelectric points will interact to varying extents with macroions of opposite net charge irrespective of whether the enzymes attack high molecular weight or low molecular weight substrates, but we shall direct our attention chiefly to enzymes whose substrates are polymeric. When inhibition occurs under conditions in which the enzyme and the macroion are carrying the same net charge, it has often been assumed that other than electrostatic interactions are involved, but this is not necessary. Let us picture an enzyme of isoelectric point pH 7 in a reaction medium of pH 6.5. The net charge on the enzyme will be positive, but interspersed with the cationic groups on the enzyme surface there will be many anionic carboxylate groups in most instances. If a macrocationic substance can orient itself on the enzyme surface so as to react with these anionic groups, perhaps because of complementary spacing of the oppositely charged groups, inhibition may result, although it will undoubtedly be less than at pH's above the isoelectric point. This is not said to minimize the importance of nonelectrostatic interactions, which certainly must occur, particularly when the inhibitory macroions contain groups capable of forming hydrogen bonds or regions contributing to the binding through van der Waals' forces.

The question of the specificity of macroionic inhibition is a difficult one and the data available do not allow us to draw general conclusions. It has been established, however, that a particular enzyme will be inhibited quite differently by various macroions of the opposite net charge, and that a particular macroion exerts very different effects on a group of enzymes. There is probably sufficient evidence, to be discussed later, that enzymes acting on macroionic substrates are likely to be inhibited rather strongly by other macroions in which the charge distribution is similar to that of the substrate, and in such cases the inhibition may indeed be competitive. A quantitative treatment of the interactions must be on a statistical basis and, as far as I know, this has not been undertaken, and would indeed be very difficult since there are several factors about which there is inadequate information.

The rate at which macroionic inhibition develops has not been studied

extensively but one can imagine some interesting phenomena in this connection. The initial binding of the macroion to the enzyme would not be expected to be that for maximal interaction because the first contact between them would be random. Inasmuch as most macroionic inhibitions are readily reversible, it is likely that the polymer would move on the enzyme surface until near maximal or maximal interaction occurs. The inhibition for this reason might increase with time, the rate depending on several factors, such as the binding affinity of the individual group interactions and the flexibility of the polymer, and progressive developments of inhibition have been experimentally observed. Inasmuch as many configurations of the macroion on the enzyme surface may be characterized by interaction energies very near the maximal, it is likely that even at equilibrium each enzyme molecule will not be inhibited to the same degree, especially for those enzymes acting on small substrates, since the inhibition will usually be due to a steric interference by a polymer chain passing over or near an active site.

Factors Determining the Degree of Inhibition

The most important factors relating to the inhibitory macroion would be (1) the molecular weight or polymer length, (2) the density of ionic groups on the polymer, or the repeat distance between them, (3) the over-all configuration of the polymer, i.e., linear, branched, or globular, and (4) the flexibility of the polymer. The last factor is perhaps very important but has been generally ignored. If one assumes an approximately globular enzyme, the net binding energy and the degree of inhibition may well depend on the ability of the polymer to conform to the enzyme surface, specifically to wrap around it so that interactions between many ionic groups can take place. Some of the ionic polysaccharides must not be too flexible and this may limit the effects they have on certain enzymes, while the synthetic macroions vary in flexibility over a wide range. Entropy factors must be very significant in the binding of macroions, and would to a great extent depend on the deviation of the bound polymer from its statistical configuration in solution. A polysaccharide or polypeptide macroion of molecular weight 10,000 would contain roughly 35 units and the total extended length would be 200–250 Å if linear. Such a macroion might be able to encircle an average enzyme 1 or 2 times or, if it is randomly distributed over the enzyme surface, would cover very roughly about 10–15% of the enzyme. Many macroions used to inhibit enzymes are, of course, much larger, often being of molecular weights of 100,000 or over.

Two characteristics of the media used in inhibition studies are particularly important, namely, the pH and the ionic strength, since the interactions between enzyme and macroion are mainly of the ion-ion type. The pH will determine the net charge on the enzyme and occasionally the ioni-

zation state of groups with which the inhibitor reacts, and in all cases in which this has been studied a marked dependence on the pH has been demonstrated. An increase in the ionic strength should reduce such inhibitions because of the competition of the small ions for the enzyme and macroion groups, and this has been repeatedly confirmed experimentally (data for the inhibition of trypsin by polyglutamate are given in Table I-15-6). Hydration of the ionic groups must also be a significant factor in reducing the inhibitions from what might be expected on the basis of interactions in a vacuum, so that anything which modified the extent of hydration of either enzyme or macroion might secondarily affect the inhibition. A final factor which can markedly reduce such inhibitions is the presence of macroionic impurities in the preparation if one is not working with pure enzymes. It has been shown many times that inhibitions by macroions can be prevented or actually reversed by other macroions of opposite charge to the inhibitor. Such results are not particularly significant since they imply only that the inhibitor can also bind to nonenzyme macroions, a fact which can be better demonstrated with other techniques, but they emphasize the possible importance of such impurities in the studies on macroionic inhibitions.

Trypsin and Chymotrypsin

The isoelectric point of trypsin is close to pH 11 and that of casein is between 4 and 4.5; thus the hydrolysis of casein involves the interaction of a macrocationic enzyme with a macroanionic substrate at pH values near neutrality. Since heparin, a strongly negatively charged sulfated polysaccharide, was known to form complexes with positively charged proteins, Horwitt (1940) examined its action on trypsin and found a rather potent inhibition at pH 7.3. Inhibition does not occur unless the enzyme is incubated with heparin before the addition of the casein, possibly indicating a competitive type of interaction. Acidification to pH 3 leads to a dissociation of the trypsin-heparin complex with restoration of full activity. The pH_{opt} for trypsin is possibly shifted from 8.4 to lower values by heparin (Glazko and Ferguson, 1940); it is not known if this means that enzyme combined with heparin can act on casein — it is difficult enough to understand the pH_{opt} of proteolytic enzymes in the absence of inhibitors. The distance between sulfate groups in heparin is 10.2 Å, which is approximately equivalent to 3 peptide residues in proteins, so it was suggested by Kornguth and Stahmann (1960) that heparin may bridge the active site by combining with cationic groups on either side. The active site appears to be covered, since the hydrolysis of benzoylarginamide by trypsin is inhibited. Poly-α-L-glutamate and polycysteate also inhibit trypsin, but poly-γ-D-glutamate does not, and this is probably correlated with the different distances between COO^- groups in these macroanions. Poly-D-lysine inhibits the tryptic hydrolysis of poly-L-lysine, equimolar concentrations giving complete inhi-

bition, showing the importance of the configuration of the polypeptide chains (Tsuyuki *et al.*, 1956). Polyacrylate at concentrations around 0.25–0.5 mg/ml inhibits trypsin at high pH and accelerates catalysis at low pH (Morawetz and Sage, 1955). Denatured hemoglobin, the substrate, has an isoelectric point around 7.8, so that above pH 7.8 the polyacrylate can combine only with the positively charged trypsin (the trypsin-polyacrylate complex has some activity), whereas at lower pH's polyacrylate also binds to the hemoglobin. Since the hemoglobin-polyacrylate complex is more susceptible to trypsin and since hemoglobin is much in excess of trypsin (thus binding most of the polyacrylate), the rate is stimulated at lower pH values. The inhibitions by polyglutamate and polyacrylate are reduced by increasing the ionic strength, as expected (Table I-15-6). It has been shown that copolymers of glutamate with other amino acids (e.g., tyrosine, phenylalanine, or leucine) are more effective inhibitors than glutamate polymers, but the copolymer of glutamate and alanine is less inhibitory (Rigbi and Sela, 1964). Ornithine polymers or copolymers with ornithine are not inhibitory and will reactivate trypsin inhibited by glutamate polymers. This is one instance in which the inhibition produced by glutamate polymers or copolymers is progressive and depends on the incubation time. From the different degrees of inhibition brought about by the various copolymers and the effects of the ionic strength, it was concluded that forces other than electrostatic are involved in the binding. It is interesting that trypsin seems to be particularly susceptible to macroanions, inasmuch as neither chymotrypsin nor papain is inhibited by heparin, although both enzymes are negatively charged at physiological pH.

The hydrolysis of acetyltyrosine ethyl ester and methylhippurate by chymotrypsin is inhibited 35–50% by various proteins (seralbumin, ovalbumin, and β-lactoglobulin) at concentrations equivalent to the enzyme and in the absence of salt (Hofstee, 1960). Addition of salts, particularly multiply charged ions, reduces or abolishes the inhibition. These proteins are as potent inhibitors as the naturally occurring chymotrypsin inhibitors, but differ in not being so sensitive to salt concentration. Carboxymethylcellulose and nucleic acids (both DNA and RNA) also inhibit chymotrypsin (Hofstee, 1961). The complexes are dissociated by 100 mM KCl. The inhibition is not complete at maximal binding of nucleic acid, indicating that the active center is not directly involved in the interaction. Methylhippurate was the substrate and if protein substrates had been used it is likely that the active center would not have been accessible.

Pepsin

The hydrolysis of hemoglobin by pepsin is rapidly inhibited by poly-L-lysine and this is readily reversible by adding heparin, which complexes with the inhibitor (Katchalski *et al.*, 1954). Cationic polyornithine and poly-

p-aminophenylalanine act similarly, but anionic polyalanine, polyaspartate, and polyglutamate do not inhibit, indicating purely electrostatic binding. The inhibition disappears at high poly-L-lysine concentrations (Dellert and Stahmann, 1955). Changes in the optical transmittance also occur (Fig. 2-15); that is, low concentrations of inhibitor complex with the enzyme to decrease the solubility, but as the inhibitor concentration rises the com-

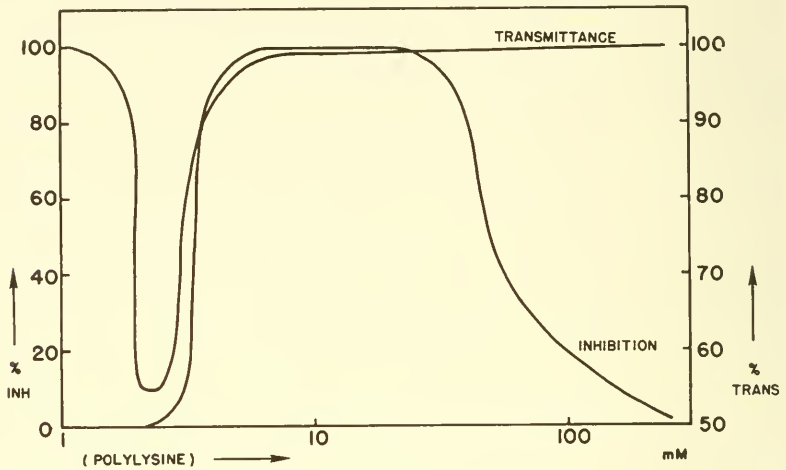


FIG. 2-15. Inhibition of pepsin by polylysine of mean molecular weight 2100 at pH 4.7. Transmittance determined at 400 mμ. (Data from Dellert and Stahmann, 1955.)

plexes become more soluble. However, the correlation between inhibition and transmittance is such as to suggest that the depression of enzyme activity is by no means directly related to the aggregate size of the complex.

Some have believed that pepsin plays a role in the genesis or maintenance of gastric ulcers and hence have looked for inhibitors that might be affective clinically. Strange to say, they have invariably used macroanions such as heparin, chondroitin sulfate, polyhydromannuronic sulfate, and various polymers formed by condensation of aldehydes with hydroquinonesulfonate (Levey and Sheinfeld, 1954; Marini and Levey, 1955; Heymann *et al.*, 1959). The isoelectric point of pepsin is around 2.8 so the relative effectiveness of macroanions and macrocations might depend on the experimental or physiological pH. Although the hydrolysis of casein is inhibited to some extent by these polymers, it is possible that this is due in part or wholly to the formation of complexes with the casein. It has been claimed that chondroitin sulfate and the polymeric sulfonates reduce the number of ulcers in Shay rats, but it is doubtful if this is related to pepsin inhibition, even if it occurs, and there are other explanations (for example, inhibition of lysozyme, which has also been implicated in ulceration).

Lysozyme

It is not surprising that this mucolytic enzyme is inhibited by a variety of macroanions since its isoelectric point is above 10.5. Heparin is a fairly potent inhibitor of lysozyme (Kaiser, 1953), and it has been stated that this is competitive with substrate (which was a dried preparation of *M. lysodeikticus*) (Kerby and Eadie, 1953). Inhibition is also exerted by hyaluronate, polysaccharide of *Pneumococcus*, polyglutamate, DNA, and RNA (Skarnes and Watson, 1955). Various synthetic polymeric sulfonates also inhibit to different degrees (Heymann *et al.*, 1959). The most potent inhibitor yet found for lysozyme is polyglucose sulfate (molecular weight around 20,000), although oxidized polyglucose (containing carboxylate groups) is likewise very active; tetraglucose sulfate is without activity (Mora and Young, 1959). These inhibitions are generally reversed by increasing salt concentration or by the addition of a macrocation, such as protamine, to bind the inhibitor. Copolymers of glutamate, tyrosine, phenylalanine, and leucine are potent inhibitors of lysozyme, and the inhibitions can be completely reversed by polylysine (Sela and Steiner, 1963). The greater inhibitory activity of the copolymers compared to the homopolymer of glutamate is attributed to nonionic bonds; although this is the most likely explanation, one must recognize that the charge distribution is quite different in the copolymer relative to the homopolymer.

Hyaluronidase

It was noted by Pantlitschko and Kaiser (1951) that hyaluronidase is not significantly inhibited by low molecular weight substances or by high molecular weight substances unless they are esterified with sulfate or are otherwise anionic, and, furthermore, that inhibitory macroanions must be filiform and not globular. Heparin and artificially sulfurated polysaccharides are inhibitory; sulfurated hyaluronate, which can with some justification be thought of as a true analog of hyaluronate, inhibits well. Hyaluronate is a high molecular weight polymer of *N*-acetylhyalobiuronate units and hence contains free COO^- groups; however, sulfuration essentially doubles the negative charge on the molecules, and prevents degradation by the enzyme. Nitrated and acetylated hyaluronates are also inhibitory. A few macroanionic inhibitors can be mentioned but require no discussion: chitin sulfates, polymers formed from formaldehyde and various phenolic sulfonates (e.g., hydroquinone, catechol, and resorcinol), polymers formed from formaldehyde and various hydroxybenzoates, polyesters of phosphate with phenols and aniline, polystyrenesulfonate, sulfated pectate, polymethacrylate, amylopectin sulfate, and heparin (Rogers and Spensley, 1954; Bernfeld *et al.*, 1961).

Alburn and Whitley (1954) suggested that the inhibition of hyaluronidase

by heparin is competitive and this was studied in detail by Houck (1957 a), who found competitive behavior from $1/v$-$1/(S)$ plots for both heparin and chondroitin sulfate B. The inhibitor constants and their variation with

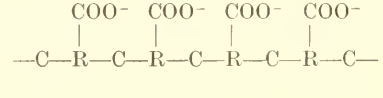
Temperature	K_m (mM)	K_i (mM)	
		Heparin	Chondroitin sulfate B
22°	0.70	6.17	6.58
27°	0.74	6.45	6.85
32°	0.78	6.68	7.10
37°	0.81	7.00	7.41

temperature are tabulated, and from these values it was possible to calculate the important thermodynamic quantities shown in the following tabulation:

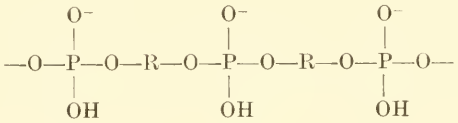
	$-\Delta F°$ (kcal/mole)	$-\Delta H°$ (kcal/mole)	$-\Delta S°$ (e. u.)
Heparin	3.07	5.0	6.8
Chondroitin sulfate B	3.0	4.9	6.2

The rather weak binding might indicate that only a fraction of the anionic groups on heparin or chondroitin sulfate B interact at close range with enzyme cation groups. It is difficult to predict the entropy changes in the interactions of such complex molecules because several factors may be involved, e.g., the restriction of polymer configuration, possible changes in enzyme structure, and release of water of hydration. The rather small changes in ΔS observed are probably the result of the balancing of larger changes in different directions. The importance of ion-ion interactions is shown by the marked reduction in the inhibitions when the ionic strength rises above 0.3.

The differences in inhibitory activity between low molecular weight substances and polymers made from them are well illustrated by Hahn and Fekete (1953). Various phenolic compounds inhibit testicular hyaluronidase weakly, but upon polymerizing these with formaldehyde it is possible to obtain potent inhibitors. Their results are expressed in terms of the potency relative to resorcinol. γ-Resorcylate has an activity of 1.5 while its polymer has an activity of 980. The most active inhibitor is the polymer



of gentisate with values around 2000–2500 (Hahn and Frank, 1953). Polyesters of phosphate with phloretin (or other polyphenols) are very inhibitory to hyaluronidase, 0.005 mg/ml completely abolishing enzyme activity (Diczfalusy *et al.*, 1953). Phloroglucinol phosphate polymer may be even more potent, 0.00013 mg/ml inhibiting 80% (Fernö *et al.*, 1953). Such polymers may be represented as:



The extent of the inhibition may depend primarily on the distance between anionic groups and the ability of the polymer to assume the appropriate configurations on the enzyme surface.

Ribonuclease

Pancreatic ribonuclease is strongly and competitively inhibited by heparin (Zöllner and Fellig, 1952, 1953) but the results by different investigators vary quite widely, due perhaps to different experimental conditions (especially pH and ionic strength), different preparations of heparin, and different techniques for measuring the enzyme activity. The competitive nature of the inhibition (at least the reduction in inhibition upon increasing RNA concentration) has been confirmed by Roth (1953) and Houck (1957 b). Increase in ionic strength reduces the inhibition as expected (Houck, 1957 b; Heymann *et al.*, 1958; Fellig and Wiley, 1959; Lorenz *et al.*, 1960), although Houck found some deviation from this at very high NaCl concentrations. The results of Lorenz *et al.* (1960) are quite typical (see accompanying tabulation):

NaCl (m*M*)	% Inhibition
0	97
30	50
50	33
100	0

Lowering the pH from around 7.5–8.0 to 5.0 progressively augments the inhibition (Zöllner and Fellig, 1953; Roth, 1953 b), which is perhaps a reflection of the increasing positive charge on the ribonuclease (isoelectric point is 9.5). The ribonucleases of rat kidney and liver (Roth, 1953 b) and rat and guinea pig serum (Rabinovitch and Dohi, 1957) are also inhibited

by heparin. It has been known for many years that heparin depresses cell division and it has been said to prevent gelation of the mitotic apparatus. Paff *et al.* (1952) found that heparin inhibits mitosis in cultured chick heart fibroblasts and after 24 hr there is a marked accumulation of granular ribonucleoprotein in the cells. It was postulated that heparin might interfere with the metabolism of nucleoproteins and thereby block mitosis.

The inhibition of ribonuclease by DNA was first clearly shown by Houck (1957 b) and this could be thought of more reasonably as a true analog inhibition. Likewise, deoxyribonuclease can be inhibited by RNA, the K_i being around 0.00001 mM for the endonuclease of *E. coli* (Lehman *et al.*, 1962 a, b). Although inhibition occurs with RNA from various sources, the most potent inhibitor is the amino acid acceptor RNA from *E. coli*, the inhibition being competitive. The other DNA-cleaving enzymes tested are not inhibited. The potency and specificity of this inhibition cannot but stimulate thoughts on the possible regulatory relationships intracellularly.

Other macroanions have variable effects on ribonuclease. Zöllner and Fellig (1952) reported no inhibition by chondroitin sulfate, hyaluronate, and alginate, but Houck (1957 b) found chondroitin sulfate A and hyaluronate to inhibit equivalently with heparin. Synthetic polyglucose sulfate is a competitive inhibitor, the effect decreasing with increase in the pH, the net charge on the ribonuclease being reduced (Mora, 1962). Vandendriessche (1956) studied the inhibitions by sulfonated pectin, poly-*p,p*-dioxydibenzyl phosphate, and poly-L-aspartate, and found them to be much weaker than heparin, while Fellig and Wiley (1959) claimed that the sulfation of a variety of polysaccharides (e.g., cellulose, amylose, amylopectin, dextran, pectate, and nitrochitin) produces inhibitors often more potent than heparin (although in this work the inhibition by heparin was unaccountably weak). Ribonuclease is also inhibited by copolymers of glutamate and tyrosine (or phenylalanine), which are more affective than polyaspartate or polyglutamate (Sela, 1962). Interactions between the benzene rings of the aromatic amino acids and certain groups on the enzyme were believed to occur in addition to the electrostatic forces. Possibly the most potent inhibitors were discovered by Heymann *et al.* (1958) in a survey of 66 macroanions of synthetic origin, some inhibiting around 50% at 0.001 mg/ml. It was noted that the inhibitory activity is markedly reduced in the presence of proteins, a point worth considering in the use of such substances in cellular preparations. A number of these polymers exhibit antiviral activity against influenzal and vaccinial infections in eggs. Sulfate groups seem to be particularly able to confer inhibitory activity on polymers and in the sulfated polysaccharides the carboxylate groups may be relatively unimportant, since Dickman (1958) showed that sulfation of pectate, pectate methyl ester, and pectic amide gives inhibitors roughly equiactive. It should finally be noted that ribonucleases of different origins may not be equally suscep-

tible to macroanions. A striking difference was demonstrated by Nishimura (1960), bovine ribonuclease being readily inhibited by polyvinyl sulfate to which *Bacillus subtilis* ribonucleases are completely resistant.

Lipoprotein Lipase

Macroions inhibit lipoprotein lipase but affect other lipases little or not at all. The enzyme from chicken fat is inhibited by macrocations in a purely noncompetitive fashion and the inhibition is rapidly reversible either by dialysis or the addition of a macroanion (Korn, 1962). Poly-L-lysines of increasing chain length are progressively more effective, which is one of the few observations relating polymer size to inhibition. The degree of inhibition by various copolymers of tyrosine and lysine depends on the content of lysine, indicating purely electrostatic binding. The enzyme is also inhibited by macroanions, which may act noncompetitively or competitively (e.g., heparin and polyglucose sulfate). The lipoprotein from mouse heart is inhibited by many polysaccharide sulfates as long as there is at least 0.6 sulfate group per repeat unit (Bernfeld and Kelley, 1963). The potency of the inhibition is independent of the configuration of the polysaccharide, whereas in the case of the chicken enzyme the highly branched polymers are less effective than the more linear ones.

Polynucleotide Phosphorylase

This enzyme, usually obtained from *Azotobacter vinelandii* or *Micrococcus lysodeikticus*, catalyzes the synthesis of polynucleotides, such as polyadenylate:

$$\mathrm{ADP} + (\mathrm{AMP})_n \rightleftarrows \mathrm{P}_i + (\mathrm{AMP})_{n+1}$$

The synthesis of a particular polynucleotide may be inhibited by another polynucleotide; thus polyuridylate inhibits the synthesis of polyadenylate, and polyuridylate, polyadenylate, and RNA inhibit the synthesis of polycytidylate (Mii and Ochoa, 1957). The formation of polyadenylate has been shown to be inhibited by variously degraded yeast RNA and polyadenylate (Hendley and Beers, 1959, 1961; Beers, 1961). Increase in substrate concentration reduces the inhibition but not in a strictly competitive fashion. Competition with primers or activators was considered to be the most likely mechanism. If the polynucleotides are too extensively depolymerized, the inhibitory activity falls, indicating a certain minimal chain length for optimal inhibition. It was also established that phosphate groups on or adjacent to the 3′-position of the terminal ribose units are necessary. It is important to remember in such systems that interactions between different polynucleotide chains, perhaps through hydrogen bonding, can occur (Warner, 1957); the possible role of such interactions in the inhibitions observed is not yet completely understood.

Amylases

Both α- and β-amylases from barley are inhibited by heparin at concentrations around 0.1 mg/ml (Myrbäck and Persson, 1953 a, b). These inhibitions show a striking pH dependence. At pH 5 or above there is no inhibition whereas below pH 5 the inhibition increases rapidly and is very marked at pH 4.5. The isoelectric points of amylases usually lie between pH 5 and 6, so it is likely that the combination of heparin with the enzymes below pH 5 is due to the positive charge arising in this range. The electrostatic nature of the binding is indicated by the protection afforded by high concentrations of NaCl during the incubation of the enzyme and the heparin. Once the inhibition is established, raising the NaCl concentration will not reactivate. The presence of substrate also protects the enzyme, pointing to a basically competitive mechanism. Human salivary amylase is also inhibited by heparin but the critical pH is at least one unit higher, possibly because of a higher isoelectric point than the barley enzymes (Astrup and Thorsell, 1954).

Enzymes Acting on Substrates Which Are Not Macroions

Macroions interact with proteins generally when the conditions are favorable (e.g., when the total charges on protein and macroion are opposite, although this is not a necessary condition, and when the ionic strength is low), so it is not surprising that many enzymes whose substrates are small molecules are inhibited. The combination is probably seldom at the active center, but more often a bridging or covering of the active center by larger molecules bound at many points and in no specific orientation. This type of inhibition is, of course, independent of structural relations with the substrate, but must always be considered in the use of macroionic inhibitors. A few examples only will be mentioned.

Prostatic acid phosphatase is potently inhibited by polyphloretin-phosphate in a noncompetitive fashion (Diczfalusy *et al.*, 1953; Beling and Diczfalusy, 1959). K_i is given as 1.55 μg/ml. Polyestradiol phosphate is even more inhibitory ($K_i = 0.55$ μg/ml). The inhibitions increase as the pH is lowered below the pH_{opt}. Alginate of 556 residues and molecular weight of 92,000 is also strongly inhibitory ($K_i = 0.0054$ mM); the mechanism is partially competition with substrate and partially interference with stabilizing or protective substances present (Jeffree, 1957). The variation of the inhibition with chain length is complex: chains of 10–100 residues inhibit less, but below 10 the inhibition rises (Jeffree, 1956). A third potent inhibitor is polyxenyl phosphate, a polydisperse, small molecular weight polymer of branched chains and random coils, 66% inhibition being given by 0.001 mM (Hummel *et al.*, 1958). Polyhydroquinone is almost as potent, polyethylenesulfonate and sulfonated polystyrene inhibit moderately, and

polyacrylate and chondroitin sulfate are relatively inactive. The inhibition by polyxenylphosphate is noncompetitive, partially reversed by raising the NaCl concentration, and maximal at pH 4.6, decreasing on either side.

β-Fructofuranosidase of yeast is inhibited by heparin and chitin disulfate at low pH's (Astrup and Thorsell, 1954). The glucuronidases from several rat tissues are inhibited by heparin and hyaluronate (Becker and Friedenwald, 1949). Fumarase is inhibited 92% by heparin at a concentration of 0.2 mg/ml in a pH range of 5.5–6.0, whereas nucleic acid and chondroitin sulfate inhibit only 26% and 11%, respectively, at 2 mg/ml (Fischer and Herrmann, 1937). These examples indicate that inhibitions of this sort are widespread. There has been only one investigation of the effects of macroions on a complex metabolic sequence, the study of Dische and Ashwell (1955) on the actions of ribonucleate and some smaller anions, such as sulfate, on anaerobic glycolysis in pigeon hemolysates. RNA inhibits lactate formation 48% at 1 mg/ml and the formation of phosphoglyceraldehyde 30% at 3 mg/ml. There would thus appear to be at least two sites of action, the major effect being on the transformation of 3-phosphoglyceraldehyde to lactate.

INHIBITIONS BY NUCLEOTIDES AND RELATED SUBSTANCES

Enzymes acting on pyrimidines, purines, nucleosides, nucleotides, or polynucleotides are frequently inhibited by analogs of these substrates. Sometimes the inhibitors are normally occurring substances and it is here that some of the most clear-cut and important examples of feedback control and metabolic regulation have been demonstrated. In other cases the inhibitors are synthetically derived abnormal analogs, which are frequently quite depressant to rapidly growing cells where nucleotide metabolism is active and have for this reason been studied with regard to carcinostasis. Many instances of inhibition have been reported, some of which are summarized in Table 2-28, but thorough quantitative work and studies of the mechanisms are rather uncommon.

Most of the inhibitions in Table 2-28 appear to be competitive and probably many of those in which the kinetics were not studied are competitive. Although the inhibitory activity of most of these analogs is low or moderate, a few analogs, particularly the abnormal aza and fluoro derivatives, are quite potent. It seems that all parts of the nucleotide structure can contribute to the binding. Where only the nature of the purine or pyrimidine component is varied, the inhibitions may be very different, indicating that the ring-substituted groups can be important. The pentose structure is also a determinant since different activities are observed in nucleotides containing ribose or deoxyribose. Finally, the number of phosphate units in the

TABLE 2-28

INHIBITIONS PRODUCED BY NUCLEOTIDES AND RELATED SUBSTANCES[a]

Enzyme	Source	Substrate	Inhibitor	Concentration (mM)	% Inhibition	K_i (mM)	Reference
Acyl 5′-nucleotidase	Pig kidney	Benzoyl-AMP	AMP	50	20		Kellerman (1959)
Adenine deaminase	*E. coli*	Adenine(2)	2-Methyladenine	2	100		Remy (1961)
			2-Amino-*N*-methyladenine	2	83		
			2-Aminoadenine	2	68		
			N-Methyladenine	2	49		
			N-Dimethyladenine	2	44		
Adenosine deaminase	Adenocarcinoma	Adenosine(0.11)	8-Azaguanine	3.3	100		Feigelson and Davidson (1956 b)
			8-Azaxanthine	3.3	31		
			2-Aminoadenine	3.3	28		
			6-Mercaptopurine	3.3	11		
Adenosine hydrolase	Brussels sprouts	Adenosine(5)	Adenine	9	38		Mazelis and Creveling (1963)
Adenylosuccinate lyase	—	Adenylosuccinate	6-Mercaptopurine nucleotide	0.1	50		Hampton (1962)
	Yeast	Adenylosuccinate	AMP	0.5	52		Cohen and Bridger (1964)
			ATP	0.5	8		
			IMP	0.5	6		
			Adenosine	0.5	1		

Adenylosuccinate synthetase	*E. coli*	Aspartate, IMP, and GTP	GDP	0.1	50		Lieberman (1956)
	E. coli	Aspartate, IMP, and GTP	Hadacidin	—	—	0.0042	Shigeura and Gordon (1962)
	E. coli	Aspartate, IMP, and GTP	Adenine	0.1	0		Wyngaarden and Greenland (1963)
			Adenosine	0.4	17		
			AMP	0.1	26	0.095	
			ADP	0.1	3		
			ATP	0.3	0		
			Guanine	0.1	0		
			Guanosine	0.1	0		
			GMP	0.1	39	0.092	
			GDP	0.04	67	0.022	
			UMP	0.3	0		
			CMP	0.1	13		
			dAMP	0.15	22		
			dGMP	0.05	25	0.079	
	—	dGMP	6-Marcaptopurine nucleotide	0.1	75		Hampton (1962)
Arginine kinase	Crayfish (*Jasus verreauxi*)	Arginine and ATP	ADP	—	—	1.5	Griffiths *et al.* (1957)
		Arginine-P and ADP(1)	AMP	1	16		
				4	26		
			ATP	1	0		
				10	54		
Aspartate transcarbamylase	*E. coli*	Aspartate(5) and carbamyl-P(5)	Cytidine	10	18		Smith and Sullivan (1960)
				20	46		
			CMP	20	57		
			5-F-cytidine	5	18		
				10	56		

TABLE 2-28 (*continued*)

Enzyme	Source	Substrate	Inhibitor	Concentration (mM)	% Inhibition	K_i (mM)	Reference
			5-F-orotate	5	10		
	E. coli	Aspartate(5) and carbamyl-P(3.6)	Cytosine	2	0		Gerhart and Pardee (1962)
			Cytidine	2	24		
			Deoxycytidine	2	26		
			CMP	2	38		
			dCMP	2	48		
			CDP	2	68		
			CTP	2	86		
			dCTP	2	88		
			UTP	2	8		
			GTP	2	35		
			ATP	2	(st)		
	Rat liver	Aspartate(5) and carbamyl-P(5)	Cytidine	13.3	17		Bresnick (1963)
			Deoxycytidine	13.3	41		
			CMP	13.3	9		
			dCMP	13.3	22		
			CTP	13.3	16		
			Uridine	13.3	10		
			UMP	13.3	20		
			UTP	13.3	21		
			Thymidine	13.3	44		
			TMP	13.3	18		

	Rat liver	Aspartate and carbamyl-P	5-F-deoxyeytidine	—	—	0.34	Bresnick (1962)
			5-Br-deoxycytidine	—	—	0.26	
			Deoxythymidine	—	—	1.5	
			dAMP	—	—	0.16	
Deoxyeytidylate deaminase	Sea urchin eggs	dCMP(2)	AMP	9	0		Scarano *et al.* (1960)
			dAMP	4	30		
			UMP	8	0		
			dUMP	2.1	65		
			GMP	0.4	50		
			dGMP	0.4	50		
			2′, 3′-GMP	0.36	24		
			CMP	9	0		
			dTMP	0.43	22		
				1	70		
	Embryo rat liver	dCMP(10)	AMP	4	10		Maley and Maley (1959)
				10	19		
			dAMP	4	28		
				10	42		
			UMP	4	15		
				10	25		
			dUMP	4	42		
				10	82		
			CMP	4	0		
				10	13		
			dTMP	4	69		
				10	94		
Deoxycytidylate kinase	*Azotobacter vinelandii*	dCMP(5.8) and ATP(4.8)	ADP	0.65	22		Maley and Ochoa (1958)
				1.3	35		

TABLE 2-28 (*continued*)

Enzyme	Source	Substrate	Inhibitor	Concentration (mM)	% Inhibition	K_i (mM)	Reference
Deoxythymidine kinase	*E. coli*	Deoxythymidine (0.85)	dTTP	0.7	88		Okazaki and Kornberg (1964)
Dihydroorotase	*E. coli*	Carbamylaspartate(0.25)	Orotate	0.5	2		Smith and Sullivan (1960)
				2	5		
				4	16		
			5-F-orotate	0.5	17		
				2	58		
				4	70		
Dihydroorotate dehydrogenase	*Zymobacterium oroticum*	Orotate(×)	2,4-Dihydroxy-6-methylpyrimidine	70×	45		Friedmann and Vennesland (1958)
	Zymobacterium oroticum	Orotate(2)	5-Methylorotate	2	50		Friedmann and Vennesland (1960)
Fructose-1,6-diphosphatase	Pig kidney	Fructose-1,6-diP(0.06)	AMP	0.01	6		Mendicino and Vasarhely (1963)
				0.05	63		
				0.1	74		
				0.3	83		
			ADP	0.3	10		
			ATP	0.3	12		
			IMP	0.3	0		
			UDP	0.3	7		

Glutamine synthetase	Sheep brain	Glutamate, NH_4^+, and ATP(×)	ADP	0.3×	50		Elliott (1955)
Guanosine-5′-P reductase	*Aerobacter aerogenes*	GMP(1)	AMP	5	60		Mager and Magasanik (1960)
				10	71		
			ATP	0.5	57		
				1	95		
			IMP	5	32		
				10	57		
Inosine hydrolase	*Lactobacillus delbrueckii*	Inosine(×)	Xanthosine	1×	50		Takagi and Horecker (1957)
			Urate riboside	1×	50		
Inosine-5′-P dehydrogenase	*Aerobacter aerogenes*	IMP(0.5)	ATP	5	9		Mager and Magasanik (1960)
			GMP	1	38		
				2	62		
				5	87		
			GDP	2	51		
			GTP	2	9		
	Ehrlich ascites carcinoma	IMP	6-ThioIMP	—	—	0.0036	Atkinson *et al.* (1963)
Inosine phophorylase	*E. coli*	Inosine(3)	2-Methyladenine	6	64		Remy (1961)
			2-Amino-*N*-methyl-adenine	9.6	49		
			Adenine	12	47		
			N-Methyladenine	12	39		
			N-Dimethyladenine	12	21		
5′-Nucleotidase	*Azotobacter vinelandii*	AMP(2)	IMP	2	40		Hurwitz *et al.* (1957)
			UMP	2	0		

TABLE 2-28 (*continued*)

Enzyme	Source	Substrate	Inhibitor	Concentration (m*M*)	% Inhibition	K_i (m*M*)	Reference
	Potato	AMP	Adenine	10	0		W. Klein (1957)
			Adenosine	10	70		
			Uridine	10	19		
			Thymidine	10	51		
			Inosine	10	53		
			Guanosine	10	52		
			Uracil	10	0		
	Rat liver	AMP(0.3)	Adenosine	25	53		Segal and Brenner (1960)
			Inosine	25	54		
			Cytidine	50	41		
			Uridine	50	70		
		AMP(1)	Adenosine	25	31		
			Inosine	25	34		
			Cytidine	50	30		
			Uridine	50	46		
Orotidylate decarboxylase	Yeast	Orotidine-5′-P (0.05)	6-Azauridine	0.05	0		Pasternak and Handschumacher (1959)
			6-Azauridine-5′-P	0.002	50		
	Yeast	Orotidine-5′-P	6-Azauridine-5′-P	—	—	0.00075	Handschumacher (1960)

	Rat liver	Orotidine -5′-P (0.1)	UMP	2	20	6.9	Blair and Potter (1961)
				8	45		
				16	75		
			UDP	—	(ni)		
			UTP	—	(ni)		
Orotate transphosphoribosylase	Yeast	Orotate	6-Uracilsulfonamide	—	—	0.007	Holmes (1956)
			6-Uracilsulfonate	—	—	0.39	
			6-Uracilmethylsulfone	—	—	0.71	
Phenol sulfokinase	Rabbit liver	3′-AMP-5′-phosphosulfate(×) and *m*-aminophenol	3′-AMP-5′-P	1×	50		Brunngraber (1958)
Phosphodiesterase	*Crotalus adamanteus* venom	Nitrophenyl-pU (0.5)	pTpTpTpT	0.0014	36		Razzell and Khorana (1959)
				0.0027	61		
				0.0068	77		
			TpTpTpT	0.0027	34		
				0.0068	49		
	Mouse leukemic cells	Poly A(3.3)	Adenosine	1.9	70		Anderson and Heppel (1960)
			pApA	0.4	6		
				1.6	62		
			AMP	1.4	0		
				2.8	30		
	Pig kidney	Nitrophenyl-pT (0.3)	AMP	0.3	16		Razzel (1961)
				1	100		
			ADP	0.3	0		
				1	40		
			ATP	1	21		
			TMP	1	60		
			dAMP	1	90		

TABLE 2-28 (*continued*)

Enzyme	Source	Substrate	Inhibitor	Concentration (m*M*)	% Inhibition	K_i (m*M*)	Reference
Phospho-fructokinase	Guinea pig heart	Fructose-6-P(1) and ATP(1)	Cyclic 3′,5′-AMP (pH = 7.6)	0.1	16		Mansour (1963)
Phosphoglucose isomerase	Rabbit muscle	Fructose-6-P	ATP	—	—	0.4	Kahana *et al.* (1960)
Phosphoribosyl-PP amidotransferase	Pigeon liver	Glutamine and PRPP	ADP	—	—	0.04	Wyngaarden and Ashton (1959)
			ATP	—	—	0.04	
Polynucleotide phosphorylase	*Azotobacter vinelandii*	ADP	dCDP	—	(i)		Maley (1958)
			dCMP	—	(ni)		
			dCTP	—	(ni)		
	Azotobacter vinelandii	ADP	dCDP	1.08	50		Maley and Ochoa (1958)
	Azotobacter vinelandii	Polyadenylate (1.5)	Adenyl-5′-methylenediphosphonate	5	60		Simon and Myers (1961)
			Adenyl-5′-methyl-phosphonate	10	0		
			ATP	10	0		
	E. coli	ADP(1.5)	6-AzaUMP	—	(ni)		Škoda *et al.* (1959)
			6-AzaUDP	0.5	18		
				1	38		
				2.5	80		
	Agrobacterium tumefaciens	ADP	GDP	—	—	0.15	Vardanis and Hochster (1961)

Pyridoxal kinase	Yeast	Pyridoxal and ATP	Adenine	—	—	0.58	Hurwitz (1953)
			Adenosine	—	—	0.63	
			AMP	—	—	0.66	
			ADP	—	—	0.23	
			ITP	—	—	2.2	
			Inosine	—	(ni)		
			IMP	—	(ni)		
	Yeast	Pyridoxal and ATP	AMP	—	—	0.06	McCormick *et al.* (1961)
Pyrophosphatase	Rat liver	Pyrophosphate (4.67)	Adenine	—	(ni)		Nordlie and Lardy (1961)
			Adenosine	—	(ni)		
			AMP	1	57		
			ADP	1	57		
			ATP	1	41		
			CMP	2	(ni)		
			UMP	2	(ni)		
			GTP	2	(ni)		
Ribonuclease	Pancreas	RNA	AMP	—	(i)		Zittle (1945)
			GMP	—	(i)		
	Pancreas	Cyclic 2′,2′-CMP	2′-CMP	—	—	0.3	Nelson and Hummel (1961)
Succinyl-CoA deacylase	Pig heart	Succinyl-CoA	ATP	0.33	72		Gergely (1955 a)
Thiamine kinase	Yeast	Thiamine(1) and ATP(1)	Adenosine	1	0		Kaziro (1959)
			AMP	1	59		
			ADP	1	35		
			IMP	1	28		
Thymidylate kinase	*E. coli* (phage-infected)	TMP and ATP	dGMP	—	(i)		Bello *et al.* (1961)

TABLE 2-28 (*continued*)

Enzyme	Source	Substrate	Inhibitor	Concentration (m*M*)	% Inhibition	K_i (m*M*)	Reference
Thymidylate synthetase	*E. coli*	dUMP(2)	5-F-uracil	—	(ni)		Flaks and Cohen (1959)
			5-F-deoxyuridine	4	40		
			5-F-UMP	0.001	0		
				0.002	32		
				0.005	87		
	Ehrlich ascites carcinoma	dUMP(0.025)	5-F-uridine	1	0		Hartmann and Heidelberger (1961)
			5-F-deoxyuridine	0.1	15		
			5-F-dUMP	0.00003	67		
				0.0002	84		
			5-F-dCMP	0.001	19		
				0.01	100		
UDP glucose:α-1,4-glucan-α-4-glucosyl-transferase	Rat liver	UDP glucose(5)	UMP	5	50		Leloir and Goldemberg (1960)
	Rat muscle	UDP glucose(4.6)	UDP	0.5	18		Leloir *et al.* (1959)
				1	31		
Xanthosine-5′-P aminase (GMP synthetase)	*E. coli*	Xanthosine-5′-P and ATP	Psicofuranine	2.5	75		Slechta (1960)

[a] The concentrations of the substrates in m*M* are sometimes shown in parentheses. X is undesignated concentration. The designations (i), (ni), and (st) indicate inhibition, no inhibition, and stimulation, respectively, when data are lacking.

nucleotide is a factor in the binding. However, the binding energy does not always increase with increase in phosphate residues or total negative charge; the potencies of the inhibition of pig kidney phosphodiesterase are AMP > ADP > ATP. Little is known about the topography of enzyme sites for nucleotides but it is clear that multiple binding sites must be involved. The relative binding energies for competitive inhibitors of yeast pyridoxal kinase (see tabulation) (Hurwitz, 1953) show that here the purine

Inhibitor	Relative $-\Delta F$ of binding (kcal/mole)
Adenine	4.60
Adenosine	4.55
AMP	4.51
ADP	5.17
ITP	3.77
Pyrophosphate	3.90

component is of primary importance, addition of ribose or phosphate residues having little effect. Yet ITP inhibits while inosine does not, and pyrophosphate is bound fairly tightly to the enzyme. The phosphate residues in ADP must not be oriented for interaction as optimally as free pyrophosphate.

Azaguanine and Azauracil

The 8-azapurines are usually inhibitory to growth and this has been generally attributed to the incorporation of these analogs to form abnormal polynucleotides which are nonfunctional or inhibitory. However, it has more recently been found that these analogs or their immediate metabolic products are potent inhibitors of certain enzymes involved in purine metabolism, and it is possible that such actions contribute to the growth depression. 8-Azaguanine has been studied the most thoroughly and has been shown to inhibit the growth of many bacteria, fungi, algae, viruses, tissue culture cells, chick embryos, epithelium, and tumors. It is usually antagonized by guanine or guanidylate, and occasionally by other purines, nucleosides, and nucleotides. The intention is not to discuss these azapurines in detail since it is a very large subject but to mention only a few observations bearing on enzyme inhibition.

Adenosine deaminase is inhibited reversibly by 8-azaguanine ($K_i = 0.28$ mM) and the inhibition was stated to be noncompetitive, although the $1/v$-$1/(S)$ plot appears to indicate uncompetitive or coupling inhibition (Feigelson and Davidson, 1956 b). Xanthine oxidase is also strongly inhibited (Table 2-2). Unfortunately, very few enzymes operative in purine

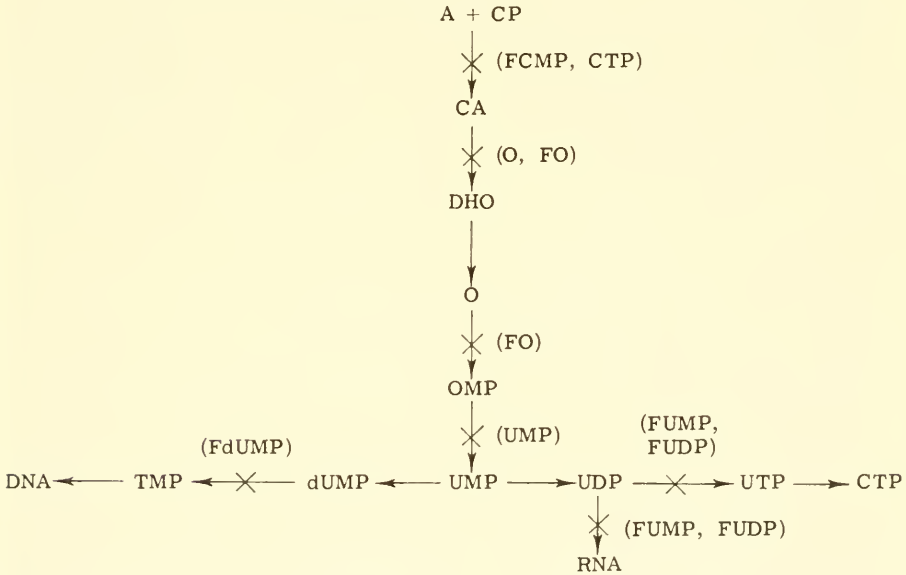
metabolism have been examined with respect to 8-azaguanine inhibition, or to inhibition by nucleosides and nucleotides of 8-azaguanine, but this type of mechanism for the growth inhibition must be borne in mind. 8-AzaGTP is formed from 8-azaguanine and can serve as a substrate for adenylosuccinate synthetase; it also inhibits competitively with respect to GTP (Cohen and Parks, 1963). Here one sees the interesting situation in which 8-azaGTP stimulates the rate when GTP is low and inhibits the rate when GTP is high. It was pointed out that in such cases one might obtain selective inhibition of an enzyme in tissues having a relatively high substrate concentration. This behavior is characterized in the double reciprocal plot by the curve for the inhibited reaction crossing the curve (or straight line) for the uninhibited reaction and being nonlinear. 8-Azaguanine suppresses the induction of liver glucose-6-phosphatase, fructose-1,6-diphosphatase, and tryptophan pyrrolase in the rat (Kvam and Parks, 1960) and the formation of catalase in yeast (Bhuvaneswaran *et al.*, 1961). These effects on protein synthesis may relate to an interference with nucleic acid metabolism, but whether it is a general depression or a more specific block is not known. Studies on 8-azaguanine in animals are complicated by the rapid deamination to the relatively noninhibitory 8-azaxanthine so that only a fraction of the quantity fed is available for either inhibition or incorporation (Mandel, 1955). Thus the usefulness of 8-azaguanine in tumoristasis is limited.

6-Azauracil is also generally growth-inhibiting and tumoristatic. It is possible that 6-azauridine-5′-P (6-azaUMP) is the true inhibitor, since it has been shown that orotidylate decarboxylase is strongly inhibited by 6-azaUMP, leading to the accumulation of orotidylate (Handschumacher and Pasternak, 1958; Pasternak and Handschumacher, 1959). 6-Azauridine is metabolized to 6-azaUMP and inhibition of the decarboxylase after administration of 6-azauracil was demonstrated, so it is likely that the block in pyrimidine metabolism is at this point and that this is an important mechanism in the tumoristatic action. The inhibition of the decarboxylase is characterized by a K_i of 0.00075 mM (Handschumacher, 1960). It is interesting to speculate that a similar mechanism might be involved in the action of 8-azaguanine.

Fluoropyrimidines and Feedback Inhibitions in Pyrimidine Pathways

The fluoropyrimidines are among the most potent inhibitors of nucleic acid biosynthesis yet discovered but the sites of action have not been completely elucidated. The 5-halogen analogs of orotate inhibit the conversion of orotate to the uridine phosphates, the most active being the fluoro compound (Stone and Potter, 1957). It was suggested that some of the action could be due to nucleotide analogs formed from these, and it was shown that 5-fluoroorotate is converted to 5-FUMP in yeast (Dahl *et al.*, 1959).

However, 5-fluoroorotate blocks earlier in the sequence, an inhibition of dihydroorotase, which forms dihydroorotate by cyclization of carbamylaspartate, having been observed (Smith and Sullivan, 1960). Orotate also inhibits but more weakly, this being an example of negative feedback. Orotidylate decarboxylase which forms UMP is inhibited by UMP, although not by uridine, UDP, or UTP, and it is possible that 5-FUMP would also inhibit at this locus (Blair and Potter, 1961). The conversion of dUMP to TMP by thymidylate synthetase, which is phage-induced in *E. coli*, is very potently inhibited by 5-F-dUMP, K_i being around 0.00005 mM, and following a competitive phase there is irreversible reaction with the enzyme (Mathews and Cohen, 1963). This illustrates the principle that analogs often simulate feedback inhibition if they are structurally similar to the compound normally exerting the inhibition. Some of the inhibitions observed in pyrimidine nucleotide metabolism are shown in the following scheme modified from Smith and Sullivan (1960).



(A = aspartate, CP = carbamyl-P, CA = carbamylaspartate, DHO = dihydroorotate, O = orotate, OMP = orotidine-5′-P, and fluorinated analogs are indicated by an inital F).

The effects of 5-fluorouracil on protein synthesis in *E. coli* and *B. megaterium* are very interesting because total protein synthesis is not altered significantly but the proteins formed have abnormal amino acid compositions (Gros and Naono, 1961). For example, the proteins contain less proline and tyrosine but more arginine. The alkaline phosphatase has normal catalytic activity but is less thermostable, whereas α-galactosidase

seems to be synthesized, as shown serologically, but is catalytically inactive. An RNA fraction into which 5-fluorouracil is rapidly incorporated was detected and it is possible that this is responsible for the changes in protein synthesis. The halopyrimidines are very potent growth inhibitors but, although much is known of their fate and actions (Brockman and Anderson, 1963), the over-all effects produced are usually so complex that the primary sites of block have not yet been determined. These analogs can be metabolized into such a variety of abnormal substances which can inhibit at many different sites that it is likely no single mechanism for the growth inhibition will be found. More investigations of the changes in the steady-state concentrations of the intermediates in these pathways, as they are affected by the analogs, would be valuable in determining the important loci attacked.

A few examples of enzyme inhibitions which may be involved in feedback regulation of pyrimidine and purine metabolism will be cited because of the importance of this type of inhibition in the control of nucleic acid and protein synthesis.* Gots and Gollub (1959) described the suppression of the formation of purine precursors in *E. coli* whenever a purine which supports growth is added, and the accumulation of 5-amino-4-imidazolecarboxamide is suppressed in certain mutants. Various purine analogs (e.g., 6-mercaptopurine and 2,6-diaminopurine) act like the normal feedback inhibitors, only more potently. These analogs can thus act on at least two sites in the biosynthetic sequence, the formation of purine precursors and the eventual utilization of the purines, their therapeutic usefulness possibly being related to this type of sequential inhibition. It may be noted, however, that Rubin *et al.* (1964) have recently shown that sequential inhibition in pyrimidine biosynthesis is not synergistic. Combinations of 5-azaorotate, which competitively inhibits the conversion of orotate to orotidylate, and 6-azauridine, which after its metabolism to 6-azauridylate competitively inhibits the conversion of orotidylate to uridylate, do not produce greater inhibitions in either isolated enzyme systems or leucocytes than are seen with the individual analogs. Almost every step in the nucleotide synthesis has been shown to be inhibited by more distal intermediates. The PRPP-amidotransferase, which catalyzes the first irreversible and specific step in purine synthesis, utilizing glutamine as the amino donor, is inhibited by AMP, ADP, ATP, GMP, GTP, other nucleotides, and some analogs (Wyngaarden and Ashton, 1959); adenylosuccinate synthetase (Wyngaarden and Greenland, 1963), aspartate transcarbamylase (Bresnick, 1963; Gerhart and Pardee, 1962), phosphoribosylformylglycineamidine synthetase (Henderson,

* Although an intermediate or product in a metabolic sequence is shown to be an inhibitor of an enzyme catalyzing a previous step, it is perhaps not justified to call it a feedback inhibitor, which implies that inhibition occurs during the *in vivo* operation of the pathway. For various reasons the substance may not play a role in regulating metabolism, even though it is a reasonably potent inhibitor.

1962), and other enzymes are inhibited in similar manner but each exhibits a unique pattern (Table 2-28); while TTP inhibits several steps in its formation, including CDP → dCDP, deoxyuridine → dUMP, and deoxythymidine → dTMP (Ives *et al.*, 1963). All of these inhibitions and many more constitute possible feedback situations, but in the cell probably only a few are important, since the concentrations of some intermediates may never rise sufficiently to exert an effect, and compartmentalization may limit the actions of these inhibitors. We have mentioned that certain enzymes appear to contain sites specially evolved for feedback inhibition (Gerhart and Pardee, 1962, 1964), the best documented case being aspartate transcarbamylase, which is inhibited particularly well by CMP, CDP, and CTP. This enzyme is normally a tetramer and it may be that these inhibitors alter the subunit interactions since the monomer is not inhibited. Another interesting example of this phenomenon is the inhibition of xanthosine-5′-P aminase by psicofuranine (9-D-psicofuranosyl-6-aminopurine), which occurs in two steps, a reversible pyrophosphate-dependent reaction with the enzyme and an irreversible xanthosine-5′-P-dependent reaction (Udaka and Moyed, 1963). The first step can be observed in a psicofuranine-resistant bacterial strain and here the inhibition is noncompetitive. It would appear that the inhibitor is bound to a different site than that at which the substrate reacts and this second site could have regulatory function.

Some interesting results have been obtained in the analysis of the inhibitions produced by the metabolites of 6-mercaptopurine, a few of which will be mentioned briefly. One product into which 6-mercaptopurine is converted is 6-thio-IMP, a potent competitive inhibitor of IMP dehydrogenase (which is involved in the formation of GMP in certain cells) ($K_i = 0.0036$ mM) (Atkinson *et al.*, 1963). The inhibition proceeds rather slowly, requiring 10–20 min for completion, and the enzyme is then inactivated (Hampton, 1963). Evidence was presented that reaction occurs with an SH group on the enzyme, a stable disulfide bond being formed with the 6-thio-IMP. This is one example where an analog turns out to be an SH reagent. On the other hand, adenylosuccinate lyase is inhibited by 6-thio-IMP only if a metal ion is present and it was postulated that the metal ion forms a bridge between the SH groups (E-S-Me-S-IMP) (Bridger and Cohen, 1963). These inhibitions create new possibilities by which analogs can inactivate enzymes. Another product derived from 6-mercaptopurine is 6-mercaptopurine riboside-5′-diphosphate, which inhibits polynucleotide phosphorylase quite potently (50% inhibition by around 0.03 mM), rapidly, and competitively (Carbon, 1962). The role this enzyme plays *in vivo* or the significance of such inhibition is not known.

These few remarks on the effects of nucleotides and related substances are made only to suggest certain interesting aspects of enzyme inhibition which broaden our concepts of how analogs may act; adequate coverage of this subject, young as it is, would require a volume of this size or more.

INHIBITIONS BY COENZYME ANALOGS

This field in which interest was stimulated by the demonstration of the mechanism of sulfonamide action is a large one because of the great amount of work done on the growth inhibition of microorganisms by these analogs, so that here the presentation will be restricted to those aspects directly related to enzyme inhibition and specific metabolic disturbance. The competition between a coenzyme analog and a coenzyme for combination with the apoenzyme for which the coenzyme is essential is basically of the same nature as the examples of substrate analogs discussed previously. However, there are usually additional complexities due primarily to the greater number of sites for antagonism. Figure 2-16 indicates some of the reactions

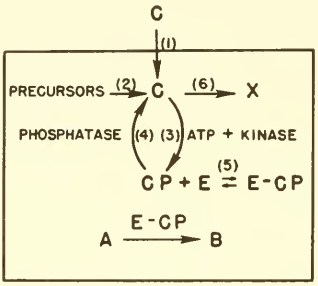


FIG. 2-16. General scheme for the formation and possible reactions of coenzymes in cells. C = a unit of the active coenzyme (e.g., nicotinamide, adenine, or thiamine), CP = the active coenzyme, E-CP = the active enzyme-coenzyme complex for the reaction A → B, and X = any derived substance from the unit C, which may be inactive, or active to some degree after phosphorylation, or capable of interfering with the formation or action of the coenzyme. Reaction 1 may be a simple diffusion into the cell or be mediated by facilitated diffusion or active transport; reaction (2) occurs in cells which synthesize the coenzyme from precursors; reaction (3) is usually a phosphorylation; reaction (4) is a dephosphorylation; and reaction (5) represents the complexing of the coenzyme with the apoenzyme.

involved in coenzyme formation, destruction, and function. Inasmuch as the catalytically active forms of most coenzymes are formed within cells from precursors, these reactions and the membrane processes responsible for entrance of the precursors must be considered as possible loci for analog interference. Furthermore, in many instances the analogs are metabolized along the same pathways as the coenzymes to form inhibitory products. Certain coenzymes are active in phosphorylated forms and the reaction immediately forming the active coenzyme is often a phosphorylation in-

volving ATP and a kinase. The analogs are occasionally phosphorylated and exert their major effects in this form. The inability of most phosphorylated substances to enter cells readily makes it necessary to use the analog of the coenzyme precursor if inhibition in cell preparations is to be obtained. Thus the initial analog or any of its metabolic products may interfere in a number of reactions involving the coenzyme, and it is this that militates against facile interpretations from superficially simple results. It should also be evident that when the reaction catalyzed by the coenzyme-dependent enzyme (A → B in Fig. 2-16) is determined, the kinetics of inhibition by an analog of the coenzyme precursor will generally not be simple and, although the fundamental block may be strictly competitive, the quantitative relationship between the analog and the precursor will not necessarily be competitive.

The direct effect of a coenzyme analog on the enzyme reaction requiring the cooperation of the coenzyme will depend on the tightness with which the coenzyme is bound to the enzyme. Some coenzymes are so tightly bound that they remain on the enzyme through numerous isolation procedures, and in such cases the addition of an analog, even though it has a high affinity for the enzyme, may not be able to replace the natural coenzyme rapidly enough to induce inhibition. It must be remembered that the analog does not actively displace the coenzyme (i.e., it does not force it from the enzyme) but only binds to the free enzyme; if essentially all of the enzyme is combined with coenzyme, there is little opportunity for the analog to act. For this reason experiments on coenzyme analogs are frequently done with reconstituted enzymes. In such cases the enzyme and coenzyme are dissociated by some means and the effect of the analog on the reconstitution of the active enzyme is investigated, this allowing the analog to act on the free enzyme and to demonstrate competitive behavior. This technique is not, of course, so applicable to cellular systems.

It has been frequently stated that coenzyme analogs are specific inhibitors. This is true in one sense inasmuch as these analogs or their metabolic derivatives appear to interfere only with those enzymes or reactions involving the corresponding normal coenzymes, in most instances. On the other hand, the coenzymes often participate in several different types of metabolic activity so that the metabolic disturbances produced by the analogs may not be specific with respect to a single reaction. For example, analogs of pyridoxal seem to interfere specifically with pyridoxal metabolism or the functions of pyridoxal phosphate, but pyridoxal phosphate plays a role in many reactions of amino acids — racemization, transamination, oxidative deamination, decarboxylation, hydrolytic cleavage — as well as being an important component of other enzyme systems, such as muscle phosphorylase, so that a deficiency of pyridoxal phosphate can induce widespread disturbances. In addition to this, a generalized depression of amino

acid metabolism can secondarily bring about changes in systems not involving pyridoxal phosphate through reduction in the concentrations of amino donors or suppression of enzyme synthesis. Whether the coenzyme analogs can be considered as specific or not will depend on the complexity and general metabolic activity of the preparation being studied.

Most coenzymes are derivatives of vitamins and it has usually been anticipated that analogs would induce vitamin-deficiency states. This has been demonstrated in some cases; that is, effective analogs have been found to produce a pattern of symptoms roughly similar to those seen in deficiency of the corresponding vitamin. Nevertheless, it should be clearly understood that the situations are basically different. A dietary restriction of a vitamin leading to a generalized depletion in the tissues would not necessarily bring about functional changes identical to those caused by an analog, which could be much more effective in interfering with certain functions of the coenzyme than simple depletion and possibly leave other functions untouched. All of the various enzymes binding a particular coenzyme do not have the same affinities for an analog. Even though the analog primarily interfered with the transport of the vitamin into the cell, or blocked its further metabolism to the active coenzyme, it is not justifiable to conclude that a state of generalized depletion will result, because these effects will presumably not be exerted equally on all tissues. The differential penetration of the analog into the various tissues will perhaps be one important factor in determining the response. Contrary to vitamin depletion, analogs often cause a rise in the renal excretion of coenzyme or its metabolites, due to the displacement of the normal coenzyme by the analog in the tissues and its release from the cells. The analog might also alter the formation of the coenzyme from its precursors, or inhibit the metabolism of the active coenzyme, or in some manner change the renal excretion or resorption of the coenzyme or its precursors, so that a variety of effects on over-all excretion is possible. If it is desired to demonstrate metabolic or functional defects due to an analog in a short period of time, it is usually necessary to restrict the intake or reduce the medium concentration of the coenzyme or its precursor, since the relationship between the analog and the coenzyme is usually competitive or pseudocompetitive, but in such cases one must use the coenzyme-depleted preparation as a control to characterize the effects of the analog.

ANALOGS OF NICOTINAMIDE AND THE PYRIDINE NUCLEOTIDES

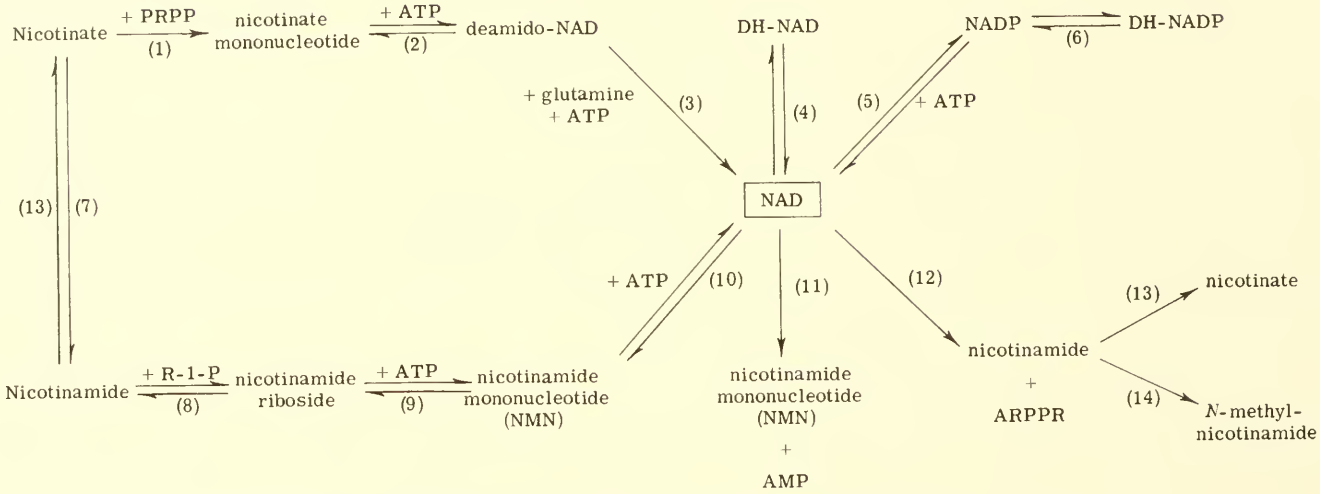
The importance of nicotinate and nicotinamide is as precursors of the coenzymes NAD and NADP, and they do not, as far as is known, act directly in any metabolic system, nor do they usually occur in significant concentrations in living cells. Some of the reactions involved in NAD syn-

thesis and breakdown are shown in the accompanying diagram. The major route of NAD formation, at least in mammalian tissues, is probably through reactions (1)–(3) since the alternative pathway (8)–(10) is kinetically and thermodynamically unfavorable. Analogs of nicotinate can thus either directly inhibit any of these reactions or enter into the reactions to form abnormal intermediates, and perhaps analogs of NAD or NADP, which are inhibitory. (See reactions on page 486).

Inhibition of NAD Nucleosidase (NADase) by Nicotinamide and Related Compounds

It will be convenient to discuss first the direct inhibitions by simple pyridine derivatives and then proceed to those substances incorporated into NAD analogs. There is a constant turnover of NAD in tissues and at least a fraction of the degradative process is attributable to NADase, and in tissue extracts or homogenates the splitting of NAD may be an important factor determining the dehydrogenase activity. Thus inhibitors of NADase might be expected under certain circumstances to protect the coenzyme. Furthermore, it will be evident later that the mechanisms of NADase inhibition are involved in the formation of abnormal NAD analogs. Mann and Quastel (1941) were the first to observe an inhibition of NAD breakdown by nicotinamide. They worked with brain suspensions and determined NAD by adding lactate dehydrogenase and lactate. Nicotinamide at 25 m*M* was found to prevent the breakdown of NAD almost completely, and addition of nicotinamide increases the respiration of various systems oxidizing lactate by preventing the destruction of NAD. Nicotinate, on the other hand, is completely inactive. Many investigators have subsequently used nicotinamide to preserve NAD in various preparations, often in very high concentrations and without regard for the other possible inhibitions it might exert. Handler and Klein (1942) soon showed that NADP splitting is also inhibited by nicotinamide.

McIlwain and Rodnight (1949) pointed out that the indiscriminate use of high nicotinamide concentrations to protect NAD in metabolic studies is unnecessary, since almost complete inhibition of NADase is seen at concentrations from 2 to 10 m*M* (actually they showed that 2.67 m*M* inhibits 73%). The problem of the proper concentration of nicotinamide to use is a difficult one because the NADases of various tissues and organisms show marked differences in susceptibility to inhibition. The early work was all done on brain NADase, which is quite sensitive, and it has been found that some other NADases are also sensitive, e.g., from beef spleen (Zatman *et al.*, 1953). However, the enzymes from rabbit erythrocytes (Alivisatos and Denstedt, 1952; Rubinstein *et al.*, 1956; Malkin and Denstedt, 1956), mouse mammary gland and tumor (Branster and Morton, 1956), and lupine seedlings (Hasse and Schleyer, 1961) are only moderately sensitive to nicotin-



(PRPP = 5-phosphoribose-1-pyrophosphate; ARPPR = adenosine pyrophosphorylribose; R-1-P = ribose-1-phosphate; DH = any dehydrogenase)

(2) NAD pyrophosphorylase
(3) NAD synthetase
(5) NAD phosphokinase
(9) nicotinamide nucleoside phosphokinase
(10) NAD pyrophosphorylase
(11) nucleotide pyrophosphatase
(12) NAD nucleosidase (NADase)
(13) nicotinamide deaminase
(14) nicotinamide transmethylase

amide (K_i's usually between 20 and 100 mM). The NADase from *Neurospora crassa* is quite resistant to nicotinamide (Kaplan *et al.*, 1951).

The inhibition by nicotinamide is competitive with respect to NAD for the weakly inhibited NADases of rabbit erythrocytes (Alivisatos *et al.*, 1956; Hofmann, 1955), lupine seedlings (Hasse and Schleyer, 1961), and *Neurospora.* However, the inhibition of the sensitive mammalian NADases from brain and spleen is noncompetitive and the elucidation of the mechanism by Zatman *et al.* (1953) has provided important information on NAD metabolism and its inhibition by a variety of agents. The binding of nicotinamide to the enzyme is, however, readily reversible upon dilution or dialysis. The following reaction mechanism was suggested as a working hypothesis:

$$\begin{array}{l} {}^{+}\text{NRPPRA} + \text{enzyme} \rightleftarrows \text{enzyme} - {}^{+}\text{RPPRA} + \text{N} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \Big\downarrow + \text{H}_2\text{O} \\ \qquad\qquad\qquad\qquad\qquad\qquad \text{enzyme} + \text{RPPRA} + \text{H}^{+} \end{array}$$

An intermediate enzyme complex which is subsequently hydrolyzed is assumed. The hydrolysis is irreversible and N + RPPRA will not form NAD. The inhibition by nicotinamide is thus a competition with water for the enzyme-$^{+}$RPPRA complex. This complex is not a Michaelis-Menten ES complex but a covalent-linked compound in which the energy of the nicotinamide—riboside bond is conserved. The free energy for the hydrolysis of this bond is —8.2 kcal/mole, and its conservation in the complex is very important for the exchange reactions catalyzed by this enzyme. If this mechanism is valid, one should observe exchange between free nicotinamide and the nicotinamide in NAD, and this was demonstrated by using nicotinamide-C^{14}. These NADases might be considered as transglycosidases and able to transfer the RPPRA group to compounds structurally related to nicotinamide to form NAD analogs (Zatman *et al.*, 1954 a). The NADases which are weakly inhibited do not operate by such a mechanism and do not catalyze exchange reactions.

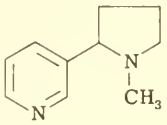
Another enzyme which is nicotinamide-sensitive and catalyzes a similar exchange reaction is the nicotinamide riboside phosphorylase of human erythrocytes (Grossman and Kaplan, 1958 a, b). $1/v$-$1/(S)$ plots showed the inhibition to be uncompetitive, which is usually interpreted as a combination of the inhibitor with the ES complex, but in this case is perhaps due to the transfer nature of the reaction. In the scheme:

$$\begin{array}{lll} & \text{ERN} \rightleftharpoons \text{E} + \text{RN} \\ \overset{+\text{N}}{\rightleftharpoons} & & \\ \text{ER} & & \\ \underset{+\text{P}}{\rightleftharpoons} & & \\ & \text{ERP} \xrightarrow{k} \text{E} + \text{RP} \end{array}$$

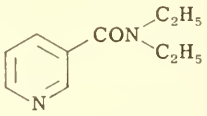
where nicotinamide riboside is the substrate and $-d(\text{RN})/dt = k(\text{ERP})$, it is seen that nicotinamide will slow the reaction by shifting the equilibrium

in favor of ERN, and that actually competition with phosphate rather than with nicotinamide riboside might be expected. A further complication is the finding that the exchange reaction and the sensitivity to nicotinamide depend on a cofactor, which was isolated and shown to be either ergothioneine or a closely related compound. It is possible that ergothioneine acts as a ribosyl acceptor and this would modify the kinetics of the nicotinamide inhibition. It is interesting that ergothioneine will make the *Neurospora* NADase inhibitable by nicotinamide.

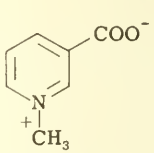
A variety of substances related to nicotinamide or other portions of the NAD molecule are inhibitory to NADases (Table 2-29). There is a good deal of variation in susceptibility between the different enzymes. In this connection it may be mentioned that Handler and Klein (1942) found that rabbit brain NADase is readily inhibited by 5–10 m*M* nicotinamide but not inhibited at all by 160 m*M* picolinate, quinolinate, benzamide, α-aminonicotinate, trigonelline, adenine, adenosine, or pyridine. These inhibitions probably involve different mechanisms. Some are not competitive and the inhibitors probably participate in the transfer reaction as does nicotinamide, while others are competitive and the inhibitors are bound reversibly to sites at the active center, thereby preventing the binding of NAD. The inhibition



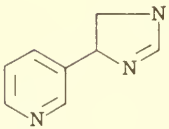
Nicotine



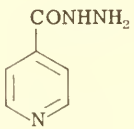
Nikethamide



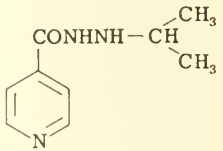
Trigonelline



4(5)-3′-Pyridyl-glyoxaline



Isoniazid



Iproniazid

of beef spleen NADase by isoniazid exhibits unique kinetics inasmuch as an increase in the NAD concentration actually increases the inhibition, this suggesting that some interaction between NAD and isoniazid occurs, the analog formed being the active inhibitor. Isonicotinamide inhibits similarly to isoniazid. 3-Substituted pyridines generally inhibit somewhat more strongly than the 4-substituted compounds. One of the most potent inhibitors is

4(5)-3′-pyridylglyoxaline but the mechanism is unknown; it is quite possible that NADases other than from brain may not be so potently inhibited by this substance since nicotine does not inhibit the beef spleen enzyme. Another surprisingly potent inhibitor is theobromine, which is bound to rabbit erythrocyte NADase around 1 kcal/mole more tightly than the other purines tested, the inhibition being competitive. Malkin and Denstedt (1956) concluded from the inhibition data that NAD is attached to the enzyme surface at the quaternary nitrogen and the pyrophosphate group. It is rather strange that adenine is a reasonably effective inhibitor, whereas adenosine or the adenine nucleotides are much weaker or completely without action, since, if adenine were bound in the same position as it is when part of the NAD molecule, one might expect ribose and phosphate groups to augment the binding. Thus the inhibition by adenine and other purines may involve interaction with the enzyme surface in a manner unrelated to the normal binding of the purine component of NAD. This is further borne out by the studies on multiple inhibition by Hofmann and Rapoport (1957) (see accompanying tabulation), inasmuch as adenine does not add to the inhibitions produced by inhibitors presumably interacting with enzyme groups binding NAD.

Inhibitors	(I)/(S)	% Inhibition
Nicotinamide	50	64
Adenine	9	20
Both		65
NMN	10	44
Adenine	9	20
Both		43
NADP	4.5	40
Adenine	9	20
Both		39

3-Acetylpyridine and the Formation of Analogs of NAD

In a search for pyridine derivatives which might have vitamin activity against black tongue in dogs, Woolley *et al.* (1938) observed that 3-acetylpyridine is not only ineffective but kills nicotinamide-deficient animals in 1 day, normal dogs being unaffected. 3-Acetylpyridine rapidly produces signs of nicotinate deficiency in mice and at the LD_{50} (around 3 mg per day) the animals succumb in 3 to 4 days (Woolley, 1945 b). The effects produced are: rapid respiration, motor incoordination followed by complete paralysis, emaciation, and inflamed skin and tongue. The mice can be completely

TABLE 2-29

INHIBITION OF NAD NUCLEOSIDASES BY NICOTINAMIDE AND VARIOUS ANALOGS

Source	Analog	NAD concentration (mM)	Analog concentration (mM)	% Inhibition	K_i (mM)	Reference
Mycobacterium tuberculosis	Nicotinamide	0.4	10	0		Gopinathan *et al.* (1964)
			50	30		
	Nicotinate	0.4	10	0		
			50	10		
	Isoniazid	0.4	1	0		
Neurospora crassa	Nicotinamide	0.5	33	0		Kaplan *et al.* (1951)
Lupine seedlings	Nicotinamide	—	—	—	28	Hasse and Schleyer (1961)
Pig spleen	Nicotinamide	0.4	1	59		Dickerman *et al.* (1962)
	Adenine	0.4	1	30		
	Adenosine	0.4	1	0		
	Adenosine-2′-P	0.4	50	Stim 10		
	ADP	0.4	1	0		
Beef spleen	Nicotinamide	0.6	1.5	50		Zatman *et al.* (1954 a)
	Nicotinate	0.6	10	20		

	Isonicotinate	0.6	10	15	
	Pyridine-2-carboxylate	0.6	10	0	
	Pyridine-3-sulfonate	0.6	10	10	
	Nicotine	0.6	5	0	
	Isoniazid	0.6	0.027	13	
			0.05	35	
			0.1	52	
			0.67	95	
Sheep brain	Nicotinamide	0.5	0.33	10	McIlwain (1950)
			0.67	40	
			1.33	56	
			2.67	73	
	Nicotine	0.5	0.17	35	
			1.67	53	
	Niketham ide	0.5	3.3	5	
	Trigonelline	0.5	8.3	Stim 5	
	Pyridine-3-sulfonamide	0.5	8.3	Stim 4	
	4(5)-3′-Pyridylglyoxaline	0.5	0.01	5	
			0.03	21	
			0.1	47	
			0.33	86	
			1.67	95	
	NADH	0.5	0.4	2	
Ox spinal cord	3-Acetylpyridine	0.5	11	65	McIlwain (1950)

TABLE 2-29 (*continued*)

Source	Analog	NAD concentration (mM)	Analog concentration (mM)	% Inhibition	K_i (mM)	Reference
Rabbit erythrocytes	Theobromine	—	—	—	0.66	Alivisatos *et al.* (1956)
	Adenine	—	—	—	2.4	
	Theophylline	—	—	—	2.5	
	Benzimidazole	—	—	—	2.5	
	Thymine	—	—	—	2.8	
	Nicotinamide	—	—	—	2.9	
	Xanthine	—	—	—	7	
	Adenosine	—	—	—	10	
	Adenosine-3′-P	—	—	—	110	
	Caffeine	—	—	—	120	
	Adenosine-5′-P	—	—	—	180	
	Ribose	—	—	—	650	
Rabbit erythrocytes	Nicotinamide riboside	0.67	0.17	0		Malkin and Denstedt (1956)
	Adenine	0.67	5	40		
	Adenosine	0.67	30	0		
	AMP	0.67	18.7	0		
	ADP	0.67	18.7	20		
	ATP	0.67	18.7	0		
	Pyrophosphate	0.67	25	0		

Rabbit erythrocytes	Nicotinamide	0.24	15	73		Hofmann and Rapoport (1957)
	Isonicotinamide	0.24	15	46		
	Nicotinate	0.24	50	10		
	Nicotinic hydrazide	0.24	15	20		
	Isoniazid	0.24	15	5		
	Nicotinic ethyl ester	0.24	15	25		
	Methylnicotinamide	0.24	15	20		
	NMN	0.24	2.16	38		
			3.6	54		
			6.48	95		
	Adenine	0.24	1.2	14		
			2.16	20		
	Thymine	0.24	2.64	40		
	Uracil	0.24	2.64	0		
	ADP	0.24	2.4	0		
	NADH	0.24	2.88	0		
Rabbit erythrocytes	Nicotinamide	0.16	40	40	18	Hofmann (1955)
Mouse mammary tumor	Nicotinamide	0.4	200	41		Branster and Morton (1956)
			400	55		
			600	93		
			800	100		
Rat brain	Nicotinamide	1.5	15	70		Kaplan *et al.* (1951)

protected by providing nicotinate or nicotinamide in the diet. On the other hand, yeast and most bacteria seem to be quite resistant to 3-acetylpyridine, although the growth of *Lactobacillus casei* in nicotinate-free medium is inhibited around 50% at 16.5 m*M*, a depression that can be reversed by nicotinate but not by nicotinamide. Chick embryos are killed by 450–600 μg 3-acetylpyridine injected into the eggs and sublethal doses cause disturbances in embryogenesis (Ackermann and Taylor, 1948). These effects can be completely reversed by nicotinamide; even 6000 μg/egg of the analog can be counteracted by 380 μg of nicotinamide, indicating a competitive relationship. Changes in the heart, characteristic of nicotinate deficiency, are produced by perfusion of 1.6–8 m*M* 3-acetylpyridine through the isolated rabbit heart, dysrhythmias and a-v block occurring within 30 min (Braun, 1949). Subsequent perfusion with nicotinamide reverses these effects but the concentration must be around 100 times that of the analog. These early observations all point to the interference by 3-acetylpyridine in the metabolism or function of nicotinate or nicotinamide. If it is assumed that the primary role of these metabolites is the formation of the NAD and NADP coenzymes, the following possible mechanisms for inhibition by 3-acetylpyridine might be imagined. (1) Inhibition of some step in the synthesis of NAD [especially reactions (1) to (3) in the scheme on page 486], (2) inhibition of the interconversion of nicotinate and nicotinamide, (3) entrance into one of the pathways of nicotinate metabolism to form inhibitory intermediates, (4) formation of an NAD analog, either through the normal pathway or by the exchange reaction catalyzed by NADase, (5) inhibition of NADases and related enzymes, and (6) direct interference with NAD or NADP to inhibit dehydrogenase activity.

It will be well to consider certain aspects of the metabolism of 3-acetylpyridine before taking up the problem of how this analog induces its inhibitory effects. 3-Acetylpyridine at doses around 0.5 g/day increases the urinary excretion of *N*-methylnicotinamide in both normal and nicotinate-deficient dogs (Gaebler and Beher, 1951). The *N*-methylnicotinamide could arise either from a disturbance of nicotinamide metabolism, since *N*-methylation is an important reaction in the elimination of nicotinamide, or directly from the 3-acetylpyridine. The oxidation of 3-acetylpyridine to nicotinate might be anticipated because benzoate is formed from acetophenone in the tissues, the entire sequence being

3-acetylpyridine → nicotinate → nicotinamide → *N*-methylnicotinamide.

It was found that the latter explanation is correct by determining labeled *N*-methylnicotinamide formed from labeled 3-acetylpyridine (Beher *et al.*, 1952). It was pointed out that in the course of its oxidation and methylation the analog might also interfere with nicotinate metabolism. Since *N*-methylnicotinamide accounts for only about 10% of the administered 3-acetyl-

pyridine, other excretory products were investigated and increased urinary nicotinate and various glucuronides were found (Beher and Anthony, 1953). No urinary 3-acetylpyridine could be detected. An interesting suggestion that the oxidation of 3-acetylpyridine may involve NAD(P) enzymes was made; this might mean that in nicotinate-deficient animals, where NAD(P) levels are low, the oxidation of 3-acetylpyridine would be impaired and the analog would be more toxic. 3-Acetylpyridine presents the strange situation wherein the analog is detoxified to the normal metabolite, and this would presumably tend to counteract the inhibitory effects. In low dosage (25–60 mg/day), 3-acetylpyridine can protect against black tongue in dogs but at higher dosage it can create a nicotinate deficiency (McDaniel *et al.*, 1955). Animals may have a limited ability to oxidize 3-acetylpyridine; small amounts are mainly oxidized and little 3-acetylpyridine is left to inhibit, whereas the larger doses exceed the metabolic capacity of the system. This is indicated by the results of Guggenheim and Diamant (1958), who determined the excretion of *N*-methylnicotinamide in rats given comparable doses of nicotinamide and 3-acetylpyridine (see tabulation). Beyond a dose of 50

Dose (mg/kg)	*N*-Methylnicotinamide excretion from:		Ratio [a]
	Nicotinamide	3-Acetylpyridine	
0	23	23	—
10	65	40	2.5
20	182	105	1.9
50	225	120	2.1
100	700	218	3.5
200	1950	376	5.5

[a] Ratio calculated after substracting endogenous excretion.

mg/kg there seems to be relatively less oxidation of the analog. Administration of 3-acetylpyridine-$C^{14}H_3$ to rats leads to 30% of the activity expired as CO_2 and 44% eliminated in the urine during 24 hr (Beher *et al.*, 1959); since the total dosage was probably around 100 mg/kg, smaller doses might be even more efficiently oxidized. 3-Acetylpyridine is also partially metabolized to NAD and the 3-acetylpyridine analog of NAD, as will be discussed shortly. Finally, nicotinamide mononucleotide excretion is augmented by 3-acetylpyridine and it is possible that this mainly originates directly from the analog (McDaniel *et al.*, 1955). The metabolism of 3-acetylpyridine and the compounds derived from it thus depend on the species, the dose, and whether the animals are normal or nicotinate-deficient.

We shall now examine the effects of 3-acetylpyridine on the tissue levels of NAD, the formation of NAD analogs, and the enzymic activities of these

analogs. Most of this work has been done by Kaplan and his associates at Johns Hopkins, and a summary of their most important results will be given. It was first demonstrated that the incubation of NAD, brain NADase, and isonicotinyl hydrazide (isoniazid, IHN) leads to the formation of the INH analog of NAD, which was isolated in good yield, and it was postulated that the antitubercular activity of isoniazid may be related to the appearance of this nonfunctional or inhibitory analog (Zatman *et al.*, 1954 b). It was soon shown that a variety of pyridine derivatives can exchange with nicotinamide in the presence of certain NADases to form NAD analogs; these include isonicotinamide, iproniazid, ethylnicotinate, and 3-acetylpyridine (N. O. Kaplan *et al.*, 1954). The formation of 3-AcPyr-NAD* in tissue homogenates and whole animals is inhibited by nicotinamide. The exchange reaction and hydrolysis may be represented as:

$$\begin{array}{ccccc} \text{NRPPRA} + \text{E} & \rightleftarrows & \text{E-NRPPRA} & & \\ & & \downarrow\uparrow +\text{N} & & \\ & & \text{E-RPPRA} & \rightarrow & \text{E} + \text{RPPRA} \\ & & \uparrow\downarrow +\text{X} & & \\ \text{XRPPRA} + \text{E} & \rightleftarrows & \text{E-XRPPRA} & & \end{array}$$

where NRPPRA is NAD, XRPPRA is the NAD analog, E-RPPRA is the relatively stable ribosyl enzyme complex, and X is the pyridine derivative exchangeable with nicotinamide. The over-all exchange reaction would be:

$$\text{NRPPRA} + \text{X} \rightleftarrows \text{XRPPRA} + \text{N}$$

Injection of 3-acetylpyridine leads to a rise in total pyridine nucleotides in most tissues; in the liver this is NAD and none of the analog is demonstrable, due presumably to the oxidation of 3-acetylpyridine to nicotinate, whereas in brain, spleen, and tumors 3-AcPyr-NAD appears. In tumors the NAD content actually decreases as 3-AcPyr-NAD increases. The equilibrium between NAD and any of its analogs, and the ratio of their concentrations in a particular tissue, will depend on (1) the ΔF between NAD and the analog, (2) the concentrations of N and X, (3) the rate of transformation of X to nicotinate, if it occurs, and (4) the relative bindings of NAD and its analogs to the dehydrogenases. The time courses for the formation of 3-AcPyr-NAD from 3-acetylpyridine and the toxic reactions led to the suggestion that the toxic and lethal actions are related to the NAD analog; whether the toxicity depends on a reduction of NAD or a rise in 3-AcPyr-NAD was undecided.

In order to determine the nature of the effects of 3-acetylpyridine on tissue metabolism, it will be necessary to consider the ability of 3-AcPyr-

* The analogs of NAD will be designated by prefixes of this type, following Kaplan, since this is convenient if not exactly accurate.

NAD to replace NAD as the coenzyme for the various dehydrogenases. 3-AcPyr-NAD can function in most NAD-dependent dehydrogenase reactions. In some cases it can be reduced more rapidly than NAD (horse liver alcohol dehydrogenase, beef liver glutamic dehydrogenase, *Lactobacillus* D- and L-lactate dehydrogenases) and in other cases proceeds more slowly (yeast alcohol dehydrogenase, beef heart lactate dehydrogenase, yeast glyceraldehyde-3-P dehydrogenase), while in a few instances the rates are approximately equivalent (rabbit muscle lactate dehydrogenase) (N. O. Kaplan *et al.*, 1956; van Eys *et al.*, 1958; N. O. Kaplan, 1959; Stockell, 1959). 3-AcPyr-NADP is reduced about one fifth as fast as NADP in the pig heart isocitrate dehydrogenase system and is inactive in erythrocyte glucose-6-P dehydrogenase (N. O. Kaplan *et al.*, 1956; Marks *et al.*, 1961). The relative rates do not necessarily reflect the relative bindings to the dehydrogenases. In those cases where coenzyme activity is low but binding is appreciable, the NAD or NADP analogs can inhibit the dehydrogenases; thus 3-AcPyr-NAD inhibits glucose-6-P dehydrogenase quite strongly (K_i= 0.03 mM) and this is competitive. NAD analogs other than 3-AcPyr-NAD are usually less active and tend to be more inhibitory. Thionicotinamide-NAD, nicotinyl-hydroxamate-NAD, and nicotinyl-hydrazide-NAD competitively inhibit lactate and alcohol dehydrogenases, whereas 3-benzoylpyridine-NAD inhibits beef heart lactate dehydrogenase uncompetitively (Anderson and Kaplan, 1959). The introduction of 3-acetylpyridine, or other pyridine analogs, can thus produce several effects on tissue dehydrogenase activity, and in the general case will bring about an imbalance of the normal relative substrate oxidations, due to altering the rates of the various dehydrogenases in different ways. Unfortunately there has not yet been sufficient study of the oxidative abilities of tissues isolated from animals treated with 3-acetylpyridine. However, it is probably safe to assume that at least a major cause of the toxic effects is the inhibition of certain dehydrogenases by the 3-AcPyr-NAD formed.

The various NAD analogs have been very useful in demonstrating differences between dehydrogenases from different tissues or species. For example, beef heart and rabbit muscle lactate dehydrogenases react better with NAD than with 3-AcPyr-NAD, but the lactate dehydrogenases from lobster heart and thorax muscle react better with the analog (N. O. Kaplan, 1959). Kaplan *et al.* (1960) have pointed out that the molecular heterogeneity of enzyme active centers has phylogenetic significance. It is possible to classify animals with respect to the affinities of their dehydrogenases for the coenzymes or their analogs, and it is hoped that further investigation along these lines will elucidade some of the evolutionary problems relative to the changes in the active center configurations.

We must now examine the evidence for other sites of action for 3-acetylpyridine and related analogs. McIlwain (1950) reported that 3-acetylpyri-

dine inhibits spinal cord NADase 65% at 11 mM. Such inhibition could be due to (1) direct competition with NAD, (2) reaction with E-RPPRA to form a relatively stable complex, thereby depleting free enzyme, or (3) inhibition by a 3-AcPyr-NAD analog formed. In the case of the INH-NAD analog, the inhibition seems to be mainly of the third type (Zatman *et al.*, 1954 b). Nicotinamide deaminase is inhibited quite well by 3-acetylpyridine; the inhibition is competitive and 50% at (I)/(S) = 20 (Grossowicz and Halpern, 1956 b). Yeast alcohol dehydrogenase is inhibited directly by substituted pyridines (van Eys, 1956; van Eys and Kaplan, 1957 a). The inhibitions are related to the pK_a's of the analogs (Table 2-30) and a

TABLE 2-30

INHIBITION OF YEAST ALCOHOL DEHYDROGENASE BY SUBSTITUTED PYRIDINES[a]

Substituted pyridine	pK_a	Concentration for 50% inhibition (mM)		
		Total base	Pyridinium ion	N-methyl derivatives
4-CH_3	6.11	20	0.013	—
3-CH_3	5.82	40	0.013	5.8
None	5.27	70	0.0066	5.5
3-$CONH_2$	3.40	230	0.00029	5.0
3-$COCH_3$	3.39	—	—	4.0
3-CHO	3.37	—	—	4.2
3-$COOC_2H_5$	2.24	300	0.000026	3.6
3-CN	1.45	600	0.0000085	3.2
3-SO_3^-	2.9	13	0.0000051	—

[a] From van Eys and Kaplan (1957 a).

straight line is obtained by plotting pK_i against pK_a. The pyridinium ions are presumably the active inhibitors. The N-methyl derivatives are relatively weak inhibitors. The pyridine N must be important for the binding, its properties being altered by the substituents (the stronger the electronegativity of the substituent, the greater the inhibition). It is thus evident from these data that the pyridine analogs can inhibit various enzymes directly; it is likely that these effects are not as important as those arising from the corresponding NAD analogs in whole animals.

If the major actions of 3-acetylpyridine are mediated through 3-AcPyr-NAD, the susceptibility of microorganisms or animals to 3-acetylpyridine

will depend primarily on the exchange activity of the NADases present, and perhaps secondarily on the ability to oxidize the 3-acetylpyridine to nicotinate.

Some of the effects of 3-acetylpyridine on tissue functions and whole animals were mentioned at the beginning of this section, and some of the more recently studied actions will now be discussed. The LD_{50} for the intraperitoneal route is 300–350 mg/kg in mice and 80 mg/kg in rats (Coggeshall and MacLean, 1958). Hicks (1955) found that the administration of 3-acetylpyridine to rats and mice at doses around the LD_{50} produces necrosis of adrenal medulla, of certain neurons in the supraoptic nucleus of the hypothalamus, and of the pyramidal layer of the hippocampus. No effects on the cerebral cortex were observed, contrary to the action of most metabolic inhibitors. These effects are not seen in nicotinate deficiency, but the picture may represent a more accelerated and acute deficiency; it is possible that the regions affected are more dependent on the pyridine nucleotides, but differential penetration might also be a factor. Coggeshall and MacLean (1958) found that single LD_{50} doses to rats lead to weakness of the extremities, inspiratory rhonchi, urinary incontinence, and other symptoms, but gross pathological examination of the organs showed nothing remarkably abnormal. Surviving mice show motor incoordination and a slight to complete loss of neurons in the hippocampal areas CA3 and CA4; some damage to other hippocampal areas and the dentate gyrus may occur, but no changes in other brain areas were detected. It was concluded that the hippocampus must be metabolically different from the rest of the brain.

Rats given 100 mg/kg of 3-acetylpyridine develop ataxia, hyperkinesis, and convulsions and it was found that 5–10% of the total brain pyridine dinucleotides is 3-AcPyr-NAD (Brunnemann *et al.*, 1962). The maximal levels of the abnormal analogs occur at 6–8 hr and various regions of the brain differ in the fraction incorporated, the highest levels being found in the hippocampus (Herken and Neuhoff, 1963; Willing *et al.*, 1964). The administration of 4-acetylpyridine does not lead to incorporation or to toxic symptoms. This Berlin group of workers favors the concept that the central neurological effects of 3-acetylpyridine are due primarily to interference in electron transport as a result of the inhibitions produced by the 3-AcPyr-NAD(P) formed in the brain.

Hollander and I have studied the effects of the acetylpyridines on the isolated rat atrium and, although the work is not yet complete, the basic actions are clear.* 3-Acetylpyridine at 1 m*M* increases the contractile tension of the atria 10% and simultaneously the resting and action potential magnitudes are increased 4–5%. The action potential duration and con-

* We have found that most commercial samples of the acetylpyridines are quite impure and redistillation under reduced pressure is necessary to obtain reliable preparations.

duction rate are either not affected or slightly decreased. The effects are somewhat greater at 5 mM, contractile tension increasing 25%. 2-Acetylpyridine and 4-acetylpyridine produce very much the same effects, and nicotinamide itself quite potently stimulates atrial contractility, although in this case the resting and action potentials tend to decrease. In view of these effects by substances not giving rise to NAD analogs, the acute action of 3-acetylpyridine on the atria must be attributed to some other mechanism. The lack of depressant activity at these concentrations is also interesting, since metabolic disturbances invariably produce certain characteristic changes. The contractile stimulation by nicotinamide increases with concentration and at 100 mM is around 100%. The mechanism is not understood but seems to be unrelated to membrane potential changes.

Inhibition of Dehydrogenases by Nicotinamide and Related Compounds

The inhibition of NADases and inhibitions dependent on the NADase-catalyzed exchange reactions have been discussed. We now turn to the inhibitions of NADP-dependent dehydrogenases by nicotinamide and other substituted pyridines. The groups and interactions involved in the binding of the pyridine coenzymes to the dehydrogenases have been discussed by Shifrin and Kaplan (1960). Sulfhydryl groups are often important and have frequently been thought to react with the pyridine N of the coenzymes, while in some dehydrogenases Zn^{++} is involved and perhaps reacts with the phosphate or adenine residues. It is apparent that the coenzymes are bound in different ways to different dehydrogenases and this will determine to some extent the ability of analogs to inhibit. The nature of the very tightly bound intramitochondrial NAD in unknown, but possibly it is much more difficult to inhibit intact mitochondrial dehydrogenases than the isolated and often reconstituted enzymes usually studied.

The first report of an inhibition of metabolism by nicotinamide was made by Baker *et al.* (1938) in connection with a study of the action of nicotine on cerebral respiration. However, the inhibition of brain slice respiration is slight, 30 mM depressing the oxidation of glucose 8% and lactate 15%. A greater differential effect on glucose and lactate oxidation is exerted by nicotinate, the inhibitions at 30 mM being 9% and 57%, respectively. Von Euler (1942) made the initial investigation of dehydrogenase inhibition and found inhibitions of both beef liver glucose dehydrogenase and heart lactate dehydrogenase (see accompanying tabulation). The generally weak inhibitory activity and the greater potencies of nicotinate and pyridine-3-sulfonate, compared to nicotinamide and pyridine-3-sulfonamide, may indicate that these substances do not actually combine with the pyridine-binding site on the enzymes, and it is even doubtful if they may be classed as NAD or NADP analogs. However, the inhibition by the pyridine-3-sulfonate appears to be competitive. Brink (1953 b) continued von Euler's work in

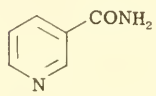
Inhibitor	Relative "K_i" [a]	
	Lactate DH	Glucose DH
Salicylate	7.7	—
p-Aminobenzoate	14.1	8.6
Salicylamide	14.2	—
Pyridine-3-sulfonate	22.1	27.6
Nicotinate	32.6	12.0
Benzenesulfonate	43.4	34.1
m-Aminobenzoate	51.7	—
Benzoate	54.2	75.2
Pyridine-3-sulfonamide	60.0	135
Nicotinamide	105	41.5
Benzamide	—	46.3
Trigonelline	No inhibition	No inhibition
Adenosine	7.2	—
Adenosine-3′-P	12.6	—
ATP	21.0	—

[a] The relative "K_i" values were calculated from the inhibitions at varying concentrations in order that the relative potencies of the inhibitors could be more readily compared; they are not absolute values.

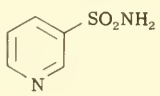
Stockholm but determined the inhibitor constants by plotting, so that the values in the accompanying tabulation for beef liver glucose dehydrogenase

Inhibitor	K_i (mM)	Relative $-\Delta F$ of binding (kcal/mole)
4-Pyridoxate	0.3	4.98
Pyridoxal	0.8	4.39
3-Hydroxypyridine	9.2	2.89
Nicotinate	21.5	2.36
2-Methylnicotinate	25	2.26
Nicotinamide	23	2.32
Isonicotinyl hydrazide	200	0.99
Pyridine	300	0.74
Isonicotinate	No inhibition	
Trigonelline	No inhibition	
ATP	1.75	3.92
Adenosine-3′-P	3.5	3.48
Adenosine	9.25	2.89
Adenine	12	2.73
Phosphate	810	0.13

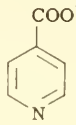
are more reliable than in the tabulation above. The K_m for NAD is 0.00428 mM (relative $-\Delta F$ would be 7.63 kcal/mole) so that none of these inhibitors are bound nearly so tightly. Substitution in the 3-position of the pyri-



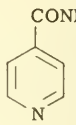
Nicotinamide



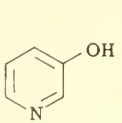
Pyridine-3-sulfonamide



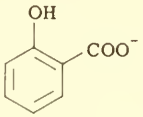
Isonicotinate



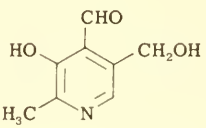
Isonicotinyl hydrazide



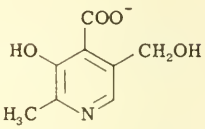
3-Hydroxypyridine



Salicylate



Pyridoxal



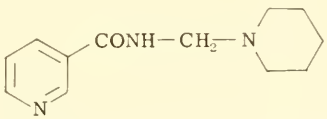
4-Pyridoxate

dine ring is necessary for significant inhibition, but the nature of this group can vary considerably and certainly no marked electrostatic interaction is involved. The pyridine N would not seem to be of much importance in the binding, since benzamide is about as inhibitory as nicotinamide, and benzenesulfonate almost as potent as pyridine-3-sulfonate, and yet N-methylation (to form trigonelline) abolishes the inhibition. The extra 2.1 kcal/mole provided by the 3-hydroxy group suggests the possibility of hydrogen bonding to the enzyme from this position, but dipolar and dispersion interactions could also account for this. It may be noticed that the results with alcohol dehydrogenase (Table 2-30) are in certain respects different than those with glucose dehydrogenase. In this connection one must remember that the ionization constants of these analogs should be considered, and it may be that the major effect of the substituent groups is by modification of the pK_a of the pyridine N. Until these problems have been treated quantitatively, it is impossible to evaluate accurately the relationship between structure and inhibitory activity. In any event, it is clear that the major binding energy of NAD is contributed by the adenine nucleotide portion of the molecule, so that pyridine derivatives might not be expected to be potent inhibitors of dehydrogenases. The relatively strong inhibitions produced by pyridoxal and 4-pyridoxate may be significant for cellular metabolic regulation and further study of the effects of these substances on various dehydrogenases is probably warranted.

Nicotinamide has been frequently used in homogenates to inhibit the splitting of NAD by NADases, as discussed above, and often at concentra-

tions sufficiently high to interfere with dehydrogenases. Feigelson *et al.* (1951) investigated this problem in liver homogenates and noted first that nicotinamide reduces endogenous respiration, an effect reversed by NAD. At 50–100 mM, nicotinamide stimulates the endogenous respiration somewhat, perhaps due to protection of NAD, but at higher concentrations inhibits quite potently. Malate dehydrogenase was partially purified and nicotinamide inhibited competitively with respect to NAD with $K_m = 0.00367$ mM, and $K_i = 113$ mM, corresponding to about 5 kcal/mole tighter binding for the NAD. Care must thus be used in the choice of nicotinamide concentration when NADase inhibition is desired. Results with lactate and glucose-6-P dehydrogenases from rabbit erythrocytes are very similar (Alivisatos and Denstedt, 1952). Nicotinamide inhibits competitively with K_i around 100 mM. It was also shown that incubation of the apoenzyme with nicotinamide in the absence of NADP leads to progressive irreversible inactivation of the dehydrogenases; this may be related to the possible location of binding sites for the coenzymes on adjacent helices of the apoenzyme, separation of these sites occurring unless they are held together by the coenzymes. Nicotinamide also inhibits 6-phosphogluconate dehydrogenase competitively (Dickens and Glock, 1951). The NADH oxidases from pigeon liver microsomes and mitochondria are inhibited 40% and 23% by 20 mM and 80% and 78% by 200 mM nicotinamide, respectively (Jacobson and Kaplan, 1957 a).

A unique nicotinamide derivative, *N*-piperidinomethylnicotinamide, which is claimed to be a specific dehydrogenase inhibitor has been reported by Matkovics *et al.* (1961). Inhibition of methylene blue reduction in liver homogenates in the presence of various substrates was studied. Unfortunately the inhibitor concentrations are not given (only the milligrams added)



N-Piperidinomethylnicotinamide

and no control experiments on endogenous activity are included. Inhibition of the oxidation of glucose, malate, lactate, and glutamate was observed. However, succinate oxidation is also inhibited, indicating that this substance is not specific for the NAD(P) dehydrogenases. Furthermore, no evidence for competition with the coenzymes was provided. Much more work must be done before this inhibitor can be accepted as having specific anticoenzyme activity.

Pyridine-3-sulfonate and Pyridine-3-sulfonamide

These analogs might be expected to inhibit nicotinate and nicotinamide metabolism. Both inhibit the growth of various bacteria and the inhibitions can be overcome by nicotinamide (McIlwain, 1940). Reference to the tabulation on page 501 shows that lactate dehydrogenase is inhibited more by these analogs than the corresponding nicotinic compounds, whereas glucose dehydrogenase behaves in the opposite fashion (von Euler, 1942). Feeding pyridine-3-sulfonate at 5% in the diet to mice produces no signs of nicotinate deficiency, but the mouse does not require exogenous nicotinate (Woolley and White, 1943 a). However, nicotinate-deficient dogs are made worse by administration of the analog (Woolley *et al.*, 1938), although Gaebler and Beher (1951) observed no effect of 0.5–2 g/day of pyridine-3-sulfonate on the excretion of *N*-methylnicotinamide, erythrocyte coenzyme level, or general health of either normal or nicotinate-deficient dogs. Hicks (1955) found hippocampal necrosis in only one animal given pyridine-3-sulfonate, so that it is presumably not as effective as 3-acetylpyridine. Brain NADase is not inhibited by pyridine-3-sulfonamide (McIlwain, 1950), and the sulfonate does not significantly inhibit either beef spleen NADase (Zatman *et al.*, 1954 a) or nicotinamide deaminase (Grossowicz and Halpern, 1956 b). The most potently inhibited enzyme examined seems to be yeast alcohol dehydrogenase, the sulfonate being the most potent inhibitor of all the substituted pyridines (Table 2-30). There is no evidence that NAD analogs can be formed from these substances. The respiration of resting *Mycobacterium phlei*, either without substrate or with glycerol, is not inhibited by 1 m*M* pyridine-3-sulfonate, but proliferating bacterial respiration is inhibited 52–85% (almost completely by 10 m*M*) (Müller *et al.*, 1960). These scattered observations do not arouse much interest in these analogs, but perhaps the proper systems have not been studied.

6-Aminonicotinamide

This analog has been called the most potent nicotinamide antagonist available (Johnson and McColl, 1955). The acute LD_{50} in mice is 35 mg/kg, although 2 mg/kg/day leads to 50% mortality by the eleventh day (Johnson and McColl, 1956). Simultaneous administration of 50 mg/kg nicotinamide raises the LD_{50} 8-fold. It is very toxic to rabbits, producing loss of motor control and paralysis, and in rats it produces these and other signs of nicotinate deficiency (Halliday *et al.*, 1957). The endogenous respiration of liver homogenates from treated mice is depressed 70% and lactate oxidation is depressed 49%; addition of NAD counteracts these depressions. However, no effect is observed when the analog is added directly to liver slices. The possibility of the formation of a NAD analog was entertained and such an analog was soon isolated following incubation of NAD, 6-ami-

nonicotinamide, and NADase (Johnson and McColl, 1956). The NAD analog was also detected spectroscopically in the livers and kidneys of treated mice. This NAD analog is completely inactive with yeast alcohol dehydrogenase. If the analyses of Shapiro *et al.* (1957) actually represent true NAD, the small decreases observed following administration of 6-aminonicotinamide at 30 mg/kg for 3 days (14% in liver, 17% in adenocarcinoma, and none in brain) would point to the NAD analog as being inhibitory to dehydrogenases. This explanation was accepted by Friedland *et al.* (1958) on the basis of decreases in tissue ADP and ATP, as well as oxidative inhibition. Although it has generally been assumed that the central effects of 6-aminonicotinamide are due to the formation of an abnormal NAD(P) analog and to reduction in normal NAD(P), Redetzki and Alvarez-O'Bourke (1962) found that the NAD level in the brain is only slightly depressed, despite the rather marked decrease of liver NAD, and obtained no evidence for the occurrence of an abnormal analog. The 6-aminonicotinamide analog of NAD inhibits creatine kinase and pyruvate kinase noncompetitively, about 40% depression occurring at 1 m*M* (von Bruchhausen 1964). It is unlikely that these actions can be important *in vivo* unless these enzymes are much more sensitive in intact cells.

Administration of the analog to adenocarcinoma-bearing mice leads to inhibition of certain enzymes determined in homogenates of the tumor: lactate dehydrogenase is not affected, glyceraldehyde-3-P dehydrogenase is inhibited 44%, the conversion of β-hydroxybutyrate to acetoacetate is inhibited 69%, and α-ketoglutarate oxidase is inhibited 83% (Dietrich *et al.*, 1958). It was believed that the NAD analog is quite tightly bound to the apoenzymes and prevents the combination with NAD.

6-Aminonicotinamide exerts a depressing action against the growth of certain lymphosarcomas and adenocarcinomas, and this is reversed by nicotinamide (Halliday *et al.*, 1957). Tumor regression occurs at 3–4 mg/kg/day but some weight loss also occurs; at lower doses the weight loss can be minimized with some reduction in carcinostatic activity, but combined at these lower doses with 8-azaguanine it is reasonably effective (Shapiro *et al.*, 1957). It was considered to represent a new class of potentially useful carcinostatic agents. It is interesting that 6-aminonicotinate is one-seventh to one-fifteenth as toxic as the amide, suggesting either that penetration of the acid is limiting or that conversion to the amide is slow.

Inhibition of NAD(P) Enzymes by Various Nucleotides and Related Substances

The study of these inhibitions has three major purposes: (1) to obtain information on the nature of the active centers and the binding groups of the coenzymes, (2) to understand better the mutual relationships between these naturally occurring substances and the possible regulatory effects

exerted in cellular metabolism, and (3) to find useful inhibitors that may specifically inhibit particular reactions in complex systems. Some of the results on different types of enzyme involving NAD or NADP are summarized in Table 2-31. It is unfortunate that in very few instances have the types of inhibition been determined and it is seldom possible to calculate accurately the K_i's or even relative K_i's, from which interesting binding energy information might be obtained.

One may first ask: Does the inhibitory activity generally increase as ribose and phosphate groups are added? The answer is roughly in the affirmative for NAD kinase, NADH pyrophosphatase, NADH oxidase, NAD : NADP transhydrogenase, alcohol dehydrogenase, and malate dehydrogenase, but in a few enzymes there appears to be no definite trend, while in some the addition of a group may reduce the binding. The addition of a phosphate to adenosine to form 5′-adenylate (AR → 5′-ARP) leads to only 0.1 kcal/mole extra binding to the alcohol dehydrogenase and 0.4 kcal/mole to the NAD kinase, but an increased binding of over 2.4 kcal/mole for the NADH pyrophosphatase. The further addition of a phosphate to form ADP increases the binding approximately 0.7 kcal/mole for the NAD kinase, 1.1 kcal/mole for alcohol dehydrogenase, and 0.6 kcal/mole for the NAD : NADP transhydrogenase, whereas the binding to NADH oxidase or NADH pyrophosphatase is unchanged or slightly reduced. Addition of another phosphate to form ATP leads to increased binding only for the liver NADH oxidase. Addition of a ribose to ADP to form ARPPR has no effect for NAD kinase but increases the binding around 0.7 kcal/mole with NADH pyrophosphatase. Final addition of nicotinamide to ARPPR to form NAD increases the binding around 1.9 kcal/mole for NAD kinase and NADPH-glutathione reductase, whereas a reduction of 1.9 kcal/mole in the binding to NADH pyrophosphatase is observed. Addition of nicotinamide to 2′-P-ARPPR to form NADP leads to a 2.3 kcal/mole increase in binding for the NADPH-glutathione reductase and to very little change for the NADP-cytochrome c reductase. The marked variation in behavior between enzymes and the uncertainty in the accuracy of the energy values make it impossible to draw definite conclusions or formulate rules for these inhibitions. It appears that all the components of the NAD and NADP molecules can participate in the binding, although not all of them need function for a particular enzyme. The rather marked inhibition occasionally exerted by NADH on NAD reactions, or by NAD(P) on NAD(P)H reactions, indicates not only the specificity of these enzymes but points to a somewhat different orientation of the oxidized and reduced forms on the enzymes.

A more interesting correlation emerges when one considers the variation of inhibitory potency with the position of phosphate groups on the adenyl ribose. In NAD the 5-position is phosphorylated and enzymes involving NAD are more readily inhibited by 5′-AMP than by 2′- or 3′-AMP (NAD

kinase and malate dehydrogenase). However, NADP is additionally phosphorylated in the 2-position and, as has been especially emphasized by Neufeld *et al.* (1955), enzymes reacting with NADP are frequently inhibited by 2′-AMP. In addition to the enzymes listed in Table 2-31 (NADPH diaphorase, NADP-cytochrome c reductase, glucose-6-P dehydrogenase, and isocitrate dehydrogenase), they found phosphogluconate dehydrogenase and NADP-activated oxalacetate decarboxylase to be inhibited by 2′-AMP more than by the other AMP's. It was suggested that 2′-AMP may be a useful inhibitor to distinguish between NAD and NADP enzymes. It must be admitted that an insufficient number of NAD enzymes have been examined. It is interesting that the NAD : NADP transhydrogenase is inhibited more potently by 2′-AMP than by 3′- and 5′-AMP.

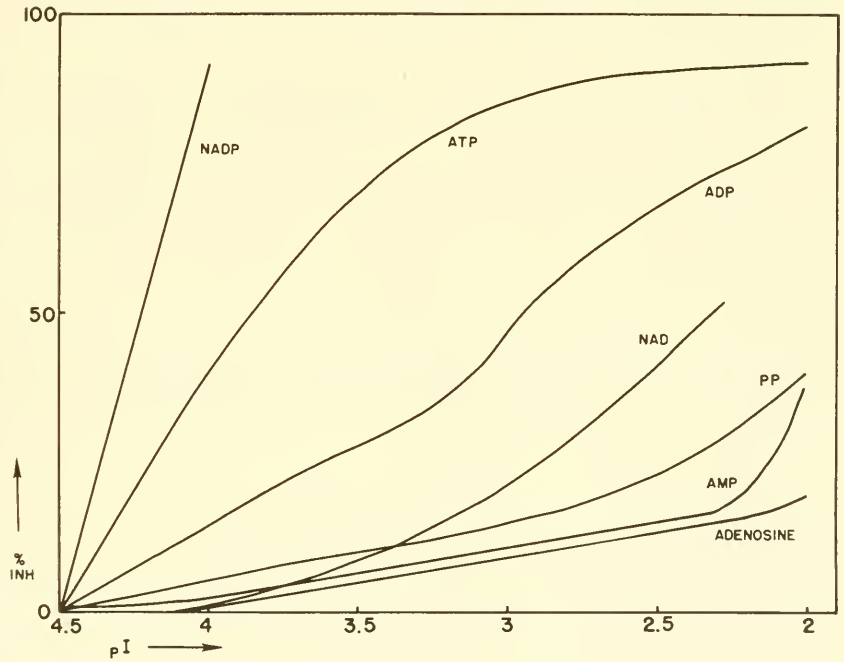


FIG. 2-17. Inhibitions of glutamate semialdehyde reductase by various nucleosides and nucleotides. (From Smith and Greenberg, 1957.)

The importance of phosphate groups for the binding of inhibitors of this type is seen strikingly in the study on glutamic semialdehyde reductase by Smith and Greenberg (1957) (Fig. 2-17). The inhibitions by AMP, ADP, and ATP are competitive, but NADP inhibits noncompetitively. Although the addition of the first phosphate to form AMP has little effect (not more than 0.1 kcal/mole), the addition of each of the next two phosphates to

TABLE 2-31

INHIBITION OF ENZYMES INVOLVING NAD OR NADP BY VARIOUS NUCLEOTIDES AND ANALOGS

Enzyme	Source	Substrate [a]	Inhibitor [b]	(I) (mM)	% Inhibition	K_i (mM)	Type [c]	Reference
Alcohol dehydrogenase	Yeast	NADH	ARPP	—	—	0.08	C	van Eys *et al.* (1958)
			5′-ARP	—	—	0.48		
			AR	—	—	0.56		
	Horse liver	NADH	3-NH_2-NRPPRA	—	—	0.097	C	Walter and Kaplan (1963)
	Horse liver	NADH	ARPP	—	—	0.026	C	Yonetani and Theorell (1964)
			5′-ARP	—	—	0.14		
			5′-ARPP	—	—	0.39		
Glucose-6-P dehydrogenase	Yeast	NADP(0.083)	2′-ARP	3	72	—	C	Neufeld *et al.* (1955)
				6	91	—		
			3′-ARP	3	15	—		
				6	24	—		
			5′-ARP	6	24	—		
Glutamate dehydrogenase	Beef liver	NADPH	GRPP	—	—	0.022	U	Frieden (1962)
			GRPPP	—	—	0.0005	U	
	Beef liver	NAD(1.13)	5′-ARP	10	4	—		Wallenfels and Sund (1957 b)
				140	50	—		
			AR	10	3	—		
				30	50	—		
	Beef liver	NADH	ARPPRN	—	—	0.13	C	Frieden (1959)
			ARPPPRN	—	—	0.10		

		NADPH	ARPPRN	—	—	0.13		
			ARPPPRN	—	—	0.12		
Isocitrate dehydrogenase	Beef heart	NAD(0.33)	2′-ARP	15	50	—	C	Chen and Plaut (1963)
			3′-ARP	15	50	—		
			5′-ARP	15	50	—		
			ARPP	0.14	50	—		
			ARPPP	0.35	50	—		
			GRPP	3	50	—		
			IRPP	3	50	—		
			IRPPP	1.7	50	—		
			URPPP	1.7	50	—		
	Pig heart	NADP(0.083)	2′-ARP	2	84	—	C	Neufeld *et al.* (1955)
			3′-ARP	2	34	—		
			5′-ARP	2	9	—		
			2′-3′-ARP	2	6	—		
			2′-HXRP	2	0	—		
Malate dehydrogenase	Pig heart	NAD	3′-ARP	4.4	0	—		Green (1936)
				8.7	16	—		
			5′-ARP	0.87	13	—		
				4.4	23	—		
				8.7	49	—		
			ARPPP	1.7	22	—		
				3.4	40	—		
	Rat liver	NAD	A	—	—	31.7		Williams (1952)
			AR	—	—	45.9		
			ARPP	—	—	16.4		
NAD kinase	Pigeon liver	NAD(2.46)	AR	5.5	8	—	C	Wang and Kaplan (1954)
		ATP(8)	2′-ARP	7.3	8	—	C	

TABLE 2-31 (*continued*)

Enzyme	Source	Substrate [a]	Inhibitor [b]	(I) (m*M*)	% Inhibition	K_i (m*M*)	Type [c]	Reference
			3′-ARP	7.3	6	—	C	
			5′-ARP	7.3	18	—	C	
			ARPP	7.8	43	—	C	
			ARPPR	7.6	38	—	C	
			ARPPRNH	0.145	21	—	C	
				1.45	84	—		
				4.5	93	—		
NAD:NADP transhydrogenase	Spinach leaves	NAD(0.33) NADPH(0.1)	2′-ARP	1.7	43	—	C	Keister *et al.* (1960)
			3′-ARP	3.3	37	—		
			5′-ARP	3.3	27	—		
			ARPP	3.3	48	—		
			ARPPP	3.3	22	—		
			a-ARPPRN	1	33	—		
NAD pyrophosphorylase	Pig liver nuclei	NMN and ATP	HXRPPP	—	—	2.1	C	Atkinson *et al.* (1961)
NADH:ferricyanide oxidoreductase	Beef heart	NADH(0.15)	FRP	3	100	—		Minakami *et al.* (1963)
			ARPPRF	0.1	25	—		
			ARPPRHX	1.4	56	—		
			ARPPRAcPy	1.4	56	—		
NADH: menadione oxidoreductase	Pig liver	NADH(0.033)	5′-ARP	1	50	—		Raw *et al.* (1961)
			ARPP	1	50	—		
			ARPPP	1	50	—		

NADH oxidase	*Xanthomonas phaseoli*	NADH(6.8)	HXRPPRN	5	7	—		Hochster and Nozzolillo (1960)
	Pig liver	NADH(0.1)	5′-ARP	1	0	—		Mahler *et al.* (1958)
			ARPP	1	0	—		
			ARPPP	1	36	—		
			ARPPPP	1	60	—		
			5′-HXRP	1	0	—		
			CRPP	1	0	—		
			GRPP	1	0	—		
			IRPPP	1	36	—		
			URPPP	1	36	—		
NADH pyrophosphatase	Pigeon liver	NADH(1.6)	AR	2	0	—		Jacobson and Kaplan (1957)
			5′-ARP	2	73	—		
			ARPP	2	54	—	C	
			ARPPP	2	46	—		
			ARPPR	0.5	47	—		
			ARPPRN	2	14	—		
NADPH: cytochrome e oxidoreductase	Liver	NADPH(0.013)	2′-ARP	1.2	73	—	C	Neufeld *et al.* (1955)
			3′-ARP	1.2	0	—		
			5′-ARP	1.2	8	—		
			ARPPR	1.4	0	—		
			ARPPPR	0.14	71	—		
			ARPPRN	1.4	0	—		
			ARPPPRN	0.025	36	—	C	
				0.07	46	—		
				0.14	81	—		
			3′-HXRP	1.5	7	—		
			HXRPPPRN	0.1	13	—		

TABLE 2-31 (*continued*)

Enzyme	Source	Substrate [a]	Inhibitor [b]	(I) (m*M*)	% Inhibition	K_i (m*M*)	Type [c]	Reference
NADPH: glutathione oxidoreductase	Peas	NADPH(×)	2′-ARP	3.3×	10	—		Neufeld *et al.* (1955)
			3′-ARP	3.3×	6	—		
			5′-ARP	3.3×	11	—		
			ARPPR	3.3×	0	—		
			ARPPPR	3.3×	5	—		
			ARPPRN	3.3×	52	—		
			ARPPPRN	3.3×	69	—		
			NRP	3.3×	17	—		
			HXRPPPRN	3.3×	5	—		
NADPH: nitrite oxidoreductase	*E. coli*	NADPH(0.25)	ARPPRN	0.066	13	—		Lazzarini and Atkinson (1961)
				0.13	35	—		
				0.27	43	—		
Nicotinamide deamidase	*Torula cremoris*	Nicotinamide	ARPPRN	—	—	0.53	N	Joshi and Handler (1962)
			ARPPPRN	—	—	0.465	N	
			ARPPRAcPy	—	—	3.2	N	
			AcPy	—	—	0.305	C	

[a] The substrate concentrations in m*M* are given in parentheses whenever they are available or necessary.

[b] The inhibitors are abbreviated in a way to show more readily the effects of structural changes: A = adenine, Ac = acetyl, C = cytosine, F = flavin, G = guanine, HX = hypoxanthine, I = inosine, N = nicotinamide, P = phosphate, Py = pyridine, R = ribose, and U = uracil. In this systems, for example, AR is adenosine, ARP in adenylate or AMP, NRP is nicotinamide mononucleotide or NMN, HXRPPRN is deamino-NAD, and ARPPRAcPy is the 3-acetylpyridine analog of NAD. The order in which the components are written is usually that in which they occur, but in certain cases the phosphates are simply gathered together.

[c] The inhibition type is designated as follows: C = competitive, N = noncompetitive, and U = uncompetitive.

form ADP and ATP leads to about 1.2 kcal/mole increase in binding energy. The addition of the 2′-phosphate to form NADP from NAD increases the binding markedly and changes the nature of the inhibition. It is rather strange that the addition of nicotinamide riboside to ADP lowers the binding energy about 1 kcal/mole.

Williams (1952) found that malate dehydrogenase is inhibited by adenine, adenosine, and ATP. From this observation he concluded that such normally occurring substances may well affect dehydrogenases and other enzymes in the cell. His work stemmed from the report of Raska (1946) that administration of 300–500 mg/day adenine to dogs on normal diets leads to the development of multiple avitaminosis after 10–20 days; signs of nicotinate deficiency, such as black tongue, were noted. The many more data now available serve to strengthen Williams' conclusion, since even more potent inhibitors have been reported. There has been much speculation concerning the regulation of oxidative reactions by adenine nucleotides mediated through coupled phosphorylation. It is quite possible that other more direct effects on dehydrogenases occur, both in the cell (particularly in the compartmentalized mitochondria) and in experimental enzyme preparations where the concentrations of added nucleotides are often high enough to inhibit appreciably. From Table 2-31 we see that five enzymes are inhibited from 22% to 50% by ATP at concentrations from 1 mM to 3.4 mM; ATP is commonly added to mitochondrial preparations at these or higher concentrations. An experimental survey of dehydrogenase inhibitions by nucleotides would be valuable. Chen and Plaut (1963), on the basis of the fairly potent inhibitions exerted by certain nucleotides on the NAD-linked isocitrate dehydrogenase (Table 2-31), felt that some regulation of cycle activity may be exerted, and if such does occur it would be a very important factor in understanding not only the effects of nucleotide analogs but also of many inhibitors which either primarily or secondarily alter the levels of cellular or mitochondrial nucleotides. Another interesting point has been brought out by Dalziel (1962) in connection with possible impurities in preparations of the coenzymes. Although one might expect in many cases an insignificant inhibitory effect of certain analogs because they have been shown to bind less tightly than the normal coenzyme to the apoenzyme, Dalziel correctly states that it is the relative values of K_i and K_m which are important, and K_m can be much higher than K_s. He calculated that the presence of an analog of NADH as a 3% impurity can produce as much as 70% inhibition of liver alcohol dehydrogenase if the analog and NADH have the same affinity for the apoenzyme.

An NAD(P) analog which would bind to the NAD(P) site on dehydrogenases and then react chemically with some group at the site might well be of some value in labeling these sites. Such an analog was investigated by van Eys *et al.* (1962) on the basis that thiazole rings often open at al-

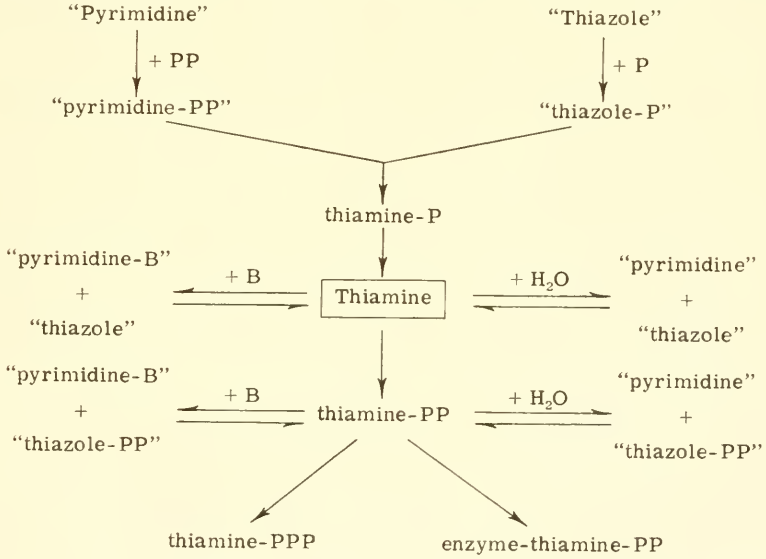
kaline pH to generate a free SH group. The NAD analog with 4-methyl-5-(β-hydroxyethyl)thiazole replacing nicotinamide was found to behave in this manner and to form a disulfide bond with SH groups at the site of dehydrogenases, this binding being competitive with NAD. In the case of horse liver alcohol dehydrogenase, 2 moles of this analog are bound tightly to each mole of enzyme.

One of the most interesting studies of dehydrogenase inhibition by nucleotides is that of the complex effects of GTP on glutamate dehydrogenase (Frieden, 1962, 1963). The K_i is 0.0003–0.0005 mM and the kinetics being uncompetitive point to different sites for NADP and GTP. Furthermore, GTP not only inhibits directly but increases the ability of NADH to inhibit. Since the NADH inhibition is due to the dissociation of the enzyme into four subunits, it is likely that GTP enhances the process, and this was demonstrated ultracentrifugally. The dissociation of the tetramer enzyme itself is not necessarily the basic cause of the loss of activity; it is possible that structural changes brought about by NADH and GTP produce both dissociation and reduced catalytic activity. The behavior can be explained adequately on the basis of three binding sites: (1) a coenzyme site, (2) a purine nucleotide site with which GTP and activating nucleotides react, and (3) a NADH-binding site. The following complexes are thus possible — EC, ECI, EC_2, EC_2I, and EI — where C represents the coenzyme. The binding of GTP to the enzyme depends on the presence of NADP at a vicinal site, the EI complex probably not being of much importance. The importance of this situation for the regulation of cell metabolism is obvious, particularly since this enzyme plays a central role in many pathways. Frieden pointed out the likely relationship between glutamate dehydrogenase and the α-ketoglutarate step in the cycle; GDP is required for the conversion of succinyl-CoA to succinate and GTP is formed, which can suppress the activity of glutamate dehydrogenase, an enzyme which under certain conditions controls the steady-state level of α-ketoglutarate in the cycle. He also suggests that ammonia formation by the liver, protein synthesis, and glyconeogenesis can all be regulated by this inhibition involving a feedback site.

ANALOGS OF THIAMINE

Thiamine functions in metabolism in the pyrophosphorylated form as the coenzyme in various reactions where a bond adjacent to a carbonyl group is broken (α-cleavage), the active complex in each case being an aldehyde-thiamine-PP-enzyme structure wherein a C—C bond is formed at the 2-position of the thiazole ring. These reactions would include (1) α-keto acid decarboxylation (e.g. pyruvate decarboxylase), (2) α-keto acid oxidation (e.g. pyruvate and α-ketoglutarate oxidases), (3) the phosphoroclastic reaction of pyruvate, and (4) α-ketol formation (e.g. transketolase and phos-

phoketolase). Thus three major metabolic sequences — the pentose-P pathway, the tricarboxylate cycle, and photosynthetic carbon dioxide fixation — are dependent on thiamine-PP, since α-cleavage occurs in all, and a variety of other metabolic processes can be secondarily affected. Thiamine deficiency, or interference with the formation or function of thiamine-PP, can produce profound metabolic and physiological disturbances. Animals require preformed thiamine, most plants can synthesize the entire thiamine molecule, and microorganisms vary widely from complete dependence on exogenous supply to complete synthetic ability. The responses of organisms to thiamine analogs will depend on these factors as well as the role of thiamine in metabolism. The pathways of thiamine biosynthesis are not completely understood and the accompanying scheme is to be taken as provisional and not necessarily applicable to all organisms. Thiaminase is apparently absent or relatively inactive in most tissues and thus the reactions catalyzed by this enzyme are probably not common or important. The most important reaction is the pyrophosphorylation of thiamine since certain analogs can interfere here or be similarly phosphorylated. Thiamine-PPP has been included because its formation from thiamine in yeast has been demonstrated (Kiessling, 1956), although it is coenzymically inactive. It may be noted that ATP is required for thiamine-PP synthesis and that



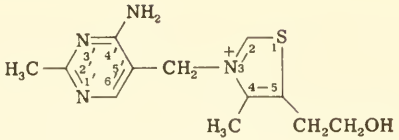
(The pyrimidine portion of thiamine is indicated in quotes and is 2-methyl-4-amino-5-hydroxymethylpyrimidine; the thiazole portion is designated likewise and is 4-methyl-5-(2-hydroxyethyl)thiazole. B is any base that can replace the thiazole in the exchange reaction catalyzed by thiaminase.)

thiamine-PP is involved in metabolic reactions leading to ATP, so that interference with thiamine-PP formation or function will tend to deplete the cells of ATP and perhaps further depress thiamine-PP synthesis.

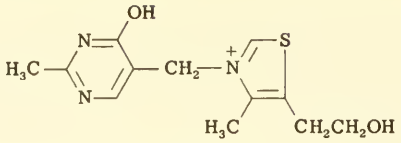
The possible sites of action for thiamine analogs can be broadly classified as (1) inhibition of thiamine-PP synthesis, either on the formation of thiamine or its pyrophosphorylation, (2) interference with the formation of complexes between thiamine-PP and enzymes, and (3) inhibition of thiaminase. In any case the inhibition may be exerted by either the analog or its phosphorylated derivatives. There is no evidence that any significant effects of any of the analogs studied can be attributed to thiaminase inhibition, so the first two mechanisms are undoubtedly the most important in the induction of thiamine deficiency symptoms. There is some evidence, which will be discussed later, that thiamine may have a function or functions unassociated with coenzyme activity, particularly in the nervous system, and, if this is true, one might consider the interference by analogs in this function.

It would appear that most of the groups in the thiamine molecule participate in either the binding or the catalysis inasmuch as the structure can not be significantly altered without loss of activity, and the number of effective analogs is rather small. The first report of enzyme inhibition by a thiamine analog was by Buchman *et al.* (1940), who found yeast pyruvate decarboxylase activity to be depressed by 4-methyl-5-hydroxyethylthiazole diphosphate (which they called "thiazole pyrophosphate"), the phosphorylated thiazole portion of thiamine. Neither the nonphosphorylated compound nor the monophosphate is inhibitory. It requires about 10 times as much analog as thiamine-PP to inhibit 50%, but if the analog is added before the thiamine-PP, the inhibition is more pronounced. These results point to the importance of the phosphate groups in the binding. They state, "We conclude that there has been demonstrated here a not hitherto recognized type of competitive inhibition of enzyme reactions, caused by competition not between substrate and inhibitor but between coenzyme and inhibitor." (See formulas on page 517).

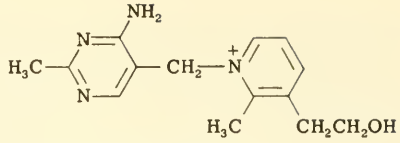
Either the pyrimidine portion or the thiazole portion of the thiamine molecule can be altered to form analogs. Replacement of the thiazole ring with a similarly substituted pyridine ring gives *pyrithiamine*, which was shown by Robbins (1941) to inhibit the growth of certain fungi, and by Woolley and White (1943 b) to produce thiamine deficiency symptoms in mice. Replacement of the pyrimidine amino group with a hydroxyl group leads to *oxythiamine*, found by Bergel and Todd (1937) to lack vitamin activity, and by Soodak and Cerecedo (1944) to be quite toxic to mice. These two analogs have been studied the most thoroughly of the thiamine-like compounds and remain the most frequently used to produce experi-



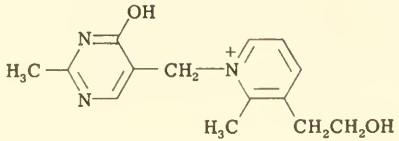
Thiamine



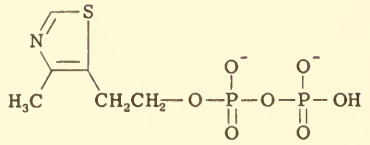
Oxythiamine



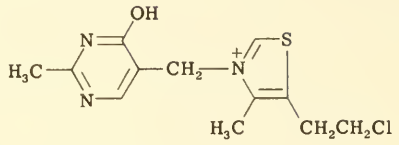
Pyrithiamine
(neopyrithiamine)



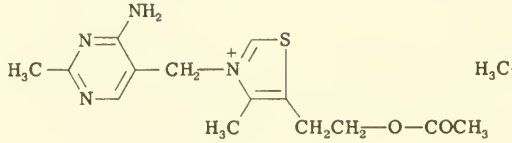
Oxypyrithiamine
(oxyneopyrithiamine)



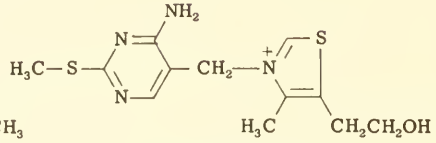
"Thiazole" pyrophosphate



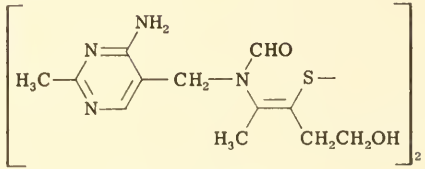
Chloroxythiamine



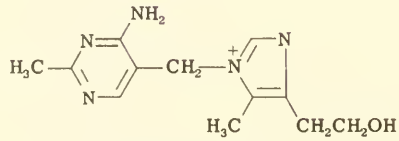
O-Acetylthiamine



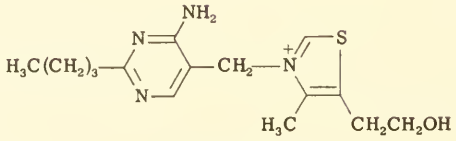
2′-Methylthio analog
of thiamine



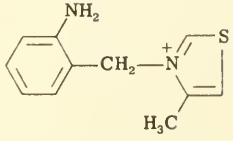
Thiamine disulfide



Imidazole analog
of thiamine



2′-*n*-Butyl analog
of thiamine



3-*o*-Aminobenzyl-4-
methylthiazole (ABMT)

mental disturbances in thiamine function.* Two other types of analog perhaps deserve more attention than they have received: the *imidazole analog* (the thiazole ring replaced with a similarly substituted imidazole ring) exerts an antivitamin effect on bacterial growth (Erlenmeyer *et al.*, 1948), and the *2′-n-butylpyrimidine analog* produces thiamine-deficiency states in rats (Emerson and Southwick, 1945), both substances being roughly of the same potency as pyrithiamine and oxythiamine. A large number of interesting analogs, such as those synthesized by Livermore and Sealock (1947) and by Mano and Tanaka (1960), have not yet been adequately examined.

Effects on Enzymes Dependent on Thiamine-PP

It is clear that neither oxythiamine nor pyrithiamine is inhibitory to pyruvate decarboxylase, but that the diphosphate esters can interfere with the binding of thiamine-PP to the apoenzyme. Thus the yeast decarboxylase is inhibited by oxythiamine-PP but not by oxythiamine (Eusebi and Cerecedo, 1950; Navazio *et al.*, 1956), and wheat germ decarboxylase behaves similarly (Eich and Cerecedo, 1954, 1955). Pyrithiamine is noninhibitory whereas pyrithiamine-PP is as effective as oxythiamine-PP (Woolley, 1951; Eich and Cerecedo, 1954). Oxythiamine-PPP is inhibitory (Velluz and Herbain, 1951; Navazio *et al.*, 1956) but it is possible that some of the action results from the splitting off of a phosphate to form oxythiamine-PP, just as the cocarboxylase activity of thiamine-PPP has been found to depend on hydrolysis to thiamine-PP (Kiessling, 1956). It is not certain if oxythiamine-P is inhibitory and contradictory results have been obtained. These data indicate the importance of the phosphate groups for the binding, and this is supported by the observation of Wiethoff *et al.* (1957) that pyrophosphate inhibits wheat germ decarboxylase competitively with thiamine-PP (the pyrophosphate must be added before the thiamine-PP).

Thiamine-PP and the phosphorylated analogs are bound fairly tightly to the apoenzyme (K_m for thiamine-PP is usually near 0.001–0.003 mM) and thus the order of addition of coenzyme and analog is important, especially as the analogs are bound about 1.5–3.0 kcal/mole less tightly (Woolley, 1951; Stewart, 1957). If thiamine-PP is added 30 min before oxythiamine-PP there is no inhibition, if they are added simultaneously there is slight inhibition, but if oxythiamine-PP is added 30 min before the coenzyme appreciable inhibition may be exerted (Eich and Cerecedo, 1954). Although

* It was found later that the material originally designated as pyrithiamine was a mixture (Wilson and Harris, 1949). The pyridine analog was synthesized in pure form and named "neopyrithiamine" but since the active compound in the early preparation was this substance, it has been generally agreed that the original name be restored. The general results of the early work are not invalidated, but the true potency of pyrithiamine is greater than indicated there.

some exchange between bound and free coenzyme and analog must occur, it is too slow for equilibrium to be obtained easily. To determine the maximal inhibiting power of an analog it is advisable to incubate the apoenzyme with the analog previous to addition of the coenzyme.

Pyruvate oxidase is inhibited similarly to the decarboxylase, as expected, and in the case of pyrithiamine-PP it would appear to be competitive (Woolley, 1951). Oxythiamine-PPP inhibits pyruvate oxidation in pigeon breast muscle extracts but this may be mediated through the diphosphate (Onrust *et al.*, 1952). The formation of acetoin from pyruvate is also inhibited by oxythiamine-PP (Eich and Cerecedo, 1954). However, Kuratomi (1959) noted that oxythiamine, like thiamine, can form acetoin from pyruvate.

Transketolase from yeast is strongly inhibited by oxythiamine-PP (Datta and Racker, 1961) but oxythiamine itself has no effect (Dreyfus and Moniz, 1962). At 0.036 m*M* and 0.072 m*M* the inhibitions are 60% and 80%, respectively, when the analog is added previous to thiamine-PP, but if the oxythiamine-PP is added 2 min after the coenzyme, no inhibition is observed. Addition of higher concentrations of thiamine-PP cannot reverse the inhibition. Thus it is difficult for either the analog or the coenzyme to displace each other from the apoenzyme. Oxythiamine-PP is bound more tightly than thiamine-PP to the enzyme but it requires 2–3 hr to inhibit 50% when the enzyme initially contains thiamine-PP. The rate of displacement for transketolase is even less than for decarboxylase, since thiamine-PP was found to reverse oxythiamine-PP-inhibited enzyme 30% in 20 min.

The inhibition of thiamine-PP-dependent enzymes by oxythiamine and pyrithiamine will depend on whether these analogs can be phosphorylated or not. Thus in the experiments of Kunz (1956), where oxythiamine and pyrithiamine at 10 m*M* inhibited pyruvate oxidation in rat liver mitochondria 95% and 35%, respectively, one is not certain if there is direct inhibition or if the depression was due to the formation of small amounts of the phosphorylated esters. Acetylthiamine inhibits to about the same degree as pyrithiamine and this analog cannot be phosphorylated (unless it is first deacetylated), so it would seem that these rather weak inhibitions may be to some extent exerted directly. It is also interesting that oxythiamine and pyrithiamine have no effect on pyruvate oxidation in brain mitochondria, this being attributed by Kunz to a different structure or permeability compared to liver mitochondria; different phosphorylative capacities might also play a role. Phosphorylation of these analogs mediated through thiamine kinase seems to occur in most tissues and it is likely, as pointed out by Woolley (1951), that the toxic reactions observed with oxythiamine and pyrithiamine in animals are produced primarily by the phosphorylated compounds.

Accumulation of Intermediates and *in Vivo* Effects

Inasmuch as the oxidation of pyruvate requires thiamine-PP, one would expect some accumulation of pyruvate in animals treated with thiamine analogs if pyruvate oxidase is indeed inhibited *in vivo*. Such has been observed in rats with oxythiamine (Frohman and Day, 1949; Gubler, 1961) and pyrithiamine (de Caro *et al.*, 1954). For example, rats injected intraperitoneally with 150 μg oxythiamine show an elevation in blood pyruvate of 1.3 to 5.1 mg%; blood lactate also increases from 11.5 to 42.8 mg%. In mice, pyrithiamine raises the blood pyruvate somewhat but oxythiamine has no effect (de Caro *et al.*, 1956), possibly indicating a species difference since in rats oxythiamine is more effective than pyrithiamine (Gubler, 1961). The administration of oxythiamine to dogs at 6 mg/kg in three doses leads to a marked rise in blood pyruvate (0.4 to 5.7 mg%) and thiamine is able to counteract this effectively (Wilson *et al.*, 1962). Simultaneously there is a severe fall in liver glycogen (13 to 0.4 mg/g). Rats and cats respond similarly but are less sensitive. Growth of *Neurospora* in the presence of oxythiamine is accompanied by pyruvate accumulation and a simultaneous reduction in pyruvate decarboxylase activity is demonstrable (Sankar, 1958). Administration of increasing amounts of thiamine partially or completely counteracts these effects on pyruvate levels, in all instances where it has been tested.

We shall now turn to evidence of enzyme inhibition in the tissues of analog-treated animals. It may be calculated from the data of Von Holt *et al.* (1955) that feeding pyrithiamine to rats at 10 mg/kg for 7–12 days results in some 63% reduction of pyruvate oxidation in liver homogenates. A thorough investigation of the changing patterns of keto acid oxidation in deficient and analog-treated rats has been made by Gubler (1958, 1961); his results are summarized in Table 2-32. It is seen that the oxidation of pyruvate is more sensitive than that of α-ketoglutarate to both dietary deficiency and the analogs; this could relate to different displacing rates in the two oxidases, or to different dependencies of enzyme activity on thiamine-PP level. Oxidation of β-keto acids, as expected, is not affected. The effects of oxythiamine and pyrithiamine are roughly the same on all tissues, with the exception of brain in which pyrithiamine is more effective. The reason for this is not understood — it would seem unlikely that oxythiamine is unable to penetrate the blood-brain barrier — but it may be correlated with the fact that only pyrithiamine is able to produce polyneuritis in rats. Another difference between these two analogs lies in the ability of thiamine-PP added *in vitro* to the mitochondrial suspensions to counteract the depression of pyruvate oxidation. The loss of activity from dietary deficiency of thiamine is readily reversed by adding thiamine-PP, as anticipated; the loss due to pyrithiamine is surprisingly well reversed (to about 90% of the control values in brain and kidney); the loss due to

TABLE 2-32

EFFECTS OF THIAMINE DEFICIENCY AND THIAMINE ANALOGS ON THE OXIDATIVE DECARBOXYLATION OF KETO ACIDS IN MITOCHONDRIA [a]

Tissue	Substrate	% Change		
		Dietary deficiency	Oxythiamine	Pyrithiamine
Liver	Pyruvate	−75	−56	−57
	α-Ketoglutarate	−38	− 1	−11
	α-Ketoisovalerate	+ 3	−22	−13
	α-Keto-β-methylvalerate	−11	−11	−12
	β-Hydroxybutyrate	+ 4	+ 2	+11
Brain	Pyruvate	−24	−18	−51
	α-Ketoglutarate	+21	+ 6	−44
Kidney	Pyruvate	−56	−52	−61
	α-Ketoglutarate	−55	+ 5	−37
Heart	Pyruvate	−32	−67	−58
	α-Ketoglutarate	−24	−37	−17

[a] The analogs were administered to rats at oxythiamine/thiamine = 200, and pyrithiamine/thiamine = 5, these doses producing polyneuritis in several days. The figures give the changes observed in the oxidation of the substrates indicated in mitochondrial suspensions. (From Gubler, 1961.)

oxythiamine is reversed poorly. The most obvious explanation of this is that pyrithiamine blocks the synthesis of thiamine-PP, so that the tissues are primarily deficient in cocarboxylase, whereas oxythiamine may exert its inhibition mainly by binding to pyruvate oxidase in the form of its diphosphate ester. These problems will be discussed after further effects of these analogs have been presented.

Feeding pyrithiamine to pigeons leads to a 50% reduction in the pyruvate decarboxylase activity in breast muscle, and this can be reversed by the addition of thiamine-PP to the homogenates (Koedam *et al.*, 1956). This is accompanied by a marked reduction in the thiamine-PP content of muscle, so that it was concluded that there is no essential difference between dietary thiamine deficiency and pyrithiamine feeding. Pyruvate dismutation and acetoin formation in breast and heart muscle are likewise depressed by pyrithiamine feeding (Koedam, 1958). A large single dose of pyrithiamine (2.5 mg) leads to a rapid inhibition of acetoin formation and even after 8 days the activity does not return to normal.

The respiratory quotient of rats treated with pyrithiamine (5 mg/day

intraperitoneally for 5–6 days) is lowered and, in contrast to normal or thiamine-deficient animals, administration of glucose does not raise it (see accompanying tabulation) (de Caro *et al.*, 1954). There is thus an inhibition

	R. Q.	
	Before glucose	After glucose
Controls	0.81	0.93
Diet-deficient	0.78	0.92
Pyrithiamine-treated	0.72	0.77

of the total oxidation of carbohydrate, which can be directly attributed to a block in pyruvate oxidation; however, it is difficult to explain the greater effect in the diet-deficient animals. The only work showing an impairment of transketolase function is that of Wolfe (1957). Rats deprived of thiamine or given oxythiamine show a depression of the pentose-P pathway in the erythrocytes, pentose accumulating, whereas pyrithiamine produces no changes in transketolase activity even when the animals are paralyzed.

Some interference with amino acid metabolism by thiamine analogs, mediated through changes in the utilization of the α-keto acids, might be expected, but little study of this has been made. Pyrithiamine inhibits the formation of aspartate and asparagine from glutamate in germinating *Phaseolus* seeds and there is an accumulation of ammonia, possibly due to the lack of oxalacetate (Sivaramakrishnan and Sarma, 1954). These effects can be attributed to a reduction in α-ketoglutarate oxidase. Oxythiamine and pyrithiamine both inhibit the growth of *Vibrio cholera* and lead to the accumulation of alanine, aspartate, and glutamate. The effects on the levels of such amino acids will depend to a great extent on the pattern of metabolism, i.e., on the over-all direction of transamination reactions.

Effects on Thiamine-PP Synthesis

Pyrithiamine has been found to inhibit thiamine kinase from chicken blood (Woolley, 1950 a), rat liver (Eich and Cerecedo, 1954; Mano and Tanaka, 1960), rat intestine (Cerecedo *et al.*, 1954), and pigeon liver (Koedam, 1958), and the inhibition appears to be competitive with thiamine. This inhibition is fairly potent: When the ratio pyrithiamine/thiamine is 1 the inhibition is around 50%, at a ratio of 5 it is 75%, and at a ratio of 10 it is 90% in rat liver and intestine (thiamine concentrations 0.01–0.1 mM). On the other hand, oxythiamine inhibits thiamine kinase much less strongly or not at all when present in similar ratios to thiamine (Eich and Cerecedo, 1954; Cerecedo *et al.*, 1954; Koedam, 1958; Mano and Tanaka, 1960). The

difference in inhibitory activity between these two analogs possibly indicates the importance of the pyrimidyl 4′-amino group. This was substantiated in the demonstration by Cerecedo and Eich (1955) that oxypyrithiamine, in which the 4′-amino group is replaced by hydroxyl group, does not inhibit rat liver thiamine kinase.

Mano and Tanaka (1960) studied a large series of thiamine analogs with respect to their abilities to be phosphorylated by a rat liver thiamine kinase system and their inhibitory potencies on thiamine phosphorylation (see accompanying tabulation). The analogs and thiamine were all at 0.1 m*M*.

Analog	Relative activity (thiamine = 100)	% Inhibition of thiamine kinase
Pyrithiamine	0	53
2′-Ethylthiamine	11	38
2′-*n*-Butylthiamine	0	32
Oxythiamine	0	6
Diacetylthiamine	3	5
Thiothiamine	0	Stim 2
Dibenzoylthiamine	2	Stim 3
O-Acetylthiamine	7	Stim 6
Thiamine disulfide	0	Stim 12

The inhibitions by pyrithiamine and the 2′-alkylthiamines are competitive and the following values of K_i were calculated: pyrithiamine 0.033 m*M*, 2′-ethylthiamine 0.041 m*M*, and 2′-butylthiamine 0.043 m*M*. The inability of the enzyme to catalyze the phosphorylation of oxythiamine and pyrithiamine is noteworthy in view of the theory that these analogs may exert their effects in the diphosphate form. It may be recalled that Woolley (1951) failed to demonstrate the synthesis of pyrithiamine-PP in chicken blood. There has been surprisingly little attention to this important problem of analog phosphorylation in organisms.

Inhibition of Thiaminase

The importance of this enzyme in the mammalian metabolism of thiamine is not known. The only evidence for its possible function is the appearance in the urine of the pyrimidine and thiazole moieties of thiamine following administration of thiamine. It would seem unlikely that inhibition of this enzyme is an important factor in the toxicity of thiamine analogs, but it could be of some significance in determining the effects on tissue levels of thiamine-PP. It was stated by Soodak and Cerecedo (1944) that oxythiamine inhibits carp thiaminase but no data were given. Pyrithiamine inhibits

this enzyme around 40% when analog and thiamine are both 0.5 mM (Sealock and White, 1949). Pyrithiamine is split by the enzyme but at a slower rate than is thiamine. Apparently it is bound more tightly than thiamine, but reacts more slowly, since in mixtures of the two only the splitting of thiamine is depressed. Thus too little is known of the effects of these analogs on thiaminase to evaluate the importance of the inhibitions.

Sealock examined the effects of a series of substituted methylthiazolium ions on fish thiaminase and found that 3-*o*-aminobenzyl-4-methylthiazole (ABMT) is a particularly potent inhibitor (see tabulation) (Sealock and Goodland, 1944). The inhibition is competitive, with $K_m = 0.0831$ mM and $K_i = 0.00197$ mM, possibly indicating that ABMT is bound 2.3 kcal/mole

Analog	Concentration (mM)	% Inhibition	Relative "K_i"
3-*o*-Aminobenzyl-4-methylthiazole	0.5	100	< 0.005
3-β-Aminoethyl-4-methylthiazole	0.5	48	0.54
3-β-Phthalimidoethyl-4-methylthiazole	0.5	6	7.8
3-*o*-Nitrobenzyl-4-methylthiazole	0.5	2	24
3-Ethyl-4-methylthiazole	10	9	101
3-Phenyl-4-methylthiazole	10	5	190
3-Phenyl-2-methyl-4-methylthiazole	5	0	>495
3-Ethyl-2-methyl-4-methylthiazole	10	0	>990

more tightly than thiamine. The amino group is probably quite important, since the replacement with a nitro group reduces the inhibition so markedly. The aminobenzylthiazoles were later studied in greater detail (Sealock and Livermore, 1949) and the position of the amino group was shown to be critical, only the *ortho* compound being inhibitory (see accompanying tabulation). On the other hand, the position of the thiazole methyl group is not critical. Thiamine was 0.5 mM in these experiments. Kenten (1958) has

Amino position in benzyl ring	Methyl position in thiazole ring	Concentration (mM)	% Inhibition
ortho	4	0.5	78.1
meta	4	0.5	Stim
para	4	0.5	1.7
ortho	2	0.5	89.2
meta	2	0.5	Stim
para	2	1	2.0
ortho	2,4	0.5	30.2

found the thiaminase from bracken (*Pteridium aquilinum*) to be very sensitive to ABMT, 15–20% inhibition being given by 0.002 m*M* and almost complete inhibition by 0.05 m*M*. The inhibition is probably basically competitive since it proceeds faster in the absence of thiamine. As far as I know, this interesting compound has not been tested in whole animals to determine if thiaminase inhibition can be achieved and how this will alter thiamine metabolism.

Effects on Excretion and Tissue Levels of Thiamine

If these analogs displace thiamine or thiamine-PP from the tissues in any way, or inhibit the transport or metabolism of thiamine, an increased urinary excretion of thiamine would be expected, and this has been found to occur in rats given 50 μg oxythiamine (Frohman and Day, 1949). One might also predict that tissue levels of thiamine or its diphosphate would be reduced, and this has been demonstrated for both pyrithiamine and oxythiamine in mice, pigeons, and rats. The depression of tissue thiamine-PP seems to be generally associated with a rise in blood pyruvate, so that at least part of this depletion is related to enzymes involved in the oxidation or decarboxylation of α-keto acids. Inasmuch as theories for the mechanisms by which these analogs act depend on the changes in tissue thiamine levels, it will be necessary to examine the results with some care.

Pyrithiamine markedly depletes the tissues of thiamine-PP in pigeons. Controls were fed 100 μg thiamine per day and another group was fed 623 μg pyrithiamine each day in addition; after an average survival time of 19 days, the thiamine-PP levels in the tissues were those shown in the following tabulation (Koedam *et al.*, 1956). The pyruvate decarboxylase activity in muscle is reduced around 50% and adding thiamine-PP restores activity.

Tissue	Thiamine-PP content (μg/g)		% Change
	Controls	Pyrithiamine-fed	
Heart	4.55	1.00	−78
Brain	2.61	0.78	−70
Liver	3.54	1.09	−69
Breast muscle	4.42	1.80	−59
Kidney	4.36	2.08	−52

The fall in thiamine-PP level is quite rapid; at 4 days it is mainly complete in most tissues, and from the data on pyruvate utilization it would appear that a marked decrease occurs within 1 day (Koedam, 1958). Pigeons given a single large dose of pyrithiamine (10 mg) and examined 64 days later show

no permanent effect on the tissue thiamine-PP levels. An important observation was that pyrithiamine induces a more rapid depletion of tissue thiamine-PP than does elimination of exogenous thiamine. A comparison between dietary deficiency and pyrithiamine administration in rats was reported by de Caro *et al.* (1954). The results in the accompanying tabulation were

Tissue	Total thiamine content (μg/g)		
	Controls	Avitaminotic	Pyrithiamine-fed
Liver	7.42	2.82	1.33
Muscle	1.60	0.80	0.71
Brain	3.38	2.89	0.57

obtained from rats injected with 5 mg pyrithiamine daily for 5–6 days and rats subjected to a thiamine-free diet for a comparable time. In all cases pyrithiamine produces a greater effect than simple elimination of thiamine intake; the effect in brain is particularly striking and possibly correlated with the polyneuritic symptoms produced by pyrithiamine.

Oxythiamine, on the other hand, does not seem to be so active in reducing the tissue levels of thiamine-PP (Steyn-Parvé, 1954). This analog at 1 mg/day for 15 days to pigeons produces the changes summarized in the accompanying tabulation. No deficiency symptoms were noted and none would

Tissue	Thiamine-PP content (μg/g)		
	Controls	Oxythiamine-fed	% Change
Heart	6.70	3.40	−49
Muscle	6.25	4.60	−26
Cerebrum	4.15	3.75	−10
Liver	5.35	4.90	− 8

be expected at these tissue levels. The author believed that the change in the liver is not significant; it is also possible that the large drop in the heart thiamine-PP is too great, since in another experiment with twice the above oxythiamine dosage the level decreases only 34%. The relative ineffectiveness of oxythiamine was confirmed by de Caro *et al.* (1956) in mice, where 0.5–2 mg/day certainly produces little effect on the thiamine levels in muscle and brain, although some decrease in liver is observed. There are likewise no significant change in blood pyruvate. These results were confirmed and extended by Gurtner (1961), who administered pyrithiamine at 250 μg/day

and oxythiamine at 10 mg/day to rats intraperitoneally for 29 days; a thiamine-deficient group was also included (see accompanying tabulation).

	Pyrithiamine	Oxythiamine	B_1-deficient
Weight (% change)	−15	−43	−44
Cardiac rate (% change)	− 5	−18	−28
Paralysis (% occurrence)	93	0	46
Convulsions (% occurrence)	73	0	20
Blood pyruvate (% change)	+15	+288	+95
Tissue thiamine-PP (% change)			
Liver	−35	+26	−97
Heart	−76	−20	−95
Brain	−86	− 6	−85

The differences in the actions of the two analogs is well illustrated here; pyrithiamine produces marked neurological symptoms without much effect on weight, cardiac rate, or blood pyruvate, although there is a very significant fall in tissue thiamine-PP, whereas oxythiamine causes bradycardia and weight loss without neurological effects, while the blood pyruvate is elevated greatly without significant changes in tissue thiamine-PP.

Tissue Levels of Pyrithiamine

The pyrithiamine in rat tissues following the injection of 1 mg intraperitoneally was determined microfluorimetrically by Rindi and Perri (1961) and Rindi *et al.* (1961) and the results are plotted in Fig. 2-18. These rats had been maintained on a thiamine-deficient diet and within 1 day the pyrithiamine content of the tissues corresponded closely to the normal thiamine content, indicating that the analog probably occupies the binding sites normally occupied by thiamine or its diphosphate. It was also shown that practically all of the pyrithiamine in the liver is phosphorylated. The concentration in the brain increases progressively throughout the 12 days of the experiment and it is likely that this reflects a transference of pyrithiamine from the liver, the analog gradually replacing the thiamine in the brain. Daily oral administration of 33 μg thiamine and 210 μg pyrithiamine leads to a slow but very definite rise in tissue pyrithiamine over 20 days, the levels eventually reached being higher than following the single intraperitoneal injection. It is unfortunate that we have no data on oxythiamine distribution in the tissues, since this might well help to answer some of the problems as to why its effects are often quite different from those of pyrithiamine.

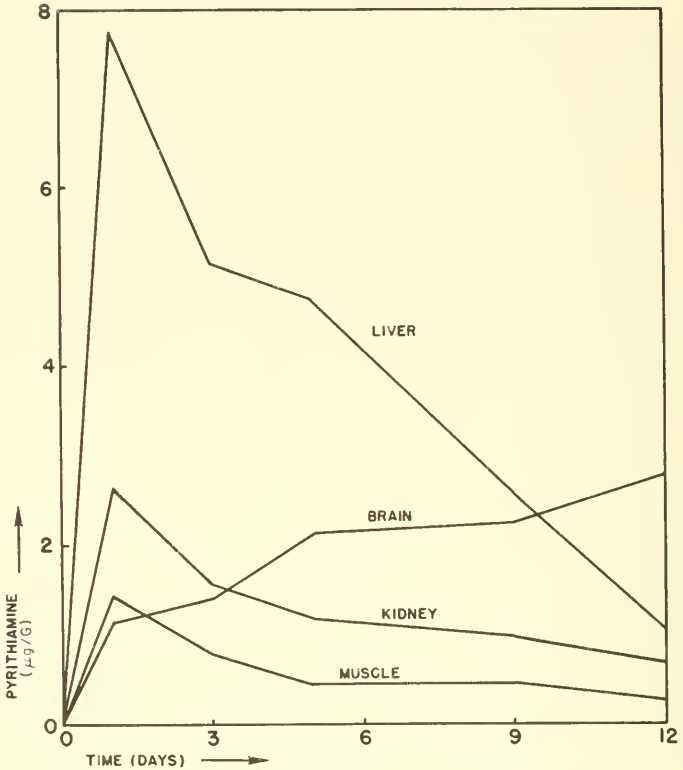


FIG. 2-18. Pyrithiamine concentrations in rat tissues after intraperitoneal injection of 1 mg. (From Rindi and Perri, 1961.)

Effects on the Growth of Microorganisms

The degree of inhibition of various bacteria and fungi by pyrithiamine has been related to the pattern of thiamine biosynthesis (Robbins, 1941; Woolley and White, 1943 c). Sensitivity to pyrithiamine was correlated with a requirement for intact thiamine, whereas those organisms able to synthesize thiamine completely are poorly inhibited. If the organisms require only part of the thiamine molecule, the growth depression by pyrithiamine is intermediate. If pyrithiamine interferes with either the formation or enzymic function of thiamine-PP, it would be difficult to understand how the manner of obtaining thiamine could determine sensitivity to the analog. However, another factor must be considered. It was shown that pyrithiamine-resistant organisms possess an enzyme capable of cleaving pyrithiamine, probably a thiaminase, while sensitive thiamine-requiring organisms do not. At least part of the resistance might be attributed to the ability of these organisms to inactivate the analog; the pyridine portion split from pyrithiamine would not be inhibitory and the pyrimidine portion can ac-

tually be utilized in thiamine synthesis. Supporting the importance of a pyrithiaminase in resistance is the observation by Woolley (1944 a) that a pyrithiamine-resistant strain of *Endomyces vernalis*, obtained by subculturing in increasing concentrations of the analog and capable of withstanding 25 times the concentration initially depressing growth 50%, contains such an enzyme. Indeed, pyrithiamine is capable of stimulating growth in the absence of thiamine since the pyrimidine portion (which is all that is required by this organism) is provided by the splitting reaction. However, destruction of pyrithiamine is not the only factor involved, since enough of the analog remains unsplit to inhibit completely the parent strain. This enzyme may be functional in the pathway biosynthesizing thiamine, which would be the reason for the correlation with thiamine requirements. It might also be well to consider another possible mechanism of inhibition, a block of the transport of thiamine into the cells; only those organisms requiring intact thiamine would be susceptible. A strain of *S. aureus* adapted to pyrithiamine exhibits a variety of changes: the pigment color changes from orange to lemon yellow, glucose utilization is severely depressed, and acetate utilization is increased (Das and Chatterjee, 1962). A partial blocking of the pentose-P pathway was also observed. These results indicate the complex alterations occurring during the development of resistance.

Some of the effects on microorganisms will be briefly summarized, since most of this work has no direct bearing on the mechanism of inhibition. In most cases the growth depression by the analogs is counteracted by thiamine, as in the inhibition of *Neurospora crassa* by oxythiamine (Sankar, 1958), and, at least in some cases, the inhibition is formally competitive with thiamine (Quesnel, 1956). Growth depression can depend on various factors. For example, *Phycomyces blakesleeanus* becomes more resistant to pyrithiamine with culture age, the concentration required for 50% inhibition being 8 times greater at 13 days than at 4 days (Fluri, 1959). Is this due to an alteration of thiamine metabolism with age, or to different metabolic requirements for thiamine? No change in sensitivity to oxythiamine with age was noted. Furthermore, oxythiamine seemed to induce a thiamine deficiency, determined by changes in carbohydrate content, whereas pyrithiamine did not. *Euglena gracilis* occurs in a normal green form and a white form (chlorophyll-deficient from streptomycin treatment): The white form is about 5 times more sensitive to pyrithiamine than is the green form (Schopfer and Keller, 1951). Thiamine analogs have been considered as possibly useful in certain infections. The growth of *Microsporum audouini* is very strongly inhibited by 0.0012 m*M* pyrithiamine and the use of the analog in tinea capitis was suggested (Ulrich and Fitzpatrick, 1951). The infection of wheat with leaf rust (*Puccinia*) might be controlled with oxythiamine inasmuch as this substances exerts a selective action on the fungus when isolated infected leaves are tested (Samborski and Forsyth, 1960).

A concentration of 0.75 m*M* inhibits rust development completely and does not exhibit phytotoxicity. *Vibrio cholera* is inhibited moderately by both oxythiamine and pyrithiamine (Chatterjee and Halder, 1960), *Lactobacillus fermentum* is inhibited by the imidazole analog of thiamine (Erlenmeyer *et al.*, 1948), and *E. coli* is inhibited 50% by the methylthio analog of thiamine at a ratio of 100 with respect to thiamine (Ulbricht and Gots, 1956). Growth inhibition by thiamine analogs has been reviewed by Rogers (1962).

Toxic and Thiamine-Deficiency Effects in Animals

Both pyrithiamine and oxythiamine are toxic to animals and produce states apparently related to thiamine deficiency. These analogs are of comparable potency; in most species pyrithiamine may be slightly more active on a weight basis. The usual daily doses to induce the characteristic toxic reactions and eventual death are usually between 0.01 and 0.1 mg, but this depends on the thiamine intake, the effective ratios of analog/thiamine being around 5 to 50. The sequence of reactions following administration of pyrithiamine to mice or rats may be summarized as: decreased food intake (this may be noted within 24 hr), inactivity and a hunched position, nervousness, tremors and occasional convulsions, spasticity followed by weakness of the legs, incoordination, and paralysis. Death usually occurs within 24 hr after the development of polyneuritis. These are essentially the symptoms seen in thiamine deficiency but they occur more rapidly after the analogs. Full polyneuritis and death may be produced within 5–12 days depending on the dose. Pyrithiamine also produces typical thiamine-deficiency polyneuritis in pigeons. The effects of oxythiamine in mice and rats are somewhat different, although death may occur in approximately the same time as from pyrithiamine. There is also anorexia and weight loss, and the animals may become nervous, convulsive, and incoordinated during the first 24 hr, but the later characteristic symptoms of polyneuritis do not occur. Descriptions of the later reactions to oxythiamine have generally been inadequate. In chicks apparently both analogs can induce polyneuritic states. The above summary is derived mainly from the work of Woolley and White (1943 b), Eusebi and Cerecedo (1949), Daniel and Norris (1949), Frohman and Day (1949), Cerecedo *et al.* (1951), Naber *et al.* (1954), and Wolfe (1957).

The differences between the effects of pyrithiamine and oxythiamine in mice and rats have been emphasized by several workers, particularly the absence of polyneuritis during treatment with oxythiamine, and have initiated speculations on the different mechanisms of action. It must be made clear that the toxic effects of oxythiamine are not nonspecific and unrelated to thiamine function, since the reactions to both analogs may be counteracted by administration of thiamine (Woolley and White, 1943 b; Jones *et al.*, 1948; Daniel and Norris, 1949; Cerecedo *et al.*, 1951; and others).

Oxypyrithiamine reduces the survival time of mice but does not produce polyneuritis, so that it appears to behave like oxythiamine, indicating the importance of the 4′-amino group on the pyrimidine ring for the effects on the nervous system (Cerecedo and Eich, 1955). The 2′-*n*-butyl analog of thiamine suppresses the growth of rats and leads to polyneuritis, these effects being antagonized by increased thiamine administration, so that this analog superficially acts like pyrithiamine (Emerson and Southwick, 1945). Another substance possibly interfering with thiamine metabolism is 2,4-diamino-5-phenylthiazole (amiphenazole, Daptazole), a drug used as a respiratory stimulant. Rats on a thiamine-free diet given injections of amiphenazole and the pyrimidine portion of thiamine in low doses do not show deficiency, indicating some ability to replace the normal thiazole component, but at higher doses deficiency signs appear sooner (Shulman, 1956). Amiphenazole alone even at high doses produces no effects. An abnormal thiamine analog is apparently synthesized in the animals. The 2-trifluoromethyl analog of thiamine (trifluorothiamine) administered at 100 mg/kg/day to mice on the thiamine-deficient diet leads to weight loss, paralysis, convulsions, and an inhibition of the growth of transplanted carcinoma (Barone *et al.*, 1960). It inhibits the growth of *Bacillus subtilis* more potently than does oxythiamine or pyrithiamine; this effect is antagonized by thiamine, but is enhanced by either the pyrimidine or thiazole moieties of thiamine. Further study of this interesting analog will be awaited with anticipation.

A few miscellaneous observations relative to the pharmacological effects of thiamine analogs on neuromuscular function will be summarized because of the importance of such effects in developing theories of the mechanisms of action. It has long been known that thiamine is involved in the formation of acetylcholine (providing the acetyl radical from pyruvate), and that brain acetylcholine concentration falls during thiamine deficiency; it is possible that some of the effects of the analogs are mediated by a depression of acetylcholine synthesis at synapses. Some have claimed that thiamine is functional in axon conduction through its role in the synthesis of acetylcholine, and others have reported a release of thiamine during nerve stimulation. It is also established that thiamine in rather high concentration inhibits cholinesterase and can, under certain circumstances, augment the action of acetylcholine. Pyrithiamine at 1–3 m*M* decreases the rates of depolarization and repolarization during the action potential in frog nerve, whereas oxythiamine at these or higher concentrations produces no effect (Kunz, 1956). This was interpreted as a blocking of the Na^+ carrier mechanism and as evidence for the participation of thiamine in Na^+ transport. Depolarization is associated with Na^+ entry, but repolarization in nerve is not connected directly to Na^+ flux. These concentrations are much higher than occur following administration to animals. The tibialis twitch response

in the cat is depressed by thiamine, pyrithiamine, and any of the pyrithiamine analogs having a hydroxyl group on the pyridinium ring; furthermore, the neuromuscular blocks produced by tubocurarine and decamethonium are antagonized by these compounds (Ngai *et al.*, 1961). In the absence of a hydroxyl group, there is a potentiation of twitch tension. Changes in blood pressure parallel those in twitch tension. These compounds, of course, may bear some relationship to acetylcholine because of the quaternary nitrogens and other functional groups an appropriate distance away, and it is likely that these acute effects are unrelated to the metabolic aspects of thiamine. It is interesting, however, that oxythiamine is without activity on the neuromuscular junction and respiration, although it causes a fall in blood pressure. No interference by the analogs in the actions of thiamine was reported. Injections of thiamine, thiamine-PP, pyrithiamine, and oxythiamine into frogs cause a miosis, which was interpreted as a direct effect on the iris (Ber and Singer-Altbeker, 1961). It is possible that this is mediated through inhibition of cholinesterase, and it is not necessary to postulate special neural or muscular functions for thiamine. It would be more valuable to study the possible changes in neuro-muscular activity during administration of the analogs chronically and when there are evident motor disturbances.

Mechanisms of Action and Comparison of Pyrithiamine and Oxythiamine

Pyrithiamine has been shown to do the following: (1) produce polyneuritis as in dietary thiamine deficiency, (2) deplete various tissues of thiamine-PP and increase its renal excretion, (3) inhibit α-keto acid metabolism *in vivo*, which is reversed by adding thiamine-PP, (4) cause elevation of blood pyruvate, (5) inhibit the phosphorylation of thiamine (thiamine kinase), (6) apparently be phosphorylated in the tissues to pyrithiamine-PP, (7) in the diphosphate form inhibit pyruvate decarboxylase, pyruvate oxidation, and probably transketolase, and (8) be picked up by the tissues to about the same extent as is thiamine normally. Most of these effects are counteracted by the administration of sufficient thiamine. It is, therefore, not difficult to establish possible sites of pyrithiamine inhibition; most of the reactions to pyrithiamine can be explained on the basis of either a block in thiamine phosphorylation or a direct inhibition of the enzymes utilizing thiamine-PP through its diphosphate ester. If the site were only on the kinase, the fundamental effect would be a depletion of thiamine-PP such as occurs in dietary deficiency, and addition of thiamine-PP to tissue preparations should restore the activity of pyruvate-metabolizing enzymes completely. The reversal is, however, only partial (Gubler, 1961) and by no means as great as in diet-deficient animals. It is thus likely that both mechanisms play a role. The appearance of pyrithiamine mainly in the diphosphate form in tissues (Rindi and Perri, 1961) also points to the im-

portance of direct α-keto acid enzyme inhibition. The rapidity with which pyrithiamine exerts its toxicity (Eusebi and Cerecedo, 1949), relative to dietary deficiency, would indicate an effect other than a block of thiamine-PP synthesis. The exact role of thiamine kinase inhibition cannot be evaluated at this time.

When we turn to oxythiamine the problem becomes more complex. The major differences from the actions of pyrithiamine may be summarized as follows: (1) typical polyneuritis is not produced, (2) it is not as effective in reducing tissue thiamine-PP levels, particularly in the brain, (3) it does not produce a significant depression of pyruvate oxidation in the brain *in vivo*, (4) inhibition of thiamine kinase is slight or absent, and (5) its toxic effects are more readily overcome by thiamine. The most obvious explanation would be that oxythiamine has generally the same actions as pyrithiamine in most tissues but for some reason does not interfere readily with thiamine function in the nervous system. Failure to penetrate into nerve tissue would adequately account for this but there is no direct evidence for this, unless the failure of oxythiamine to affect the membrane potentials of frog nerve, although pyrithiamine is effective, is interpreted in this way. One aspect that has usually not been considered is the metabolism of these analogs in the tissues, although Cerecedo *et al.* (1951) felt that oxythiamine is more rapidly metabolized than pyrithiamine. Just as in resistant bacteria, resistant tissues may contain a thiaminase-like enzyme capable of destroying the analog, and it is possible that a particular tissue can inactivate one of these analogs more than the other.

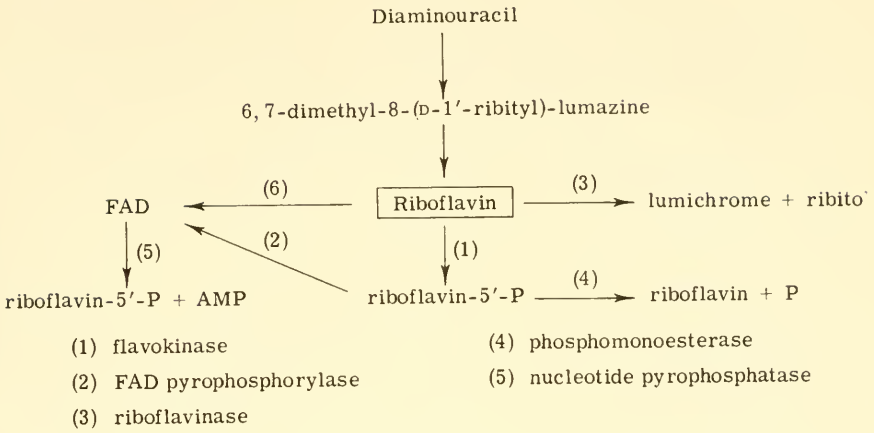
If the major pathway of thiamine in animal tissues is simply (1) the transport of thiamine into the cells, (2) its phosphorylation, and (3) the combination of the thiamine-PP with the apoenzymes, the analogs must act on one of these steps. Pyrithiamine inhibits steps (2) and (3), while oxythiamine affects only (3). No examination of interference with transport systems has been reported but this should be done. The question of whether thiamine has actions additional to its function in α-cleavage, especially in nervous tissue, must arise (Woolley and Merrifield, 1952; Gubler, 1961), but I doubt if the evidence from the use of analogs is sufficient at the present time to imply mechanisms other than on the established systems. One cannot judge the state of a tissue function from changes in blood pyruvate (which reflects changes throughout the whole animal and perhaps particularly in the liver); if central nervous system effects are to be evaluated, alterations in the metabolism in the nerve cells must be determined. Also one must always consider the relationship between cell function and thiamine-PP level. To what degree must thiamine-PP in the brain fall before symptoms occur? This has often been judged by experiments in diet-deficient animals, but in analog-treated animals it is not necessary that thiamine-PP levels be reduced to the same degree to obtain the same functional disturbances.

Finally, the arguments of Gubler (1961), that pyrithiamine must induce disturbances other than in α-keto acid metabolism, I feel are not entirely valid. He states that since the α-keto acid oxidase activities are appreciably lower in thiamine-deprived rat livers than in the livers of analog-treated rats, some other disturbances in physiological function must contribute to the deficiency symptoms and death. However, it is unlikely that the changes in liver metabolism have much to do with either the symptoms or death, and he actually found that the α-keto acid oxidase activities in the brain are lower in pyrithiamine-treated rats than in thiamine-deprived rats. The other argument, that pyrithiamine causes a polyneuritis that is difficult or impossible to reverse by administration of thiamine whereas α-keto acid oxidase activities can be readily restored in tissue extracts by adding thiamine-PP, may be significant, but these data could be just as easily explained by an inhibition of thiamine kinase (preventing the synthesis of thiamine-PP in the animal) or a very slow rate of exchange between thiamine and enzyme-bound pyrithiamine-PP in the intact nervous tissue.

ANALOGS OF RIBOFLAVIN AND FAD

Riboflavin functions in metabolism as riboflavin-5′-phosphate (flavin mononucleotide, FMN) and flavin-adenine dinucleotide (FAD) in various oxidizing enzymes and electron transport. The flavin coenzymes are usually very tightly bound to their respective apoenzymes and are not dissociated during extraction of the enzyme preparations. Indeed, in some cases, such as succinate oxidase, the flavin component can be liberated only by proteolytic digestion, with fragments of peptides attached, and the activity cannot be restored by addition of any flavin compound. In most cases it is thus difficult for analogs to replace or compete with the flavin coenzyme, particularly in preparations from animal tissues, although in microorganisms the flavoenzymes are generally more readily dissociable. The binding of FAD seems to involve the isoalloxazine ring (perhaps the imino group at position 3), possibly the ribityl portion, the phosphates, and the adenine ring. Chelation to apoenzyme-bound metal ions, such as iron, is likely because most flavoenzymes contain such metal ions, but there is still some doubt as to whether the metal ions function primarily in binding or in electron transfer.

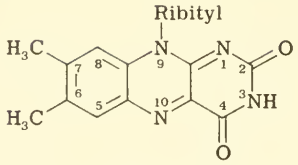
Animals and a few bacteria depend on exogenous riboflavin but it is synthesized in plants and most microorganisms. The pathway of riboflavin biosynthesis is not well understood and has been studied mainly in a few microorganisms used for the commercial production of riboflavin; the reactions by which riboflavin is transformed into active coenzymes are better documented. An abbreviated scheme of biosynthesis and breakdown is represented here to facilitate discussion of the actions of analogs.



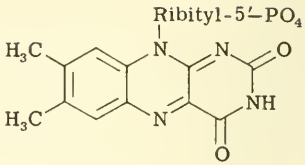
(Reaction (6) represents a possible formation of FAD directly from riboflavin and ATP, as postulated by Masuda (1955) in *E. ashbyii*; reactions (1) and (2) also require ATP)

Many analogs, in which various parts of the riboflavin or FAD structure have been modified, have been examined and very few are able to replace riboflavin, possibly due both to inability to form the coenzyme analogs and to relative inactivity of the coenzyme analogs when formed. Most of these analogs are not significantly inhibitory to riboflavin function, indicating the high degree of specificity in these reactions. Beinert (1960) has classified the structural modifications producing most of the interesting analogs into those where (1) the riboflavin is altered (as by replacement of ribitol by other sugar alcohols or alkyl groups, replacement of the isoalloxazine ring by other ring systems, or substitutions in the isoalloxazine ring), (2) riboflavin esters other than the 5′-phosphate are synthesized (as the diphosphate, acetylphosphate, or sulfate), and (3) dinucleotides of riboflavin have the AMP replaced by other nucleotides (such as GMP, IMP, CMP, or UMP).

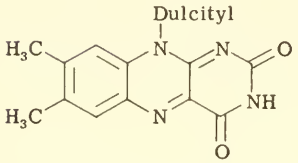
In this chapter we shall be concerned with the rather simple and obvious analogs of riboflavin and FAD. There are several types of drug and metabolic inhibitor that probably interfere in one way or another with riboflavin metabolism or function — such as the promazines, the antibiotic tetracyclines, the acridine antiseptics, certain antimalarials, and perhaps some of the chelators of the phenanthroline and bipyridine types — and these will be discussed for the most part in future chapters. Quinacrine only will be treated in some detail at the end of this section because it is the best studied riboflavin analog.



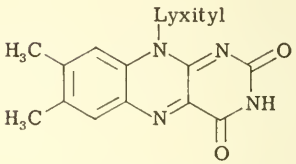
Riboflavin



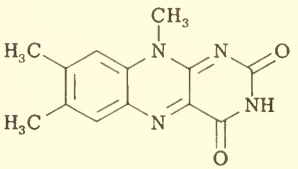
Riboflavin-5′-P



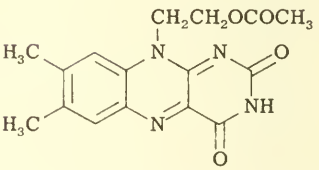
Galactoflavin



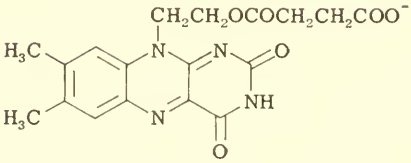
Lyxoflavin



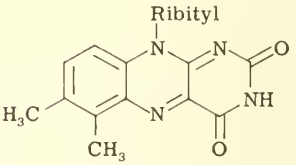
Lumiflavin



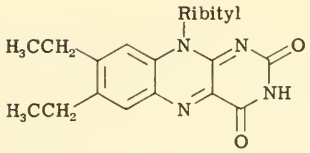
U-2112



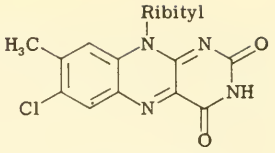
U-6538



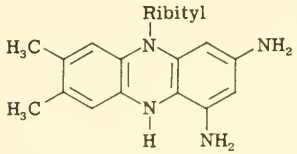
Isoriboflavin



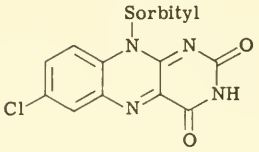
6, 7-Diethyl analog
of riboflavin



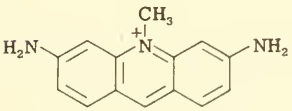
6-Chloro analog
of riboflavin



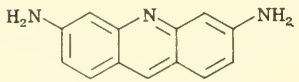
2, 4-Diamino-7, 8-
dimethyl-10-
ribityl-5, 10-dihydrophenazine



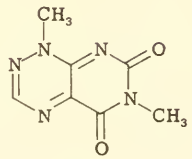
Flavotin



Acriflavine
(trypaflavine, euflavine)



Proflavine



Toxoflavin

Effects on Growth

A number of analogs have been found to inhibit the growth of *Lactobacillus casei*, the standard test organism: isoriboflavin, the 6-chloro and 7-chloro analogs of riboflavin (Lambooy, 1955), the 2,4-diaminophenazine analog (Woolley, 1944 b), and riboflavin-5′-sulfate (Egami *et al.*, 1956). Other bacteria are occasionally inhibited, for example *S. aureus* and *Str. plantarum* by dichlororiboflavin (Lambooy, 1955). The acridines, proflavine and 5-aminoacridine, suppress the growth of *L. casei* but this is not counteracted by increasing the riboflavin concentration, so the mechanism is

not clear (Madinaveitia, 1946). Toxoflavin is a poisonous substance from *Pseudomonas cocovenenans* and responsible for some fatal food poisonings in Java. It quite potently inhibits the growth of *E. coli*, *S. aureus*, *B. subtilis*, and *Shigella*, as well as being toxic to experimental animals (Latuasan and Berends, 1961). The mechanism is unknown but it was postulated that toxoflavin is an effective electron-acceptor and removes electrons from the transport chains to form hydrogen peroxide, possibly competing with the natural flavin coenzymes (or accepting electrons from them). Relatively little has been done on the induction of riboflavin deficiency in animals. Isoriboflavin almost stops the growth of young rats at 2 mg/day and riboflavin is able to reverse this (Emerson and Tishler, 1944), and galactoflavin appears to produce very similar effects (Emerson *et al.*, 1945). The 6-chloro and 7-chloro analogs of riboflavin (Lambooy, 1955) and the dinitrophenazine analog (Woolley, 1944 b) produce mild deficiency states in rats and mice. Effects have usually been determined by growth rates, and typical symptoms of riboflavin deficiency have seldom been noted, mainly because it requires a fairly long time to deplete the tissues of enzyme-bound coenzymes.

Some interesting and suggestive reports on the carcinostatic activity of certain analogs have appeared. The 6,7-dichloro-9-(1′-D-sorbityl)isoalloxazine analog causes regression of mouse lymphosarcoma, the ribityl compound has slight activity, and the other sugar alcohol derivatives are inactive (Holly *et al.*, 1950). Replacement of the ribityl group with nonsugar residues gives riboflavin antagonists which inhibit *L. casei* and produce deficiency states in rats. The 9-hydroxyethyl analog of riboflavin (U-2113) weakly suppresses mouse adenocarcinoma (Shapiro *et al.*, 1956), and the 9-acetoxyethyl analog (U-2112) behaves similarly (Lane *et al.*, 1958). However, U-2112 given to patients with various types of cancer (0.25–6 g/day for 5–84 days, with total doses 1.25–226 g) exhibits no beneficial action and no evidence of riboflavin deficiency is seen; this was attributed to the rapid hydrolysis of this substance in man. The 9-hemisuccinoxyethyl analog (U-6538) does not inhibit *L. casei* but depresses growth in rats at 10 mg/kg/day, which is reversible with riboflavin (Lane *et al.*, 1959). It is quite effective against lymphosarcoma in rats and at 5 mg/kg/day leads to a 66% inhibition of tumor growth without depressing the over-all growth rate. Two of four patients receiving the compound showed changes suggestive of riboflavin deficiency but no evidence of carcinostasis was observed, perhaps due to the terminal nature of the disease and the metabolism of the analog. Galactoflavin is known to cause tumor regression in rodents. It is tolerated by patients at a dose of 1 g every 8 hr for 2–5 months, and deficiency symptoms do not occur unless the diet is low in riboflavin (Lane and Brindley, 1964). Presumably reports on its carcinostatic activity will be published.

Metabolism of Riboflavin Analogs and Effects on Riboflavin Metabolism

Certain analogs, such as the 6,7-diethyl derivative, are able to replace riboflavin to some extent at low concentration but are inhibitory at higher concentration. This analog supports the growth of *L. casei*, and Lambooy (1950) believed that it must be phosphorylated. This was demonstrated in the rat where 6,7-diethylriboflavin-5′-P was found in the liver although no FAD analog was demonstrable (Aposhian and Lambooy, 1955). *L. lactis* is able to incorporate lyxoflavin into lyxoflavin-5′-P and the corresponding dinucleotide (Huennekens *et al.*, 1957 b). Scala and Lambooy (1958) were able to modify *L. casei* by prolonged riboflavin deficiency and high concentrations of the 6-chloro analog so that the organism could use either riboflavin or the analog. They believe that the analog inhibits the phosphorylation of riboflavin. It is interesting that the adapted organism cannot use the 7-chloro analog.

Flavokinase catalyzes the phosphorylation of riboflavin and shows a high degree of specificity toward substrates. The yeast enzyme phosphorylates dichlororiboflavin as well as riboflavin, arabitylflavin poorly, and all other analogs tested not at all (including isoriboflavin, galactoflavin, dulcitylflavin, and sorbitylflavin) (Kearney, 1952). The only analog that inhibits the enzyme is lumiflavin (35% at 0.18 mM with riboflavin 0.051 mM) and this occurs only when the analog is in excess of the riboflavin. McCormick (1962) has extended this work to partially purified rat liver flavokinase and found similar behavior, only riboflavin, dichlororiboflavin, and arabitylflavin being phosphorylated (all with K_m's between 0.012 and 0.017 mM). Four analogs were found to be inhibitory: lumichrome ($K_i = 0.048$ mM), lumiflavin ($K_i = 0.031$ mM), the 9-formylmethyl analog ($K_i = 0.0097$ mM), and the 9-(2′-hydroxyethyl) analog ($K_i = 0.0068$ mM). The following are not phosphorylated and do not inhibit: isoriboflavin, galactoflavin, sorbitylflavin, dichloroarabitylflavin, 7-methylmannitylflavin, and 7-methyldulcitylflavin. The fact that most analogs are not attacked by flavokinase is perhaps the primary reason for the failure of these compounds to replace riboflavin. It is also clear that the data are insufficient to draw conclusions relative to the possibility of some of the most commonly used analogs inhibiting the phosphorylation of riboflavin, but what evidence we have would indicate that such inhibition is unlikely to be important. The synthesis of riboflavin from 6,7-dimethyl-8-(1′-D-ribityl)lumazine by an enzyme system from *Ashbya gossypii* is potently inhibited by a variety of analogs of this precursor, of which the 6,7-dihydroxy derivative is the most active ($K_i = 0.000009$ mM) (Winestock *et al.*, 1963). It is interesting that 5′-deoxyriboflavin is fairly inhibitory ($K_i = 0.019$ mM) and, indeed, it was concluded that the sugar moiety is necessary for inhibition.

Very little information on the effects of analogs on the tissue levels of riboflavin compounds is available. Rats given galactoflavin for 10–28 days

show a 70–75% depression of liver mitochondrial flavin, and dietary riboflavin restriction also reduced the level (Beyer *et al.*, 1961). The evidence from depression of enzyme activity will be discussed later. The thorough analysis of the effects of riboflavin deficiency on rats by Burch *et al.* (1956) has shown that various tissues differ markedly in ability to retain FMN and FAD (see accompanying tabulation). Deficiency of 23-day duration

Tissue	% Change in deficient rats				
	FMN	FAD	NADH oxidase	D-Amino acid oxidase	Xanthine oxidase
Brain	−24	−19	0	+ 8	—
Liver	−86	−61	+10	−66	−22
Kidney	−41	−19	−11	−17	−19
Heart	0	−32	−23	+ 6	0

has little effect on brain flavins while liver levels drop rapidly. No necessary correlation between total FMN or FAD and enzyme activity is evident, indicating that some enzymes will lose their FMN or FAD much more readily than others and that a fraction of the cellular flavin may be nonenzymically bound. On the basis of these results, one might expect riboflavin analogs, when active, to exert differential effects on the various flavoenzymes, and it is likely that analyses for total flavins will not provide metabolically significant figures.

Effects on Flavoenzymes

The enzymes most commonly used to test the inhibitory activity of riboflavin analogs are those with dissociable flavin coenzymes, such as the old yellow enzyme and D-amino acid oxidase, and in such cases an inhibition of a competitive nature is not unexpected. However, there are many instances of the inhibition of enzymes which have very tightly bound coenzymes and these are more difficult to interpret. Some inhibitions of both types are shown in Table 2-33. Inhibitions by riboflavin, FMN, and FAD are also included because these show that it is not always necessary to consider inhibition as resulting from structural analogs.

Most of these inhibitions appear to be noncompetitive. Thus the inhibitions of L-galactono-γ-lactone dehydrogenase by riboflavin, L-amino acid oxidase by riboflavin and its analogs, and D-amino acid oxidase by riboflavin are not reduced by increasing concentrations of FMN or FAD. However, the inhibitions of kidney D-amino acid oxidase by FMN and riboflavin-5′-sulfate are competitive with respect to FAD, and the inhibition

TABLE 2-33

INHIBITION OF ENZYMES BY FLAVINS AND RIBOFLAVIN ANALOGS

Enzyme	Source	Inhibitor	Concentration (mM)	% Inhibition [a]	Reference
D-Amino acid oxidase	Pig kidney	Riboflavin	0.54	25	Walaas and Walaas (1956)
		FMN	0.4	50	
				($K_i = 0.03$)	
	Pig kidney	Riboflavin-5′-sulfate	—	($K_i = 0.031$)	Egami and Yagi (1956)
	—	Riboflavin	—	($K_i = 0.07$–0.2)	Crandall (1959)
		FMN	—	($K_i = 0.07$–0.2)	
L-Amino acid oxidase	Silkworm eggs	Riboflavin	0.1	77	Kotaka (1963)
			1	99	
		Isoriboflavin	0.1	72	
			1	95	
	Moccasin venom	Riboflavin	0.04	26	Singer and Kearney (1950)
			0.4	61	
		Isoriboflavin	0.04	7	
			0.4	49	
		Dichlororiboflavin	0.9	10	
		Alloxazine	1	24	

TABLE 2-33 (*continued*)

Enzyme	Source	Inhibitor	Concentration (mM)	% Inhibition [a]	Reference
	Cobra venom	Isoriboflavin	1	48	Singer and Kearney (1950)
	Copperhead venom	Riboflavin	1	32	Singer and Kearney (1950)
		Isoriboflavin	1	17	
FAD pyrophosphorylase	Rat liver	Riboflavin	—	($K_i = 4.1$)	McCormick (1964)
		Isoriboflavin	—	($K_i = 0.68$)	
L-Galactono-γ-lactone dehydrogenase	Cauliflower	Riboflavin	0.1	44	Mapson and Breslow (1958)
			0.2	59	
			0.5	66	
D-Gluconate oxidase	*Aerobacter aerogenes*	Riboflavin	0.1	9	Blakley and Ciferri (1961)
Glutamate racemase	*Lactobacillus fermenti*	Riboflavin	0.1	26	Tanaka *et al.* (1961)
		FMN	2	10	
D-Lactate oxidase	*Aerobacter aerogenes*	Riboflavin	0.1	21	Blakley and Ciferri (1961)
L-Lactate oxidase	*Aerobacter aerogenes*	Riboflavin	0.1	15	Blakley and Ciferri (1961)

	Mycobacterium smegmatis	Riboflavin	—	(ni)	Cousins (1956)
		FMN	0.049	24	
		FAD	0.019	18	
Lysolecithin oxidase	*Mycobacterium avis*	FAD	1	25	Hoshino (1959)
NADPH: methemoglobin oxidoreductase	Human erythrocytes	Riboflavin	10	0	Huennekens *et al.* (1957)
Protein disulfide reductase	Yeast	Riboflavin	0.008	75	Asahi *et al.* (1961)
Riboflavin synthetase	*Ashbya gossypii*	6,7-Dihydroxy-7-*n*-pentyl-8-(1′-ribityl)lumazine	—	($K_i = 0.000009$)	Winestock *et al.* (1963)
Riboflavin transglucosidase	*E. coli*	Ribityllumazine	0.2	36	Tachibana *et al.* (1958)
		FMN	0.2	90	
		Lumiflavin	0.2	82	
Succinate oxidase	*Bacterium tularense*	Riboflavin	5	(i)	Wadkins and Mills (1956)
		FMN	5	0	
		FAD	5	(i)	
	Tetrahymena pyriformis	Riboflavin	3	8	Eichel (1956 b)
		FMN	2.4	20	
		Galactoflavin	1.8	13	
		Flavotin	0.9	24	

[a] The designation (i) indicates that inhibition was observed but degree not stated; (ni) no inhibition. Values for K_i in mM.

of glutamate racemase by riboflavin is reduced by FAD. It is clear that there is not much information on the inhibition of enzymes by riboflavin analogs, especially by their phosphates, or FAD analogs. The mechanism for the inhibition of enzymes in which the flavin coenzyme is tightly bound is not known. However, it is possible to suggest three mechanisms. It is now known that various flavins and their nucleotides form molecular complexes with one another, and the formation of such complexes with bound FMN or FAD may occur, preventing the normal interactions of the coenzymes in oxidation. In some instances the inhibitors may interfere with the experimental electron acceptor, particularly when this is a dye. Lastly, one must consider the possibility of nonspecific binding of these polyheterocyclic compounds to the enzymes; one might predict that a number of enzymes not involving flavins would be inhibited by such analogs, but few have been examined. None of the riboflavin analogs in Table 2-33 is a potent inhibitor and it is unlikely that these inhibitions are responsible for any of the *in vivo* effects observed.

The coenzyme of the old yellow enzyme is FMN, and riboflavin-5′-sulfate does not interfere with its binding to the apoenzyme, which Theorell *et al.* (1957) explain by the less negative charge on the sulfate group. Riboflavin-5′-sulfate, however, inhibits D-amino acid oxidase (Egami and Yagi, 1956), so that the structural requirements for binding must be different in these two enzymes. Yagi and Nagatsu (1960) have studied the effects of riboflavin-5′-sulfate on rat liver mitochondrial oxidations of α-ketoglutarate, succinate, malate, and D-alanine, and found that no inhibition is exerted at 0.1 mM, which they interpret as due to the tight binding of the FAD in the mitochondria. Aged mitochondria are stimulated by FAD and here inhibition by riboflavin-5′-sulfate can be demonstrated. The FAD analogs of the various flavins have not been studied often but Huennekens *et al.* (1957 b) found lyxoflavin-5′-phosphate to be active in the NADPH-cytochrome c reductase (although less than FMN) and lyxoflavin dinucleotide to be active in the D-amino acid oxidase (but less than FAD). We have seen that riboflavin deficiency leads to reduction in the activities of certain enzymes in the tissues. Administration of galactoflavin to rats for 15–28 days leads to an approximately 40% reduction in glutamate and β-hydroxybutyrate oxidation in liver mitochondria, but no change in succinate oxidation or in the P : O ratios (Beyer *et al.*, 1961). U-2113, the 9-hydroxyethyl analog of riboflavin, causes a slight (15%) decrease in tumor xanthine oxidase in mice. It is not known if this is due to FAD depletion or a more direct inhibition. 5-Hydroxytryptamine (serotonin) is metabolized by monoamine oxidase to 5-hydroxyindoleacetate; both riboflavin deficiency and galactoflavin increase the urinary excretion of this product, indicating that one of the other metabolic pathways for serotonin is depressed by interference with flavin function (Wiseman and Sourkes, 1961). These mis-

cellaneous observations do not provide a satisfactory basis for understanding the metabolic effects of riboflavin analogs.

We have been discussing analogs of the riboflavin portion of FAD and some mention of the adenine nucleotides as inhibitors should be made. The D-amino acid oxidase of sheep kidney is inhibited competitively by various purines and nucleotides (see accompanying tabulation) (Burton, 1951 a).

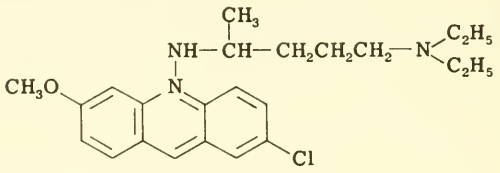
Inhibitor	K_i (mM)
5′-AMP	1.05
3′-AMP	No inhibition
ADP	1.3
ATP	11
Adenosine	45
Adenine	22
Hypoxanthine	24
Caffeine	11

It was shown that complexes between riboflavin and purines are formed and have the following dissociation constants: caffeine 10 mM, adenosine 30 mM, AMP 40 mM, ADP 37 mM, and ATP 39 mM. The formation of such complexes might account for the enzyme inhibition in the case of adenosine and caffeine, but cannot account for the more potent effects of AMP and ADP, these latter substances competing with FAD for the apoenzyme site. The D-amino acid oxidase from pig kidney is likewise inhibited: 50% inhibition is given by 0.4–0.6 mM AMP, ADP, ATP, and IMP; by 6 mM adenine, adenosine, and hypoxanthine; and by 15–20 mM uracil, cytosine, and ribose-5′-P (FAD 0.00014 mM in all cases) (Walaas and Walaas, 1956). The K_i for the competitive 5′-AMP is 0.64 mM. Crandall (1959) determined K_i for AMP as 0.1 mM for this enzyme. Flavokinase is inhibited strongly by 5′-AMP ($K_i = 0.025$ mM), and it is possible that the adenine portion of the nucleotide competes with the alloxazine ring of riboflavin for the active center (Kearney, 1955).

Quinacrine (Mepacrine, Atabrine, Atebrin)

Quinacrine is an acridine derivative introduced by the Germans in 1932 for malaria therapy as a suppressive agent and is more effective than quinine on the asexual forms of the plasmodia. Since the observation by Wright and Sabine (1944) that FAD counteracts the inhibition of tissue respiration by quinacrine, it has been commonly assumed that quinacrine exerts its

primary effects by interference with flavoenzymes, and has come to be the most widely used substance to detect the participation of a flavin component in an enzyme or metabolic system. Quinacrine not only inhibits the



Quinacrine

malarial organism but, in common with other acridines, suppresses the growth of various bacteria. For example, *Lactobacillus casei* is inhibited and this seems to be related to flavin metabolism since the maximal concentration at which growth occurs is 0.12 m*M* when riboflavin is 0.00066 m*M* and 0.49 m*M* when riboflavin is 10 times the previous concentration (Madinaveitia, 1946). Spore germination of *B. subtilis* (Falcone *et al.*, 1959) and *B. coagulans* (Amaha and Nakahara, 1959) induced by L-alanine is inhibited 58% by 0.1 m*M* quinacrine and nearly completely by 1 m*M*.

The toxic effects observed in experimental animals and man — for example, gastrointestinal (abdominal pain, diarrhea, nausea), dermatological (eczematoid dermatitis, lichen planus), central nervous system (psychoses), and others — do not appear to be related to riboflavin deficiency, and the typical syndrome of deficiency has never been produced by quinacrine. Thus one must assume that other actions are probably of more importance in animals. One characteristic of quinacrine is its remarkable ability to be accumulated in the tissues during chronic administration, and eventually the tissue levels are hundreds or thousands of time higher than in the serum (see Table I-8-1). This is evident from the yellow coloration of the tissues. These levels, of course, do not represent free quinacrine and the accumulation is due to the high affinity of various tissue components for quinacrine. It is bound in the cytoplasm, the mitochondria, and the nucleus; equilibration of isolated nuclei with quinacrine results in a 200-fold concentration differential (Reiner and Gellhorn, 1956). Proteins, nucleic acids, and nucleoproteins bind quinacrine strongly, and some of the inhibitory, mutagenic, and carcinostatic effects have been attributed to such binding. It is thus clear that quinacrine can be bound at many loci in the cell.

Effects of Quinacrine on Enzymes

Many enzymes are inhibited by quinacrine (Table 2-34). In some cases the inhibition is competitive (or at least reduced by increasing the concentration of FMN or FAD) and in others it is not. Quinacrine has come to

be the most commonly used detector for the participation of flavins in enzyme systems, but before this is subjected to analysis we shall discuss some of the results which have been obtained. The following summary presents the data reported on antagonism but does not in any case imply a truly competitive mechanism.

Enzymes in which inhibition is reduced by FMN or FAD

Adenosinetriphosphatase (mitochondrial NAD-activated): FMN and FAD (Löw, 1959)

Aldehyde oxidase (rat and monkey liver): FAD (Lakshmanan *et al.*, 1964; Mahadevan *et al.*, 1962)

Aliesterase (liver): FMN (Hemker and Hülsman, 1960)

D-Amino acid oxidase (lamb and sheep kidney): FAD (Hellerman *et al.*, 1946; Burton, 1951 a)

Catechol oxidase (spinach): FAD (Nair and Vining, 1964)

Choline dehydrogenase (rat liver): FAD (Bargoni, 1963)

Cytochrome reductase: FMN (Haas, 1944)

Hydroxylamine: cytochrome c oxidoreductase (*Nitrosomonas*): FMN and FAD (Aleem and Lees, 1963)

Lactate dehydrogenase (*Lactobacillus* and yeast): FMN and FAD (Snoswell, 1959; Iwatsubo and Labeyrie, 1962)

NADH: nitrite oxidoreductase (*Neurospora*): FAD (Nicholas *et al.*, 1960)

NADH oxidase (*Azotobacter*, *Clostridium*, and *Lactobacillus*): FAD (Repaske and Josten, 1958; Dolin, 1959; C. F. Strittmatter, 1959)

Nitrate reductase (*Pseudomonas*): FAD (Fewson and Nicholas, 1961)

Succinate oxidase (*Tetrahymena* and *Xanthomonas*): FMN and FAD (Eichel, 1956 b; Madsen, 1960)

Enzymes in which inhibition is not reduced by FMN or FAD

Adenosinetriphosphatase (mitochondrial sonicate) (Beyer, 1960)

Allohydroxy-D-proline oxidase (*Pseudomonas*) (Yoneya and Adams, 1961)

L-Amino acid oxidase (moccasin venom) (Singer and Kearney, 1950)

Ethanolamine oxidase (*Arthrobacter*) (Narrod and Jakoby, 1964)

L-Galactono-γ-lactone dehydrogenase (cauliflower) (Mapson and Breslow, 1958)

Lactate dehydrogenase (*Propionibacterium*) (Molinari and Lara, 1960)

Nitrate reductase (*E. coli*) (Heredia and Medina, 1960)

Old yellow enzyme (yeast) (Kistner, 1960)

TABLE 2-34

INHIBITION OF ENZYMES BY QUINACRINE

Enzyme	Source	Reaction components[a]	Concentration (m*M*)	% Inhibition	Reference
Adenosine-triphosphatase	*Bacillus megaterium*	ATP(5)	25	64	Greenawalt *et al.* (1962)
	Spinach chloroplasts	ATP(4)	5	40	Wessels and Baltscheffsky (1960)
	Mouse muscle	ATP(4)	1	35	Pennington (1961)
			5	75	
			10	87	
	Rat liver mitochondria	ATP	2	94	Löw (1959 b)
	Rat liver mitochondria	ATP(5)	0.4	33	Beyer (1960)
			4	76	
	Rat brain microsomes	ATP(5)	2	30	Järnefelt (1962)
			8	61	
	Rabbit myosin	ATP	2	10	Löw (1959 b)
	Rabbit myosin	ATP(1)	1	50	Kaldor (1960)
	Rabbit myosin	ATP(1)	2.5	72	Kaldor and Gitlin (1963)
	Beef heart mitochondria	ATP(6)	0.05	0	Penefsky *et al.* (1960)
Alcohol dehydrogenase	Yeast	Ethanol and NAD	0.015	10	Frimmer (1961)
			0.11	50	
			0.2	69	
	Horse liver	Ethanol and NAD	0.2	19	Frimmer (1961)

Aldehyde oxidase	Rabbit liver	*N*-Methylnicotinamide	—	(K_i = 0.0015)	Rajagopalan and Handler (1964)
			0.1	83	Rajagopalan *et al.* (1962)
	Pig liver	Acetaldehyde	0.01	57	Mahler *et al.* (1954)
			0.1	94	
	Monkey liver	Vitamin A	0.01	10	Lakshmanan *et al.* (1964)
			0.1	45	
			1	100	
Aliesterase	Rat liver	Tributyrin	5	50	Hemker and Hülsman (1960)
Allohydroxy-D-proline oxidase	*Pseudomonas striata*	Allo-OH-D-proline	0.5	69	Yoneya and Adams (1961)
D-Amino acid oxidase	Lamb kidney	FAD(0.00011)	1	70	Hellerman *et al.* (1946)
		(0.00041)	1	36	
		(0.0011)	1	8	
L-Amino acid oxidase	*Neurospora crassa*	L-φ-alanine(0.0112)	10	15	Burton (1951 b)
	Moccasin venom	—	2	48	Singer and Kearney (1950)
Apyrase	Potato	ATP	2	3	Löw (1959 b)
Catechol oxidase	Spinach leaves	Catechol(1)	0.5	87	Nair and Vining (1964)
Choline dehydrogenase	Rat liver	Choline	0.2	50	Bargoni (1963)
			0.33	70	
Cholinesterase	Human plasma	Acetylcholine(3)	0.003	50	Wright and Sabine (1948)
Cytochrome c-554 reductase	*Micrococcus*	Succinate	1	61	Hori (1963)
Cytochrome c reductase	Heart	—	0.04	14	Haas (1944)
			0.2	49	
			1	82	

TABLE 2-34 (*continued*)

Enzyme	Source	Reaction components[a]	Concentration (m*M*)	% Inhibition	Reference
Cytochrome oxidase	Heart	Cytochrome c	0.2	22	Haas (1944)
Ethanolamine oxidase	*Arthrobacter*	Ethanolamine(5)	0.2	48	Narrod and Jakoby (1964)
L-Galactono-γ-lactone dehydrogenase	Cauliflower mitochondria	L-Galactone-γ-lactone	0.1	15	Mapson and Breslau (1958)
			1	62	
			2.5	89	
Galactose oxidase	*Polyporus circinatus*	Galactose(1) and FMN(1)	0.1	9	Cooper *et al.* (1959)
Glucose dehydrogenase	Lamb liver	Glucose	2	95	Eichel and Wainio (1948)
Glucose-6-P dehydrogenase	—	Glucose-6-P and NADP	0.5	77	Haas (1944)
	Erythrocytes	Glucose-6-P and NADP	5	50	Hemker and Hülsman (1960)
Glutaminase	*Clostridium welchii*	Glutamine(5)	10	0	Hughes and Williamson (1952)
	Guinea pig liver	Glutamine	0.5	36	Guha (1962)
	Dog kidney	Glutamine(0.83)	0.93	60	Archibald (1944)
			9.3	100	
Glutaminase I	Guinea pig kidney	Glutamine(10)	0.65	100	Van Baerle *et al.* (1957)
			6.5	100	
		(30)	0.65	45	
			6.5	45	

Glutaminase II	Guinea pig kidney	Glutamine	0.65 6.5	0 85	Goldstein *et al.* (1957)
Hexokinase	Yeast	Glucose(1.25) and ATP(1)	4	100	Fraser and Kermack (1957)
	Yeast	Glucose and ATP	2–7	Stim	Löw (1959 b)
	Plasmodium berghei	Glucose(1.25) and ATP(4)	4	32	Fraser and Kermack (1957)
Hydrogenase	*Desulfovibrio desulfuricans*	—	4	78	Ishimoto *et al.* (1958)
L-α-Hydroxy acid oxidase	Pig kidney	Glycolate(3.3) α-OH-isocaproate(4) Phenyllactate(33)	1 1 1	76 68 72	Robinson *et al.* (1962)
Hydroxylamine: cytochrome c oxidoreductase	*Nitrosomonas europaea*	Hydroxylamine(0.33)	0.5	60	Aleem and Lees (1963)
Iron oxidase	*Ferrobacillus ferrooxidans*	Fe^{++}(2)	0.01 0.1 0.5	45 50 60	Blaylock and Nason (1963)
Inosinetriphosphatase	Rabbit muscle	ITP(5)	2.5	70	Kaldor and Gitlin (1963)
Lactate dehydrogenase	*Lactobacillus arabinosus*	Lactate and NAD	0.003 0.03 0.1 0.3 0.6	0 11 24 62 72	Snoswell (1959)
	Propionibacterium pentosaceum	Lactate and NAD	0.1 1	30 83	Molinari and Lara (1960)

TABLE 2-34 (*continued*)

Enzyme	Source	Reaction components[a]	Concentration (m*M*)	% Inhibition	Reference
D-Lactate oxidase	Rabbit kidney	D-Lactate	5	0	Tubbs and Greville (1961)
L-Lactate oxidase	Yeast	Lactate and cytochrome b_2	1	20	Boeri *et al.* (1955)
	Tetrahymena pyriformis	Lactate	3	10	Eichel and Rem (1959)
Lactate oxidative decarboxylase	*Mycobacterium phlei*	Lactate	1	5	Sutton (1954)
Malate dehydrogenase	Rabbit muscle	Malate	0.24	7	Frimmer (1961)
Malate: vitamin K oxidoreductase	*Mycobacterium phlei*	Malate and vitamin K	2	83	Asano and Brodie (1963)
NADH: cytochrome c oxidoreductase	*E. coli*	NADH	1	70	Brodie (1952)
	Bacillus cereus	NADH	0.1	31	Doi and Halvorson (1961)
	Mytilus edulis	NADH	1	38	Ryan and King (1962 b)
	Beef liver	NADH	0.6	0	Penn and Mackler (1958)
NADH: hydroxylamine oxidoreductase	Soybean leaves	Hydroxylamine and NADH	0.3	0	Roussos and Nason (1960)
NADH: menadione oxidoreductase	Pig liver	NADH(0.033) and menadione(0.1)	2	38	Raw *et al.* (1961)

	Pig liver	NADH and menadione	0.71	82	Frimmer (1961)
NADH: methylene blue oxidoreductase	Pig liver	NADH and methylene blue	0.79	62	Frimmer (1961)
NADH: nitrite oxidoreductase	Soybean leaves	NADH and nitrite	0.3	29	Roussos and Nason (1960)
	Neurospora crassa	NADH and nitrite	2	61	Nicholas *et al.* (1960)
NADH oxidase	*Bacillus cereus*	NADH	0.1	15–20	Doi and Halvorson (1961)
	Clostridium perfringens	NADH(0.1) and FAD(0.13)	0.1	21	Dolin (1959)
			0.33	35	
			0.66	57	
			1	99	
	Azotobacter vinelandii	NADH	0.005	50	Repaske and Josten (1958)
	Xanthomonas phaseoli	NADH(6.8)	1	15	Hochster and Nozzolillo (1960)
			5	55	
	Lactobacillus casei	NAD(2) and FAD(0.01)	1	44	C. F. Strittmatter (1959)
	Beef heart	NADH	1	0	Mackler (1961)
NADH: tetrazolium oxidoreductase	Rat liver	NADH	4	3	Slater (1959)
			8	22	
NAD(P)H diaphorase	Beef brain	NADH	6.6	38	Harper and Strecker (1962)
NAD(P)H: menadione oxidoreductase	*Phaseolus aureus*	NADH and menadione	0.01	26	Shichi and Hackett (1962)
	Spinach leaves	NADH(0.17) and menadione(0.03)	0.05	0	Lazzarini and San Pietro (1964)
			0.5	28	
NAD(P)H: methemoglobin oxidoreductase	Rabbit erythrocytes	NADPH	0.4	73	Bide and Collier (1964)
	Human erythrocytes	NADPH	1	40	Huennekens *et al.* (1957 a)

TABLE 2-34 (*continued*)

Enzyme	Source	Reaction components[a]	Concentration (m*M*)	% Inhibition	Reference
Nitrate reductase	*E. coli*	Nitrate	2	60	Heredia and Medina (1960)
	Micrococcus sp.	Nitrate	1	55	Hori (1963)
	Pseudomonas aeruginosa	Nitrate	0.5	37	Fewson and Nicholas (1961)
	Rhodospirillum rubrum	Nitrate	0.61	28	Katoh (1963)
Nitroreductase	*Aspergillus niger*	*m*-Dinitrobenzene and NADH(2)	1	33	Higgins (1961)
	Pig liver	*p*-Nitrophenol	10	32	S. Otsuka (1961)
Nitrosoreductase	Pig liver	*p*-Nitrosophenol	10	26	S. Otsuka (1961)
3′-Phosphoadenosine-5′-phosphosulfate reductase	*E. coli*	PAPS(0.2)	1	10	Fujimoto and Ishimoto (1961)
Protein disulfide reductase	Yeast	NADPH	0.01	63	Asahi *et al.* (1961)
Retinene oxidase	Rat liver	Vitamin A aldehyde	0.01	10	Mahadevan *et al.* (1962)
			0.1	50	
			1	90	
Riboflavin transglucosidase	*E. coli*	Maltose(25) and riboflavin (0.1)	0.1	10	Tachibana *et al.* (1958)
			1	27	
			10	44	

RNA nucleotidyl-transferase	*Agrobacterium tumefaciens*	RNA	0.17 0.33 0.67 1 3.3	3 24 70 91 99	Hochster and Chang (1963)
Steroid hydroxylase	Beef adrenal	17α-OH-progesterone and NADP	0.1	1	Ryan and Engel (1957)
Succinate dehydrogenase	*Bacterium tularense*	Succinate and DCPIP	10	Inh	Wadkins and Mills (1956)
Succinate oxidase	*Micrococcus* sp.	Succinate	1	73	Hori (1963)
	Xanthomonas phaseoli	Succinate	3.3	69	Madsen (1960)
	Tetrahymena pyriformis	Succinate	3 7.2	74 90	Eichel (1956 b)
	Rat liver	Succinate	1 2 4 8	11 44 45 64	Löw (1959 a)
Sulfite reductase	*Desulfovibrio desulfuricans*	Sulfite(4)	1	19	Ishimoto *et al.* (1958); Ishimoto and Yagi (1961)
Thiosulfate reductase	*Desulfovibrio desulfuricans*	Thiosulfate	1	80	Ishimoto *et al.* (1958); Ishimoto and Yagi (1961)
Thyroxine deiodinase	Rabbit muscle	Thyroxine, FMN(0.04), and Fe^{++}(0.04)	2	60	Tata (1959)
Xanthine oxidase	Milk	Hypoxanthine	0.1	>50	Mackler *et al.* (1954)
	Milk	Sulfite	1	80	Fridovich and Handler (1957)

[a] The mM concentrations of certain reactants when important or when stated are given in parentheses.

Some of the enzymes inhibited by quinacrine do not involve flavin coenzymes, as emphasized by Hellerman *et al.* (1946) and Hemker and Hülsman (1960), so that no direct antagonism with FMN or FAD would be expected. Nevertheless, reversal of the inhibition is sometimes seen and may be due to the formation of a complex between the quinacrine and the added coenzyme, as was shown to occur between quinacrine and FMN in the inhibition of aliesterase, this presumably removing some of the quinacrine from the enzyme. In any case, the degree of reversal or even whether reversal occurs with FMN or FAD will depend on the way in which the experiment is run and the relative concentrations. If interaction of the enzyme and quinacrine is allowed to occur, the chance of reducing the inhibition by adding coenzyme is less than if both are added together, since it may be difficult to reach equilibrium due to the tight binding of quinacrine.

The effects of quinacrine on ATPases and oxidative phosphorylation are interesting and perhaps important in explaining some of the metabolic changes. Myofibrillar ATPase does not depend on any flavin component and yet is inhibited rather well by quinacrine (Kaldor, 1960). Furthermore, increasing the ATP concentration from 1 mM (where 1 mM quinacrine inhibits about 50%) to 7 mM almost abolishes the inhibition. The inhibition is potentiated by Mg^{++} and counteracted by Ca^{++}, so that a quinacrine-Mg complex was postulated as the possible active inhibitor. Interactions of Mg^{++} and ATP with quinacrine were shown by fluorescence changes. Irvin and Irvin (1954) had found that quinacrine forms complexes with AMP and ATP at physiological pH's. The dissociation constant for the ATP complex is 1.38×10^{-3}, so that 1 mM quinacrine would lower the ATP concentration from 1 mM to 0.67 mM, whereas it would have a negligible effect on ATP around 7 mM. Increasing the ATP might also reduce the Mg^{++} available for a quinacrine complex, and if this is the active inhibitor, the inhibition would be lessened. The ATPase activity in mitochondrial preparations may be quite different from the myofibrillar enzyme and could involve a flavin component. Quinacrine inhibits the ATPase of beef heart mitochondria and simultaneously uncouples oxidative phosphorylation even more potently (Penefsky *et al.*, 1960), while the DNP-stimulated ATPase of rat liver mitochondria is stimulated by quinacrine at lower concentrations (0.75 mM) and inhibited by higher (3 mM) (Löw, 1959 a), the P_i-ATP exchange reaction and oxidative phosphorylation being depressed. Löw felt that this provides evidence for the participation of flavin in such ATPase activity, especially, as FMN and FAD can reverse the inhibition, but other interpretations are possible (e.g., complexes between quinacrine and ATP or the flavins). There is no doubt, however, that DNP alters the response of mitochondrial ATPase to quinacrine, but no DNP effect is observed in muscle ATPase (Pennington, 1961). Uncoupling by quinacrine was first reported by Loomis and Lipmann (1948) and recent work seems

to establish a true uncoupling action, although by no means so specific as with DNP since O_2 uptake is usually reduced simultaneously with the P : O ratio.

Baltscheffsky (1960 b) found that light-induced phosphorylation in spinach chloroplasts is strongly inhibited by quinacrine, 0.04 m*M* producing almost complete block, and in cell-free extracts of *Rhodospirillum rubrum* less potently (Baltscheffsky and Baltscheffsky, 1958; Baltscheffsky, 1960 a). The inhibition is reversed by FMN and FAD in the the bacterial extracts, but not at all in the chloroplasts; indeed, in the latter FMN and FAD are quite potent inhibitors. It was suggested that an endogenous flavin is a necessary component of the system. Photophosphorylation has recently been found to be very sensitive to quinacrine. In *Rhodospirillum* chromatophores quinacrine begins to depress the photophosphorylation at 0.0001 m*M*, inhibits 65% at 0.028 m*M*, and blocks completely at 0.1 m*M*, the K_i being 0.003 m*M* (Horio and Kamen, 1962 a). The characteristic response to riboflavin is, however, not prevented and it was postulated that quinacrine binds at some locus in the respiratory chain. Quinacrine at concentrations around 0.05 m*M* uncouples all types of photophosphorylation in Swiss chard chloroplasts and simultaneously stimulates the photoreduction of dichlorophenolindophenol (Gromet-Elhanen and Avron, 1963). Similar effects were observed in spinach chloroplasts, quinacrine at 0.02 m*M* inhibiting photophosphorylation 61% and at 0.05 m*M* inhibiting completely, at the same time stimulating the photoreduction of trimethyl-1,4-benzoquinone (Dilley and Vernon, 1964). Changes in light absorption and scattering indicate a relationship between photosphosphorylation and structural alterations in the chloroplasts, but it is not known if quinacrine modifies directly these structural changes.

The determination of the inhibitor constant, K_i, for quinacrine and similar substances is somewhat more complex than with most inhibitors, due to the fact that equilibrium is difficult to achieve and mutual depletion kinetics must be applied, the free concentrations of both quinacrine and FAD being much lower than the total concentration. Hellerman *et al.* (1946) considered these problems relative to the inhibition of D-amino acid oxidase and described a very useful technique with the appropriate equations for the calculation of K_i. The K_{FAD} is 0.00057 m*M*, and K_i for quinine is 0.67 m*M* (means for two enzyme preparations). The quinine inhibition is quite competitive but quinacrine behaves atypically and its K_i varies with the experimental conditions (it is somewhat smaller than the K_i for quinine).

An interesting example of the effects of pH on inhibition was reported by Molinari and Lara (1960) for the lactate dehydrogenase of *Propionibacterium pentosaceum* (Fig. 2-19). Increase of pH augments the inhibition by quinacrine whereas the opposite effect is seen on Dicumarol inhibition,

but these relationships are reasonable if one assumes that the negatively charged Dicumarol reacts with positively charged enzyme groups, and the positively charged basic quinacrine reacts with enzyme anionic groups.

We must finally evaluate the reliability of quinacrine as an indicator of flavin participation in enzyme reactions. Certainly the mere inhibition of an enzyme by quinacrine does not imply involvement of a flavin coenzyme.

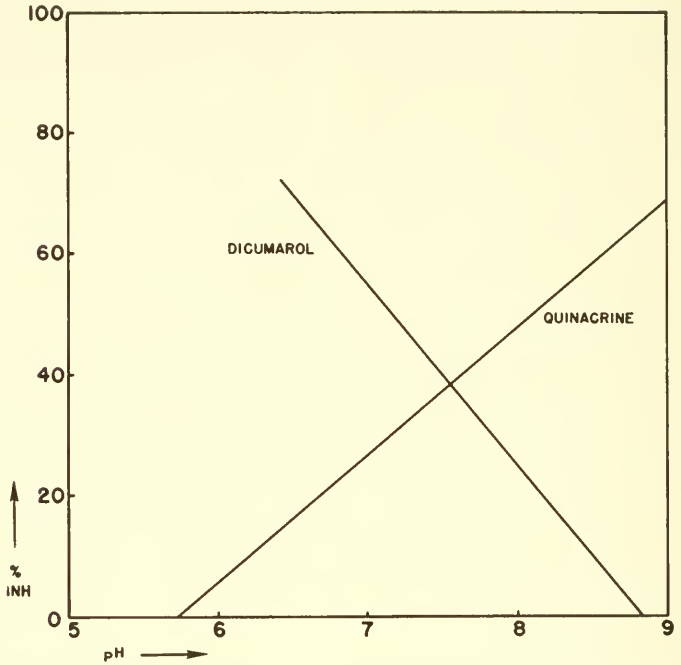


FIG. 2-19. Effects of pH on the inhibitions of lactate dehydrogenase from *Propionibacterium pentosaceum* by quinacrine and Dicumarol at 0.1 m*M*. (From Molinari and Lara, 1960.)

The observations of Hemker and Hülsman (1960) support the opinion of Hellerman *et al.* (1946) that quinacrine is a relatively nonspecific inhibitor, due to its affinity for proteins in general. If a reversal of the inhibition by FMN or FAD is demonstrated, there is more likelihood for the participation of a flavin, but even here one must consider the possible complexing of the quinacrine by the reversing flavin. Also it seems that flavin nucleotides which are not coenzymically active are often as good reversors as the coenzyme itself, indicating some other mechanism than competition for the active center. It would seem to me that quinacrine would be one of the least likely specific antagonists of FMN or FAD, since structurally it is not as close as many other analogs. An ideal indicator for flavins would be phos-

phorylated or in the form of a dinucleotide analog, and such has not yet been found.

Many flavin-dependent enzymes bind FAD or other flavin coenzymes very tightly and it is difficult to understand how quinacrine could displace these, or how exogenous FMN or FAD could antagonize the action of quinacrine if this is the case. If an enzyme after extraction is flavin-dependent and is catalytically active, it must have very tightly bound coenzyme; perhaps quinacrine can react with the bound coenzyme but there is no evidence for this. Certainly the failure of quinacrine to inhibit should not be taken as evidence for the absence of a flavin component. Actually it must be said that many of the experiments with quinacrine have not been done properly. In some instances one concentration of quinacrine has been used and, if inhibition of any degree is noted it is stated that this is evidence for a flavoenzyme, even though no antagonism has been demonstrated; it should be obvious that no conclusions can be drawn from these results. In other cases two experiments have been run, one with quinacrine alone and one with both quinacrine and either FMN or FAD; if the inhibition is less in the presence of the FMN or FAD it is concluded that quinacrine is interfering with the normal function of a flavin coenzyme. A control with FMN or FAD alone must also be run, since in many cases these substances will stimulate activity. The results of Bargoni (1963) are difficult to interpret in that FAD would prevent the inhibition by quinacrine only if the FAD were incubated with the enzyme for 30 min before the inhibitor is added. The binding of quinacrine to some enzymes is readily reversible, but yeast lactate dehydrogenase is irreversibly inactivated; FAD will slow this inactivation but will not reactivate (Iwatsubo and Labeyrie, 1962). Several suggestions as to the design of such antagonism tests may be made: (1) use a flavin derivative which is the most likely coenzyme involved, (2) use coenzyme-dissociated and reconstituted enzymes whenever possible, (3) always have a control with the reversor alone, and (4) attempt to establish competitive relations between the quinacrine and the reversor.

Effects of Quinacrine on Metabolism

Quinacrine and other antimalarials were found by Fulton and Christophers (1938) to depress the respiration of trypanosomes. Concentrations as low at 0.004 m*M* exert inhibitory effects on multiplication but it requires 0.3 m*M* to inhibit the respiration 11.5%, at which concentration the count is reduced 26.3%. These observations and several others afterward demonstrate that concentrations presumably higher than are present *in vivo* must be used to depress the respiration of these organisms. Wright and Sabine (1944) studied the effects of quinacrine on the respiration of rat tissue slices. In most instances there is an initial stimulation followed by a slowly developing inhibition, often not complete after 100–200 min. Liver

slice respiration is depressed only slightly by 0.5 m*M*, even after 2 hr, while 1–2 m*M* produces a maximal depression of around 65% after 1 hr. Brain respiration is more sensitive and is reduced around 75% by 0.5 m*M*. Following inhibition by quinacrine, addition of pyruvate, lactate, citrate, fumarate, or malate does not restore oxygen uptake, but addition of succinate brings about a rapid rise in respiration, indicating only that succinate oxidase is not blocked significantly at these concentrations. Because of the reversal of D-amino acid oxidase inhibition by coenzyme, they suggested that the block may be around the flavoenzyme locus in the respiratory chain, but actually there is no evidence for this. The respiration of *Plasmodium lophurae* with different substrates is depressed 15–24% by 0.1 m*M* quinacrine and somewhat above 50% by 1 m*M* (Bovarnick *et al.*, 1946). Actually it is very difficult to compare *in vitro* and *in vivo* effects and concentrations because of the progressive binding and accumulation of quinacrine; in other words, the free plasma concentration of quinacrine means very little, nor does total tissue concentration necessarily relate to any enzyme effects.

A glycolytic inhibition by quinacrine was suggested by the early work of Marshall (1948), who found a depression of glucose utilization, a decrease in glucose-1-P, an increase in glucose-6-P, some decrease in triose-P's, and decreases in pyruvate and lactate in washed chick erythrocytes parasitized by *Plasmodium gallinaceum*. It is difficult to separate the metabolic effects on parasite and erythrocyte, but it is probable that the major fraction of the glucose utilization was due to the parasites. The most marked effect of quinacrine is an accumulation of ATP, which Marshall attributed to an inhibition of hexokinase.

The only analysis of the effects of quinacrine on metabolism was made by Bowman *et al.* (1961). The glucose utilization of *P. berghei* free parasites, parasitized reticulocytes, and reticulocytes was determined, and low concentrations of quinacrine (claimed to be near those found therapeutically) exhibit a selective action on the parasites. The glucose utilization over 1 hr is reduced 34% by 0.0125 m*M* and 94% by 0.035 m*M* quinacrine. There is no effect on the pattern of glucose-1-C^{14} and glucose-6-C^{14} distribution. The amount of lactate formed from glucose is reduced and there is an accumulation of hexose-6-P, so it was concluded that quinacrine inhibits some enzyme which is involved in the utilization of hexose-6-P and is normally rate-limiting in the free parasites; this enzyme may be phosphofructokinase. Depression of respiration could thus, in part, be attributed to a glycolytic inhibition and, if so, is probably not related to a flavoenzyme.

There is probably need for more investigation of the effects of quinacrine on tissues and parasites in animals given the drug for varying times, because of the difficulty in estimating the proper *in vitro* concentrations to use. It may well be that some enzyme system not previously examined is

most potently inhibited. With regard to the inhibition of growth, it might be well to consider more carefully the changes resulting from complexes formed between quinacrine and nucleotides or nucleic acids.

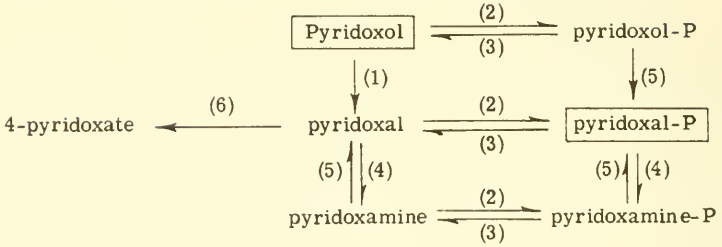
ANALOGS OF PYRIDOXAL

A group of substances, including pyridoxol, pyridoxal, pyridoxamine, and their phosphates, possess vitamin B_6 activity and these will be designated as pyridoxine in accordance with Braunstein (1960) and the Commission on Chemical Terminology of the International Union of Pure and Applied Chemistry (the substance previously called pyridoxine now being pyridoxol). These substances are converted to pyridoxal which is metabolically functional in the form of its phosphate. Pyridoxal-P is the coenzyme for a large number of enzymes involved in the decarboxylation, transamination, oxidative deamination, racemization, α,β-cleavage, and β- and γ-substituent replacement in amino acid metabolism, and, in addition, may be active in amino acid transport. Disturbances in pyridoxal metabolism or functions will thus bring about primarily alterations in the biosynthesis and degradation of amino acids, and indirectly will affect protein synthesis and a variety of other metabolic pathways. Possibly the most important biochemical defect will be the reduction in transaminations involving glutamate, inasmuch as these reactions are central in amino acid metabolism. More recently it has been found that phosphorylase *a* contains pyridoxal-P, perhaps bound in an aldamine linkage, and, although initially it was believed that it is enzymically nonfuctional, the demonstration by Illingworth *et al.* (1958) that pyridoxal and 5-deoxypyridoxal will prevent the binding of pyridoxal-P and enzyme activity points to some role of the pyridoxal-P in the catalysis.

Animals generally require pyridoxine whereas plants and most microorganisms can synthesize pyridoxal. Little is known about the biosynthesis of pyridoxal, but the rather complex interrelationships between the pyridoxines and their phosphates are now fairly clear. The pathways and the enzymes involved are summarized in the accompanying diagram (Braunstein, 1960; Wada and Snell, 1961). It is believed that the major pathway for the formation of pyridoxal-P is

Pyridoxol → pyridoxol-P → pyridoxal-P

The primary excretory metabolite of pyridoxal is 4-pyridoxate and its lactone. Most of the pyridoxine in tissues is present as pyridoxal-P bound quite tightly to enzymes and other proteins. Certain analogs can inhibit the formation of pyridoxal-P and may act partly in this way, while other analogs may be phosphorylated and compete with pyridoxal-P.

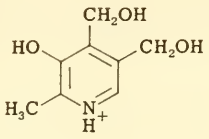


(1) pyridoxol oxidase
(2) pyridoxal kinase
(3) phosphatase
(4) transaminase
(5) pyridoxol-P oxidase
(6) aldehyde oxidase

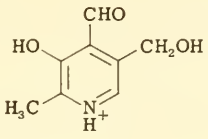
Two general types of analog are theoretically possible — those resulting from alteration of the pyridine ring and those in which the substituent groups are modified, replaced, or eliminated — but practically it has been found thus far that only analogs of the second type are effective. Actually, not many really effective analogs have been found. The most commonly used analog has been deoxypyridoxol* and we shall limit our discussion mainly to this substance. Unless otherwise noted, the name deoxypyridoxol will refer only to the 4-derivative.

Deoxypyridoxol was found to have no vitamin B_6 activity by Unna (1940) and to be an antagonist of pyridoxine in the chick by Ott (1946). Chicks on a low pyridoxine diet can be killed by as little as 16 μg deoxypyridoxol, whereas normal chicks on an adequate pyridoxine diet can withstand as much as 600 μg. By varying the relative doses of both vitamin and analog, Ott showed that approximately 2 molecules of analog can counteract 1 molecule of pyridoxine. Deoxypyridoxol has since been found to inhibit certain microorganisms and to produce symptoms of vitamin B_6 deficiency in animals, including man. 4-Methoxymethylpyridoxol (usually called methoxypyridoxine) was found by Unna to have slight vitamin activity in the rat, but Ott (1947) demonstrated a potent inhibitory effect in the chick. The ability of rats to use this analog is related to its transformation to pyridoxal in these animals (Porter *et al.*, 1947). In the chick it is about 25 times as effective as deoxypyridoxol. These are apparently the only analogs so far tested that can produce rather typical pyridoxine-deficiency symptoms in animals, although several others can inhibit microbial growth by disturbing pyridoxal function. Toxopyrimidine is undoubtedly toxic to animals and can be antagonized by pyridoxine, but it is debatable whether

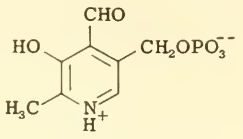
* This is 4-deoxypyridoxol and has previously been called desoxypyridoxine or deoxypyridoxine. However, if we are to conform to the modern nomenclature, the specific compound must be deoxypyridoxol. Deoxypyridoxine might be used to refer to the entire group of deoxy substances exhibiting vitamin B_6 activity antagonism.



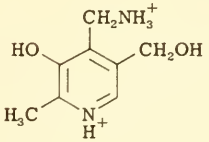
Pyridoxol
(pyridoxine)



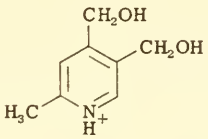
Pyridoxal



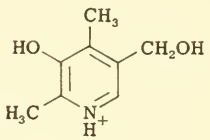
Pyridoxal-P



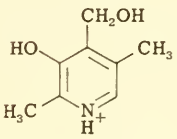
Pyridoxamine



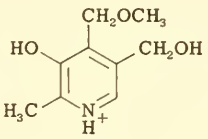
3-Deoxypyridoxol



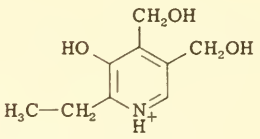
4-Deoxypyridoxol
(deoxypyridoxine)



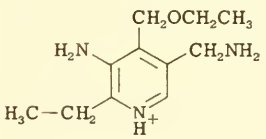
5-Deoxypyridoxol



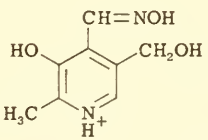
4-Methoxymethyl-
pyridoxol



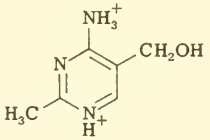
ω-Methylpyridoxol



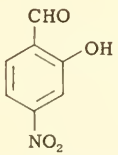
2-Ethyl-3-amino-
4-ethoxymethyl-5-
aminomethylpyridine



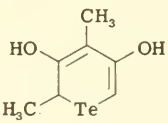
Pyridoxal oxime



Toxopyrimidine



4-Nitrosalicylaldehyde



2, 4-Dimethyl-*cyclo*-
telluropentane-3, 5-dione

it can be termed a true pyridoxine analog; this substance will be discussed separately at the end of this section.

Effects on Pyridoxine Metabolism and Tissue Levels of Pyridoxine

The only analog found in the early work to inhibit pyridoxal kinase strongly is 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine (Hurwitz, 1952). This substance cannot be phosphorylated because of the lack of a hydroxyl group at position 5, but has a rather high affinity for the yeast enzyme ($K_i = 0.073$ mM) and competitively inhibits the phosphorylation of pyridoxal. Certain analogs can be phosphorylated by this enzyme (e.g., deoxypyridoxol, 3-deoxypyridoxol, and the 3-amino analog of pyridoxol) (Umbreit and Waddell, 1949; Hurwitz, 1955 b), and presumably could reduce the phosphorylation of pyridoxal through substrate competition. Phosphorylation was shown to require a hydroxymethyl group at the 5-position and the absence of a substituent at position 6. The more recent work of McCormick and Snell (1961) demonstrated other potent inhibitors and made it clear that the kinases from different sources vary markedly with respect to affinity for the analogs (Table 2-35). Furthermore, the relative affinities are fairly well correlated with the abilities to inhibit growth of the various bacteria. The inhibitions are not always competitive and, in some instances, increase with pyridoxal concentration. The 3-hydroxy group can be replaced by an amino group or omitted without affecting affinity adversely, but substitution in the 6-position reduces the affinity without necessarily abolishing it. It is interesting that *N*-methylpyridoxal is completely inactive as an inhibitor. The most potent inhibitors of pyridoxal kinase are derivatives obtained by the reaction of pyridoxal with various carbonyl agents. The beef brain kinase is inhibited 50% by 0.00005 mM pyridoxal semicarbazone and by 0.000065 mM pyridoxal azine (the product of the reaction of 2 pyridoxals with hydrazine), but the discussion of such inhibitions is more pertinent to the subject of the carbonyl agents.

Various enzymes oxidizing pyridoxol, pyridoxol-P, or pyridoxamine-P have recently been found in liver, and are occasionally inhibited by analogs. The oxidation of pyridoxol is competitively inhibited by deoxypyridoxol (67% when pyridoxol is 10 mM and the analog is 12.5 mM) (Morino *et al.*, 1960), while the oxidation of pyridoxol-P is competitively inhibited by deoxypyridoxol-P ($K_m = 0.02$ mM, and $K_i = 0.35$ mM) (Morisue *et al.*, 1960). These enzymes thus follow the general rule that nonphosphorylated analogs inhibit the reactions of nonphosphorylated substrates, and phosphorylated analogs inhibit the reactions of phosphorylated substrates. The oxidative deamination of pyridoxamine-P is inhibited by pyridoxamine (the latter is also deaminated at a slower rate) and rather weakly by pyridoxol (Pogell, 1958). Wada and Snell (1961) examined the competitive inhibitions of pyridoxol-P oxidase by a variety of substances (see accompanying tab-

TABLE 2-35

INHIBITION OF PYRIDOXAL KINASE BY VARIOUS ANALOGS[a]

Analog	K_i (mM)				
	L. casei	*S. faecalis*	*S. carlsbergensis*	Rat liver	Beef brain
4-Deoxypyridoxol	2.0	4.5	0.04	0.015	0.09
5-Deoxypyridoxol	0.045	0.02	0.25	0.15	0.035
ω-Methylpyridoxal	0.035	0.20	0.15	0.045	0.25
2-Methyl-3-hydroxy-5-hydroxymethylpyridine	1.5	>1.0	—	0.2	0.15
Pyridoxal[b]	0.03	0.015	0.15	0.015	0.05
Pyridoxol[b]	4.0	1.5	0.025	0.025	0.20

[a] From McCormick and Snell (1961).

[b] The values for pyridoxal and pyridoxol are K_m's.

Inhibitor	Concentration (mM)	% Inhibition of oxidation of:	
		Pyridoxol-P (0.3 mM)	Pyridoxamine-P (0.3 mM)
Pyridoxal	1	6	7
Pyridoxol	1	<3	<3
Pyridoxamine	1	<3	<3
4-Pyridoxate	1	3	5
4-Pyridoxate-P	0.2	23	52
	1	33	62
Deoxypyridoxol	1	0	0
Deoxypyridoxol-P	0.2	34	54
	1	42	70
Pyridoxal oxime	0.001	17	31
	0.01	57	67

ulation) and noted that only the phosphorylated derivatives are significantly inhibitory. Whether inhibition of these oxidases by analogs plays a role in the depressant or toxic effects produced is at present unknown, but it must be admitted that for deoxypyridoxol and its phosphate none of the inhibitions is probably potent enough to be important *in vivo*.

The effects of analogs on the tissue levels of the vitamin B_6 group are particularly important in certain arguments relative to the mechanisms by which these analogs are toxic. Umbreit (1955 a) believes that deoxypyridoxol exerts actions other than the antagonism of vitamin B_6 function. The basis for this is principally that deoxypyridoxol accelerates the appearance of deficiency symptoms when animals are on a diet lacking pyridoxine and yet does not reduce the tissue levels of pyridoxal coenzymes. He has also pointed out that in some cases there is also no fall in transaminase or decarboxylase activity during the "acute" deficiency produced by deoxypyridoxol. Nevertheless, it is admitted that the toxic effects of deoxypyridoxol can be readily counteracted by pyridoxine administration. Actually there is very little published on tissue levels of vitamin B_6 as affected by deoxypyridoxol. Stoerk (1950) reported that dietary deficiency lowers liver pyridoxine content but that deoxypyridoxol produces no further lowering despite a more rapidly appearing deficiency syndrome. Similar results were obtained by Beaton and McHenry (1953) in rats exhibiting acrodynia following deoxypyridoxol feeding (see accompanying tabulation). Umbreit also cites unpublished data supporting these results. Effects of deoxypyridoxol on enzyme activity *in vivo* will be taken up in the following

section, but there is now sufficient evidence that the activities of certain pyridoxal-P-dependent enzymes are reduced.

Pyridoxine	Deoxypyridoxol	Liver vitamin B_6 (μg/g)
−	−	6.3
+	−	11.0
−	+	6.3
+	+	9.6

Interpretation of this apparent discrepancy between toxic reactions and insignificant changes in liver vitamin B_6 during deoxypyridoxol treatment can be made along several lines. In the first place, it is generally believed that much of the tissue pyridoxal is bound to nonenzyme protein, possibly in part through the aldehyde group, so that analyses of total tissue levels do not necessarily reflect changes in coenzyme concentration. The fact that enzyme activity is often depressed without significant changes in total vitamin B_6 suggests that this can be an explanation. That bound to nonenzyme protein may not be displaced by deoxypyridoxol since this analog contains no aldehyde group. In the second place, analyses have been made only in the liver and it is unlikely that changes in liver pyridoxal function are responsible for any of the common toxic symptoms of deficiency. It is even possible that during treatment with deoxypyridoxol there is a transfer of vitamin B_6 substances from one tissue to another. It is conceivable that deoxypyridoxol has actions other than interference with pyridoxal function, but the fact that its toxicity can be reduced by pyridoxine administration points to a close relationship between its effects and pyridoxal. I think that more emphasis must be placed on the changes in enzyme activity rather than on the tissue levels of vitamin B_6 for the reasons given above.

The most complete investigation of the effects of deoxypyridoxol on the concentrations of tissue B_6 vitamers is that of Bain and Williams (1960), who utilized chromatographic separation. The results are summarized in Table 2-36. The extreme fall in brain pyridoxal-P they believe must in some way be related to the convulsions. The rapidity with which the analog can deplete the brain coenzyme is surprising. Is the pyridoxal-P replaced on the apoenzymes by deoxypyridoxol-P? It seems unlikely that interference with transport or metabolism of pyridoxol could produce such marked effects so soon. Also the displaced pyridoxal-P must be metabolized or leave the tissue. It is not known why pyridoxamine-P does not fall comparably. Dietary vitamin B_6 deficiency for 37–51 days does not cause such marked losses of pyridoxal-P or total B_6 vitamers from the brain as the single dose of deoxypyridoxol. The severe drop in pyridoxal-P in brain following deoxy-

TABLE 2-36

EFFECTS OF DEOXYPYRIDOXOL ON THE CONCENTRATIONS OF B_6 VITAMERS IN MOUSE TISSUES AND FLUIDS[a]

Tissue	Treatment	Concentration of B_6 vitamers (mμg/100 g wet weight)						
		Pyridoxol	Pyridoxal	Pyridoxamine	Pyridoxol-P	Pyridoxal-P	Pyridoxamine-P	Total
Brain	Control	t	4	t	t	102	170	316
	Deoxypyridoxol	5	39	t	0	8	100	180
	% Change		+975			−92	−41	−43
Adenocarcinoma	Control	0	12	12	0	246	239	598
	Deoxypyridoxol	t	t	t	0	164	120	335
	% Change					−33	−50	−44
Ascites tumor	Control	t	3	t	0	486	144	720
	Deoxypyridoxol	1	7	2	0	272	187	542
	% Change		+133			−44	+30	−25
Ascites fluid	Control	t	128	t	0	125	4	257
	Deoxypyridoxol	t	59	t	0	150	6	315
	% Change		−54			+20	+50	+23

[a] The dose of deoxypyridoxol was 250 mg/kg intraperitoneally. The animals were sacrificed in convulsion 60–90 min later. Trace amounts indicated by t. (From Bain and Williams, 1960.)

pyridoxol must be reflected in depression of the activity of enzymes requiring this coenzyme, but not necessarily in all equally. The failure of pyridoxamine-P to fall as much as pyridoxal-P might indicate that transaminases are not depleted as readily as other pyridoxal-P enzymes; but interpretation is made difficult by the fact that we do not know what fractions of these substances are bound to apoenzymes and to nonenzyme protein. The effects on the tumors are similar but of less magnitude, and the changes in pyridoxamine-P are again variable, even increasing in ascites tumor. The rise in pyridoxal-P and total vitamers in ascites fluid may reflect the loss of these substances from the cells.

Effects of Deoxypyridoxol on Pyridoxal-P-Dependent Enzymes *in Vivo*

The results reported on transaminase activity during administration of deoxypyridoxol are variable. Transamination in hamster hearts is reduced 30–40% in animals with a dietary deficiency, but injecting deoxypyridoxol at 50 μg per animal 3 times a week does not lower the activity further (Shwartzman and Hift, 1951). However, there is some growth inhibition beyond that shown in the deficient animals, although no specific symptoms were noted. Deoxypyridoxol at 100 μg/day in rats does not alter the aspartate-glutamate transaminase and actually seems to increase the alanine-glutamate transaminase activity in liver compared to animals on a deficient diet (Caldwell and McHenry, 1953). Since the animals receiving deoxypyridoxol had severe dermatitis, it was justifiably concluded that the production of dermatitis is unrelated to liver transaminase. On the other hand, Dietrich and Shapiro (1953 a) found a greater fall in liver transaminase when mice were injected with deoxypyridoxol at 150 mg/kg/day than in simple dietary deficiency (-49% and -37%, respectively). Indeed, transaminase levels in several tissues fall very markedly in mice on 175 mg/kg/day of deoxypyridoxol (Shapiro *et al.*, 1953). There is not much difference in the rates of decrease in the various tissues (Fig. 2-20). It is difficult to reconcile all of these results unless it is a matter of species variation, which is unlikely. Transaminases are not directly inhibited very potently by deoxypyridoxol; the K_i is 0.12 mM for the alanine-pyruvate transaminase of *Pseudomonas*, for example (Dempsey and Snell, 1963). The very high inhibitory activity of pyridoxyl-L-alanine ($K_i = 0.00018$ mM) is surprising.

The results on decarboxylases are similar. Dietary pyridoxine deficiency causes a 50% fall in rat brain glutamate decarboxylase, but administering deoxypyridoxol in addition produces no further depletion (Roberts *et al.*, 1951). More recent studies, summarized in Table 2-37, clearly indicate a lack of correlation between the brain decarboxylase levels and the occurrence of convulsions; e.g., 3-deoxypyridoxol convulses without a significant change in enzyme activity, whereas ω-methylpyridoxol lowers the enzyme level without producing convulsions. Liver dopa decarboxylase is decreased

moderately in mice from both deficiency and injections of deoxypyridoxol (Dietrich and Shapiro, 1953 a). Despite the lowered levels of transaminase and decarboxylase, cysteine desulfhydrase (also dependent on pyridoxal-P) is not affected by deoxypyridoxol, due to the fact that the apodesulfhydrase has little affinity for deoxypyridoxol-P, no inhibition of the binding of pyridoxal-P being observed (Dietrich and Borries, 1956).

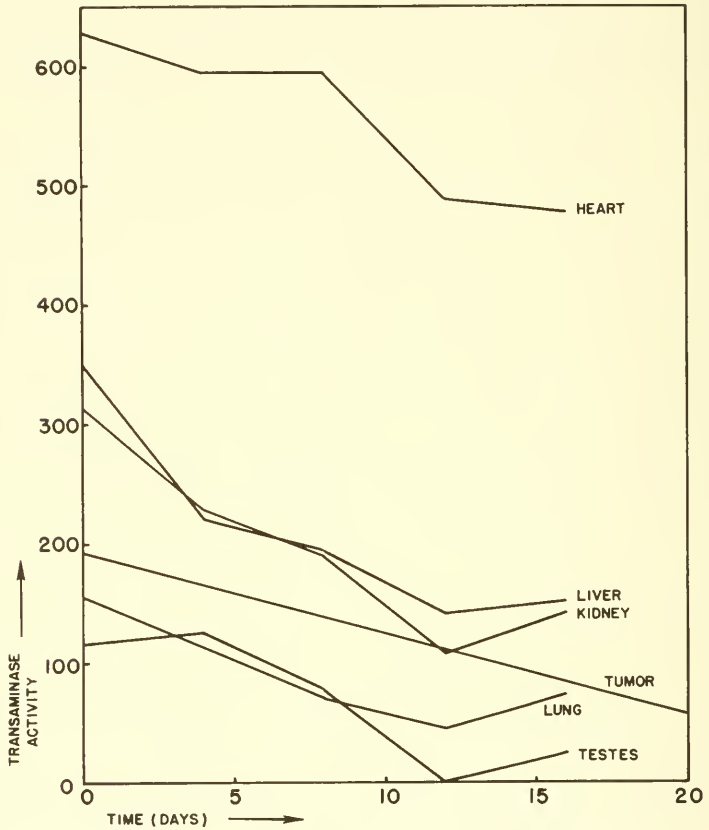


FIG. 2-20. Effects of deoxypyridoxol given intraperitoneally at 150 mg/kg/day on the aspartate: α-ketoglutarate transaminase of mouse tissues. (From Shapiro *et al.*, 1953.)

Serine biosynthesis from formate and glycine, involving serine transhydroxymethylase, is dependent on pyridoxal-P. The incorporation of formate-C^{14} into serine in chick liver extracts is much reduced when these are obtained from deoxypyridoxol-treated animals, the depression being around 50% and reversible with pyridoxal-P *in vitro* (Sakami, 1955). Renal

TABLE 2-37

EFFECTS OF PYRIDOXINE ANALOGS ON RAT BRAIN GLUTAMATE DECARBOXYLASE [a]

Treatment	Dose (mg/kg)	Convulsions	Glutamate decarboxylase (μmoles/g/hr)	
			Endogenous	+Pyridoxal-P
Pyridoxine-deficient				
Control			36	218
Toxopyrimidine	15	+	40 (+11%)	202 (− 7%)
Control			54	235
Toxopyrimidine	25	+	56 (+ 4%)	233 (− 1%)
Normal diet				
Control			88	274
Toxopyrimidine	700	+	50 (−43%)	266 (− 3%)
3-Deoxypyridoxol	150	−	56 (−36%)	245 (−11%)
ω-Methylpyridoxol	200	−	31 (−65%)	261 (− 5%)
Pyridoxine-supplemented [b] ($100\ \mu$g%)				
Control			70	276
3-Deoxypyridoxol	100	+	72 (+ 4%)	252 (− 9%)
4-Deoxypyridoxol	100×7	−	71 (+ 2%)	296 (+ 7%)
5-Deoxypyridoxol	100×7	−	82 (+18%)	283 (+ 3%)
ω-Methylpyridoxol	100×7	−	30 (−57%)	233 (−16%)

[a] From Rosen *et al.* (1960).

[b] The analogs were injected intraperitoneally for 7 days, except for 3-deoxypyridoxol, which convulsed the animals after a single dose.

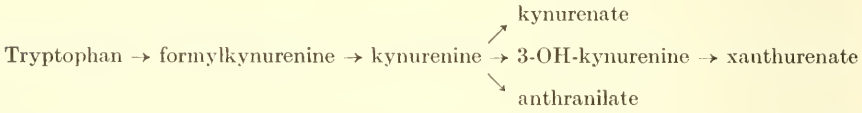
glutaminase is also reduced by deoxypyridoxol at 21 days, at which time dietary deficiency produces no change (Beaton and Goodwin, 1955).

It is impossible to evaluate the importance of these changes in enzyme levels for the toxic effects of deoxypyridoxol. The toxic effects are manifested principally in the central nervous system, skin, and hematopoietic system, and no enzyme determinations during deoxypyridoxol treatment have been reported in any of these tissues, except for brain glutamate decarboxylase. There is no reason to attribute the toxic effects to reduction of transaminase rather than to the possible reduction of many other enzymes dependent on pyridoxal-P, most of which have never been examined in this connection. It is more likely that the growth inhibition observed

with both deficiency and deoxypyridoxol is related to lowered transaminase activity and protein synthesis. Analyses of total brain do not, of course, necessarily reflect local changes in amino acid metabolism, and the site of origin of the convulsions has yet to be determined. It must be remembered that transaminase, glutamate decarboxylase, and γ-aminobutyrate levels vary in different regions of the central nervous system and, furthermore, the functional dependence of various regions on pyridoxal-P-dependent metabolism must also vary. The displacement of pyridoxal-P from apoenzymes by deoxypyridoxol-P *in vivo* depends on several factors: (1) rate of penetration of the analog into the cells, (2) ability of the tissue to phosphorylate the analog, and (3) relative affinities of the apoenzyme for the coenzyme and the phosphorylated analog. Thus one might for several reasons expect the pattern of enzyme depression from deoxypyridoxol to be different from that produced by simple dietary deficiency.

Effects on Metabolism

Kynurenate is a normal metabolite of tryptophan in the rat, but in pyridoxine-deficient animals one finds kynurenine and xanthurenate also. The metabolic pathway involved here may be summarized as follows:



This is an interesting situation since the formation of all three of these products involves pyridoxal-P enzymes, namely, kynurenine transaminase for the formation of kynurenate and xanthurenate, and kynureninase for the formation of anthranilate. The administration of deoxypyridoxal to otherwise normal rats produces no particular effect, but if tryptophan is given to deoxypyridoxol-treated animals there is an increase in the appearance of kynurenine and xanthurenate, just as in dietary deficiency (Porter *et al.*, 1947). The rise in kynurenine would be expected because two of its degradative pathways are depressed (including the normally most important one), and the increase in xanthurenate excretion must be due to a diversion of the metabolic flow through the remaining pathway. However, it is difficult to understand why xanthurenate excretion should increase relative to kynurenate, since both are presumably formed with the same enzyme, unless a rise in kynurenine concentration increases relatively more the rate of the xanthurenate pathway. It would be interesting to know what happens to the level of 3-hydroxykynurenine during deoxypyridoxol administration.

Pyridoxine-deficient rats have higher blood urea than normal animals and this has been attributed to an impaired utilization of amino acids,

since it is not of renal origin and hence due to an increased urea formation. Administration of 100 μg/rat/day of deoxypyridoxol for 28 days apparently increases urea formation in liver slices in both deficient and pyridoxine-fed animals (see accompanying tabulation), although the authors stated that

Pyridoxine	Deoxypyridoxol	Q_{urea}
−	−	4.32
+	−	3.38
−	+	4.76
+	+	4.00

deoxypyridoxol lessens the deficiency abnormalities rather than accentuating them (Beaton *et al.*, 1954). The cycle of urea formation does not require pyridoxal directly, but the aspartate to condense with citrulline must be formed by transamination reactions, so that one might expect impaired pyridoxal function to depress urea formation by this mechanism, and perhaps it counteracts to some extent the effect of an increased supply of amino acids for catabolism.

Convulsive seizures in mice are produced by the injection of 4-methoxymethylpyridoxol, and are completely prevented by pyridoxol in a dose 3 times that of the analog (Gammon *et al.*, 1960). The convulsions can also be prevented by prior administration of γ-aminobutyrate but not by any of the other related amino acids or products of glutamate metabolism tested. Since glutamate decarboxylase requires pyridoxal-P, the most obvious explanation would be that the analog reduces brain γ-aminobutyrate, thereby initiating convulsions, and that the administration of γ-aminobutyrate simply restores the normal level or prevents depletion. Others have suggested that certain carbonyl reagents, such as thiosemicarbazide, produce seizures by reducing brain γ-aminobutyrate, and many now believe that central motor activity is controlled by the levels of such amino acids and their corresponding amines. Analyses of mouse brains obtained during convulsions from 4-methoxymethylpyridoxol and other analeptics were thus made, and it was found that γ-aminobutyrate drops 50–70% when the analog is used but shows no significant change when the convulsions are due to Metrazol, picrotoxin, or electroshock (Gammon *et al.*, 1960; Kamrin and Kamrin, 1961). Despite the coherence of these observations in supporting the role of γ-aminobutyrate in antipyridoxine convulsions, one additional fact is difficult to fit in: the administration of pyridoxol, which blocks the seizures, does not alter the fall in brain γ-aminobutyrate. It has also been shown that administration of γ-aminobutyrate to animals with seizures produced by the analog does not reduce the seizures although the γ-amino-

butyrate content of the brain increases (Purpura *et al.*, 1960). These discrepancies might be removed if one could determine local changes in γ-aminobutyrate in the central nervous system. On the other hand, it is possible that other disturbances are more pertinent to the convulsive state, and, as Rosen *et al.* (1960) have pointed out, perhaps more thought should be given to the interference with the transport of amino acids into the brain cells. Variations in the metabolism of amino acids other than glutamate and the levels of physiologically active amines have not been studied. In this connection it is interesting that Schrodt *et al.* (1960) administered deoxypyridoxol to two patients with malignant carcinoid syndrome at doses of 100–200 mg/day, and found in one patient a fall in the urinary excretion of 5-hydroxyindoleacetate, which is the primary product of serotonin metabolism, along with symptoms of vitamin B_6 deficiency.

The role of pyridoxal in lipid metabolism is not yet clear but there is some evidence in animals for a requirement in fatty acid synthesis. When deoxypyridoxol was administered to seven subjects at 300 mg/day, six developed symptoms of pyridoxine deficiency (Mueller *et al.*, 1959). There was a general decrease in the polyunsaturated fatty acids of the blood, but no change is phospholipids or cholesterol. This was believed to be evidence for the involvement of pyridoxine in maintaining blood fatty acids through participation in the synthetic reactions, but the data do not indicate a role in the interconversion of the unsaturated fatty acids.

Effects on Active Transport

Although relatively little has been done with respect to the actions of pyridoxine analogs, there is accumulating evidence that amino acid transport is often related to pyridoxal-P function, and it is likely that some of the toxic effects of the analogs will be explained on this basis. Part of the transport of amino acids across the intestinal wall is active and is inhibited by deoxypyridoxol (Fridhandler and Quastel, 1955). A 41% inhibition of L-alanine transport was observed with 10 m*M* deoxypyridoxol, which is certainly a very high concentration; however, pyridoxol at the same concentration has no effect. The inhibition is not antagonized by pyridoxol, pyridoxal, or pyridoxal-P, which may indicate that phosphorylation is not rapid enough intracellularly and that the coenzyme itself cannot penetrate, or that the inhibition is not an antagonism of pyridoxine. A rather disturbing fact is that glucose and fructose transport is also inhibited by deoxypyridoxol to about the same extent as alanine absorption, so that this is not a specific effect on amino acid transport.

The transport of D- and L-methionine across rat intestine is depressed by deoxypyridoxol injected at 200–400 μg/day (Jacobs, 1958; Jacobs and Hillman, 1958). The effect appears within an hour after intraperitoneal injection and can be abolished by injection of pyridoxol (see accompanying

tabulation) (Jacobs *et al.*, 1960). These results definitely implicate pyridoxal in amino acid transport but do not prove that it functions directly in the transport mechanism, since the effect could be an indirect one.

Pyridoxol	Deoxypyridoxol	% Change in transport
−	−	−56
+	−	+25
−	+	−35
+	+	+ 6

The uptake of glycine by ascites carcinoma cells is inhibited by 5–25 m*M* deoxypyridoxol (Christensen *et al.*, 1954). Since pyridoxine deficiency reduces the accumulating ability and this is restored by pyridoxal *in vitro*, it would appear that pyridoxal functions here in some manner, although again not necessarily in the membrane transport system.

Effects on Growth

Deoxypyridoxol suppresses the growth of a variety of microorganisms. Rabinowitz and Snell (1953 a) emphasized that sensitive organisms are those requiring an exogenous source of pyridoxine, and in these the inhibition can be counteracted by pyridoxine; those synthesizing their own pyridoxal can effectively resist the analog. The situation is quite complex, however, and when different analogs are examined a marked variability in susceptibility is found (Rabinowitz and Snell, 1953 b). For example, ω-methylpyridoxol is inhibitory to yeast but not at all to *Streptococcus faecalis* or *Lactobacillus helveticus*, the 5-deoxypyridoxol derivatives being the most effective in these latter organisms; in *L. helveticus*, only 5-deoxypyridoxol is inhibitory, 4-deoxypyridoxol, 5-deoxypyridoxol, and 5-deoxypyridoxamine being without action. A factor that is very important in determining the susceptibility of bacteria to these analogs is the nature of the exogenous amino acids supplied. *Streptococcus faecalis* grows well if all amino acids are provided even though pyridoxine is absent, but a requirement for pyridoxine and a sensitivity to analogs are created by restriction of the amino acids in the medium (Olivard and Snell, 1955). Under certain circumstances the growth can be limited by conversion of L- to D-alanine by alanine racemase, which involves pyridoxal-P and is quite sensitive to 5-deoxypyridoxol ($K_i = 0.089$ m*M*) and ω-methylpyridoxol ($K_i = 0.53$ m*M*). The inhibition of growth by these analogs can be explained on the basis of the inhibition of this enzyme under these conditions. On the other hand, in most circumstances the inhibition must be on amino acid metabolism, as in *Vibrio*

cholera where alanine and aspartate accumulate when growth is suppressed 55% by 0.53 m*M* deoxypyridoxol (Chatterjee and Halder, 1960). Perhaps the first pyridoxine analogs to be recognized as inhibitors of bacterial growth were the tellurium compounds studied by Morgan, Cooper, and their colleagues between 1923 and 1926. Gulland and Farrar (1944) postulated that 2,4-dimethyl-*cyclo*-telluropentane-3,5-dione is toxic because of its structural similarity to pyridoxine, but no further work or attempts to counteract the inhibition with pyridoxine have come to my attention. An *in vivo* effect on bacteria has been demonstrated in at least one case for deoxypyridoxol, which prolonged the survival time of mice infected with *Toxoplasma gondii* from 5 days to 12.1 days when it was incorported into the diet at 0.1%, although some toxic symptoms were noted (Summers, 1957). Pyridoxine, can counteract both the beneficial and toxic effects.

Chick embryogenesis is disturbed by deoxypyridoxol and other analogs. When 1 mg of deoxypyridoxol is injected into eggs, there is 100% mortality of the embryos and this can be prevented by injecting pyridoxine (Cravens and Snell, 1949). However, after 4 or more days the embryos become less sensitive, and although higher doses are toxic they are not counteracted by pyridoxine. Similar effects are noted for 4-methoxymethylpyridoxol but it is at least 25 times more toxic than deoxypyridoxol to the early chick embryo (Karnofsky *et al.*, 1950). Mammalian fetal development is also disturbed by deoxypyridoxol — fetuses resorbed, still-births, and abnormal young — but administration of estrone and progesterone together prevents these effects and pregnancy is maintained in the majority of the animals, indicating that the action of the analog is primarily on the maternal tissues rather than the embryo (Nelson, 1955).

Some of the observations relative to the inhibition of tumor growth by deoxypyridoxol will be summarized since these effects are interesting in light of the fairly rapid amino acid metabolism in tumors and the generally low levels of the B_6 vitamers in solid tumors. Regression of mouse lymphosarcoma implants with deoxypyridoxol was achieved by Stoerk (1947, 1950) when the animals were on a low-pyridoxine diet, but there was also loss of body weight, suggesting an insufficiently specific inhibition. The frequency of successful fibrosarcoma implants in rats is increased by pyridoxol and decreased by deoxypyridoxol, even though in the latter case no severe deficiency symptoms are observed (Loefer, 1951). On the basis of these findings, Gellhorn and Jones (1949) gave deoxypyridoxol to patients with disseminated lymphosarcoma and acute leukemia in combination with a pyridoxine-deficient diet. Although there was some weight loss and weakness, there were no specific signs of deficiency, no changes in tryptophan metabolism, no hematopoietic depression, and no retardation in the growth of lymphoid tissue, the results being clinically insignificant. Deoxypyridoxol appears to be reasonably effective in suppressing mammary carcinoma in

mice, with a specific regression of the tumor and no weight loss (Shapiro and Gellhorn, 1951). Human carcinoma cells (Eagle's KB strain) are quite sensitive to deoxypyridoxol in tissue culture, 0.08 m*M* inhibiting the growth 50% (Smith *et al.*, 1959). It appears doubtful that deoxypyridoxol is sufficiently specific as a carcinostatic agent but its use in conjunction with other inhibitors remains a possibility, especially as Doctor (1959) has shown that deoxypyridoxol at 20 mg/kg/day in the rat has no effect on the leucocyte count, but combined with a moderately effective dose of aminopterin exerts a very marked suppression of the leucocytes, and also potentiates the action of oxythiamine.

Toxic Effects in Whole Animals

Some of the evidence that deoxypyridoxol can produce rather typical vitamin B_6-deficiency states will be summarized to emphasize that, whatever the basic biochemical disturbances, the effects are primarily related to an interference with formation or function of pyridoxal-P. It is first of all quite clear that the doses of deoxypyridoxol to induce toxic reactions must be much higher when the animals are adequately supplied with the B_6 vitamers than when the animals are subjected to a dietary deficiency, and that administration of pyridoxol can overcome the toxic reactions produced by the analog. In general the responses to deoxypyridoxol are the same as in pyridoxine deficiency, except that they appear earlier, producing an acute deficiency syndrome. Thus in rats and mice there is a dermatitis characterized by acanthosis, parakeratosis, and hyperkeratosis, sometimes with a superimposed infection, which is similar to deficiency dermatitis (Stoerk, 1950). There is atrophy and degeneration of the hematopoietic organs, evidenced by decreases in thymus and spleen weights, and these are reflected in the peripheral blood picture (Mushett *et al.*, 1947). The nervous system hyperirritability leading eventually to convulsions has been mentioned in connection with the metabolic changes in the brain.

A state resembling pyridoxine deficiency has been produced in man by Mueller and Vilter (1950). Eight individuals on a pyridoxine-poor diet were injected intramuscularly with 60–150 mg/day. Within 2–3 weeks a seborrheic dermatitis appeared around the eyes, nose, and mouth, with simultaneous glossitis and stomatitis. These symptoms disappeared in 2–3 days upon administration of pyridoxol. The total white count did not fall, nor was there evidence of anemia, but the lymphocytes dropped to around half the initial level. Schrodt *et al.* (1960) in their two carcinoid patients also observed seborrheic dermatitis and glossitis. The general pharmacology of analogs and inhibitors of pyridoxal function has been reviewed by Holtz and Palm (1964).

Although several workers have stated that reactions may be seen in acute deoxypyridoxol-treated animals which are not seen in simple dietary de-

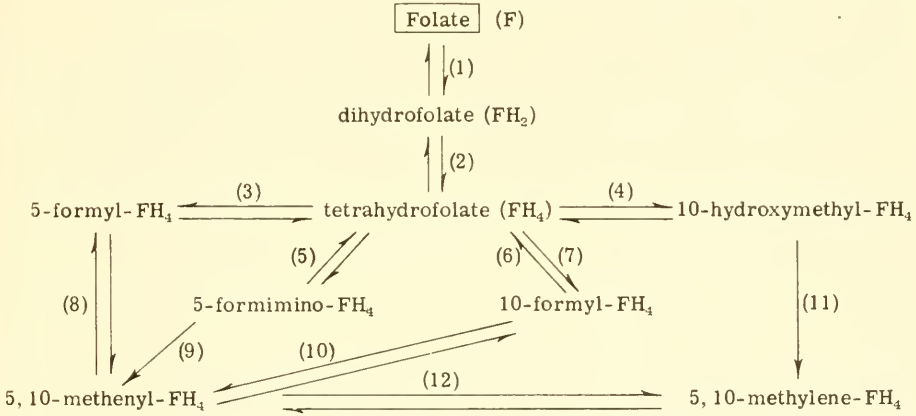
ficiency, there appears to be no really good evidence for any of these reactions being unassociated with pyridoxal function. We have discussed (p. 567) various possible reasons for different effects of deoxypyridoxol and dietary deficiency on tissue B_6 vitamer levels and, hence, on metabolic disturbances in the tissues.

Toxopyrimidine

This substance (4-amino-5-hydroxymethyl-2-methylpyrimidine), which is essentially the pyrimidine portion of thiamine, has been known for many years to produce abnormal motor behavior and convulsions, and a search for antidotes led to the discovery that the pyridoxine group is specific in preventing the toxic reactions. It was then realized that toxopyrimidine bears a structural resemblance to pyridoxal. Makino and Koike (1954 a,b) believed that toxopyrimidine acts in the phosphorylated form since tyrosine decarboxylase is not inhibited by toxopyrimidine up to 1 mM, whereas toxopyrimidine-P exerted some inhibition at 0.001 mM and almost complete inhibition at 0.1 mM. The inhibition is competitive with respect to pyridoxal-P. Haughton and King (1958) confirmed this inhibition but stated that it required an (I)/(C) ratio of 1000 to get 50% inhibition, whereas Makino and Koike found around 50% inhibition with a ratio near 3. No inhibition of tryptophanase, transaminase, glutamate decarboxylase, or arginine decarboxylase was observed, and they concluded that toxopyrimidine is not of much value in the study of pyridoxal-P enzymes. The failure to inhibit significantly the tryptophanase of *E. coli* was also reported by Wada *et al.* (1958). McCormick and Snell (1961) found no inhibition of pyridoxal kinase at concentrations of 0.01–0.1 mM toxopyrimidine. Rindi and Ferrari (1959) found that 90–120 min after the intraperitoneal injection of 125 mg/kg of toxopyrimidine in pyridoxine-deficient rats, convulsions having been produced, the γ-aminobutyrate levels in the brain have fallen some 23%, although glutamate is unchanged. Administration of pyridoxamine stops the convulsions and increases brain γ-aminobutyrate. This dose of toxopyrimidine reduces brain glutamate decarboxylase 20% but does not significantly alter transaminase activity (Rindi *et al.*, 1959). Again pyridoxamine restores activity. We have already noted (Table 2-37) that toxopyrimidine can reduce brain glutamate decarboxylase at high doses, but at lower convulsive doses in pyridoxine-deficient animals it does not. It would seem that if toxopyrimidine causes convulsions by interfering with pyridoxal function, it is not mediated through a general fall in γ-aminobutyrate or transaminase activity, and the status of the mechanism is much the same as for deoxypyridoxol, namely, uncertain.

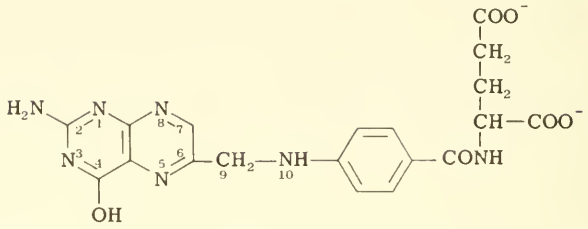
ANALOGS OF PTEROYLGLUTAMATE (FOLATE)

Tetrahydrofolate functions metabolically in the transfer of C_1 units at the oxidation level of formaldehyde or formate, and thus is important in the biosynthesis of purines, pyrimidines (thymine), certain amino acids (serine, histidine, methionine), choline, and other biologically important substances. Interference with its function leads secondarily to a depression of nucleic acid and protein synthesis and, because of this, to a general suppression of cellular growth and multiplication. The possible sites of block for folate analogs may be summarized as: (1) pathway for the synthesis of folate, (2) reduction of folate to tetrahydrofolate, (3) reactions of C_1 unit transfer, and (4) degradative reactions of folate and its derivatives. The transport of folate into cells should probably also be considered as a possible site of inhibition but little about this process is known at the present

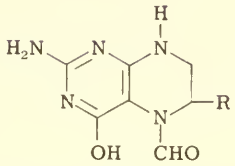


(1) folate reductase
(2) dihydrofolate reductase
(3) glutamate-FH_4 transformylase
(4) serine hydroxymethylase
(5) formimino transferase
(6) 10-formyl-FH_4 deacylase
(7) tetrahydrofolate formylase
(8) (cyclohydrolase)
(9) formimino-FH_4 cyclodeaminase
(10) cyclohydrolase
(11) (cyclohydrolase)
(12) 5, 10-methylene-FH_4 dehydrogenase

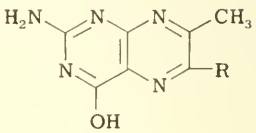
time. A number of substances can inhibit the synthesis of folate in microorganisms — e.g., the sulfonamides and certain pteridines — but they will not be treated in this chapter. There is no evidence that the important actions of folate analogs are related to inhibition of degradative reactions. We shall therefore limit the subject in this section to inhibitions of folate reduction and transformylation reactions, namely, those pathways shown in the accompanying diagram.



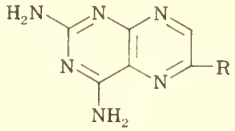
Folate



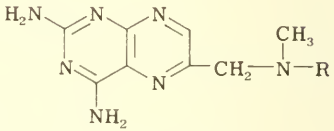
5-Formyltetrahydrofolate
(folinate, citrovorum factor)



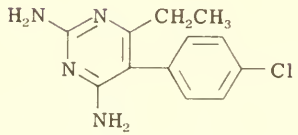
7-Methylfolate



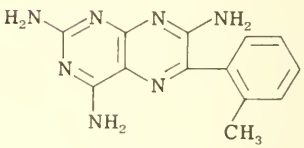
Aminopterin



Amethopterin
(methotrexate)



Pyrimethamine
(Daraprim)



2, 4, 7 Triamino-6-
o-methylphenylpteridine

The most thoroughly studied folate analogs are aminopterin (4-hydroxy group replaced by amino group) and amethopterin (10-methylaminopterin) because of their importance in the chemotherapy of cancer. These substances produce states of folate deficiency in all types of organism and, in most instances, the symptoms of this deficiency can be prevented by providing tetrahydrofolate or 5-formyltetrahydrofolate, but not with folate, indicating that the site of block lies somewhere in the pathway of the transformation of folate to its coenzymically active forms. Although this block produces a general disturbance in tetrahydrofolate function and, it appears, nucleic acid and protein synthesis, there are two reasons for some degree of specificity. In the first place, the syntheses of the various substances requiring C_1 units from folate coenzymes are not necessarily all depressed equally, since it is a general rule that a lowering of the concentration of some substance from which several pathways lead will produce varying effects on these pathways, depending mainly on the nature of the enzymes involved and the supply of reactants for each pathway (in this case C_1 unit acceptors). In the second place, those cells or tissues with the highest rates of synthesis and dependent on these synthetic reactions for growth or multiplication will be most adversely affected by the folate analogs. Here it is not a matter of degree of functional activity but of proliferation; the heart is not readily affected by these analogs whereas the hematopoietic system is. We shall not discuss the more biological aspects of the actions of these analogs, since this is covered adequately in a number of books and reviews (e.g. Holland, 1961; Delmonte and Jukes, 1962), but confine attention to the basic enzyme and metabolic effects.

Inhibition of the Reduction of Folate to Tetrahydrofolate

This reduction occurs in two steps and in most instances it appears that each step is catalyzed by a specific enzyme, but possibly in other cases a single enzyme is responsible. Most assays of folate reduction for analog inhibition have involved determination of the formation of either tetrahydrofolate or folinate, or the disappearance of folate, and it is difficult to differentiate between the two steps with regard to inhibition. In some reports the term "folate reductase" is applied to the over-all reaction. One thing is certain: Dihydrofolate reductase, which has been better purified and more thoroughly studied than folate reductase, is very potently inhibited by aminopterin and amethopterin. The enzyme from chicken liver is inhibited 74% by 0.000053 mM aminopterin (Futterman, 1957) and from human leukemic leucocytes 64% by 0.00001 mM (Bertino *et al.*, 1960), in both cases the substrate being 10,000-fold or more in excess of the analog. Osborn *et al.* (1958) calculated the K_i's for aminopterin and amethopterin to be 0.000001 mM and 0.0000023 mM, respectively, using the chicken liver enzyme, Blakley and McDougall (1961) reported a value of 0.0000024 mM

for aminopterin and the enzyme from *Streptococcus faecalis*, and Nath and Greenberg (1962) gave K_i as 0.0000023 m*M* for amethopterin and the calf thymus enzyme. The inhibitions of the full reduction of folate to tetrahydrofolate by aminopterin and amethopterin are very similar (Futterman, 1957; Futterman and Silverman, 1957; Zakrzewski and Nichol, 1958; Silber *et al.*, 1962), and point to the dihydrofolate reductase as being the more sensitive enzyme. Furthermore, the formation of folinate (citrovorum factor) from folate is very potently inhibited in rat liver slices (Nichol and Welch, 1950), *Lactobacillus casei* and *Streptococcus faecalis* (Hendlin *et al.*, 1953), mouse leukemic cells (Nichol, 1954), and chicken liver extracts (Doctor, 1958). The most potent pteridine analog was found by Doctor (1958) to be 2,4,7-triamino-6-*o*-methylphenylpteridine, although it is not as potent as aminopterin, and he showed that the site of inhibition is previous to tetrahydrofolate. There is thus much evidence that folate reduction is blocked by low concentrations of these analogs, and it is generally agreed that this must be the primary mechanism by which folate deficiency and growth depression are produced.

The inhibitions of folate reduction by aminopterin and amethopterin have been reported to be noncompetitive by several investigators, but it is very likely that the inhibitions are truly competitive, this being obscured by the much greater affinity of the enzyme for the analog than for dihydrofolate. In no case has the rate of inhibition in the presence of varying concentrations of substrate been determined, but one might predict that the competitive nature of the inhibition would be demonstrated in this way. Once the enzyme is inhibited, it is very difficult to recover the activity because the rate of dissociation of the analog from the enzyme is extremely slow. In other words, this is an example of pseudoirreversible inhibition obeying mutual depletion kinetics. This was shown by Peters and Greenberg (1959) on a sheep liver folate reductase, the inhibition at constant analog concentration being dependent on the enzyme concentration. It is thus possible to titrate this enzyme in tissues or extracts. This has been well discussed by Werkheiser (1961), who also showed that the amount of analog bound by rat liver supernates is equivalent to the amount required to inhibit folate reduction; in other words, the tightly bound analog seems to be combined only with dihydrofolate reductase. If rats are injected with amethopterin, the supernatant fraction of the liver contains most of the analog and only 10–15% of this is lost during dialysis for 6 days (Werkheiser, 1959). The amount of amethopterin or aminopterin to inhibit completely the reductase in liver extracts in 0.56 μg/g of liver, and the supernates of livers from analog-treated rats contain 0.52 μg/g of tissue. The reductase is the only protein binding these analogs significantly in chicken liver homogenates (Schrecker and Huennekens, 1964). Fountain *et al.* (1953) had found that there is a remarkable retention of amethopterin in the tissues of mice, the

concentration in the liver remaining approximately constant for at least 3 weeks after a single intravenous dose. It is noteworthy that some tissues, such as the lung and spleen, do not pick up much of the analog, and that the kidney loses the analog relatively more rapidly than the liver. Werkheiser (1960) likewise found that the liver retains aminopterin for long periods, while the intestine does not. In mice given a lethal dose of aminopterin 1 hr after a protective injection of folate, the liver folate reductase activity is depressed 96% after 24 hr and remains at this level for 7 days, following which there is a slow recovery (Werkheiser, 1962). The intestinal enzyme is similarly inhibited but recovers faster. The loss of aminopterin from the intestine is characterized by a half-life of 60 hr, but the liver shows two components with half-lives of 60 hr and 90 days, respectively. It was suggested that rapidly proliferating cells are dependent on folate reductase activity and that the disappearance of the inhibition is faster in such tissue because of the more rapid turnover of cells; in other words, the 60 hr component would arise from proliferating tissue while the 90 day component would relate to nonproliferating tissue. If this is the case, the binding of aminopterin to the enzyme *in vivo* must be essentially irreversible. These results all point to a very high degree of specificity in the binding and inhibition, and confirm the major site of attack as being on folate reduction. Further evidence comes from the reduced urinary folinate levels in rats on 25 μg/day of aminopterin (Nichol and Welch, 1950). Less direct evidence is provided by Nichol (1954) and Broquist *et al.* (1953), who showed that resistant streptococci or leukemic cells have a much greater ability to produce folinate from folate than do normal cells. It is possible that this increased activity allows enough active folinate to be formed to enable the cells to grow and multiply in the presence of the analogs, but it is probable that this is not the only mechanism of resistance to these agents.

The nature of the binding of these folate analogs to the reductase has not been fully elucidated, but Zakrzewski (1963) has determined the thermodynamic characteristics for the dissociation of the EI complexes (see accompanying tabulation). The inhibitions by the substituted pteridines are competitive. The K_i values for aminopterin were taken from Werkheiser

Inhibitor	Changes for EI dissociation			
	K_i (mM)	ΔH	$\Delta F°$	ΔS
2,6-Diaminopurine	0.0018	+6.0	+7.9	−6.4
2,4-Diamino-6-methylpteridine	0.0018	+5.0	+7.9	−9.5
2,4-Diamino-6-formylpteridine	0.0081	+4.1	+6.9	−9.5
2,4-Diamino-6-hydroxypteridine	0.37	+1.8	+4.7	−9.6
Aminopterin	10^{-7}–10^{-8}	+11.6	+13.7–15.1	−7.0–11.7

(1961) and are uncertain because it is not known whether competitive or noncompetitive inhibition occurs; it may be noted that the values are one-hundredth to one-tenth those given by previous workers. The pyrimidine amino groups are essential for tight binding and the pyrazine ring presumably does not participate in the binding. A binding mechanism was proposed in which emphasis is placed on the tautomeric state of the analogs and the formation of hydrogen bonds between the ring nitrogens and the amino groups with the enzyme, the replacement of the 4-OH group of folate with an amino group favoring greater hydrogen bonding. It may also be observed that the benzoylglutamate portion of the molecule must contribute around 6–7 kcal/mole binding energy.

Another analog with less obvious structural similarity to folate is pyrimethamine (Daraprim), an antimalarial drug that in chronic dosage produces folate deficiency in bacteria and animals (Wood and Hitchings, 1959 a; Hitchings, 1960). Pyrimethamine, like the analogs previously discussed, inhibits the reduction of folate, and does this at a concentration equivalent to that required for growth inhibition. A 35% inhibition of folinate formation is caused in extracts of *S. faecalis* by 0.000012 m*M* pyrimethamine, so that its potency is comparable to that of aminopterin. There are no effects on the biosynthesis or assimilation of folate. It is believed that the antimalarial action is due to the tighter binding of the drug to the plasmodial folate reductase than to the host enzyme. However, the uptake of pyrimethamine by bacterial cells is unique inasmuch as it is inhibited strongly by glucose, whereas the uptake of aminopterin is augmented by glucose (see accompanying tabulation) (Wood and Hitchings, 1959 b). Furthermore, aminopterin uptake is increased by a rise in temperature, whereas less pyrimethamine appears in the cells at higher temperatures. Despite the apparent similarity of site of action of these two analogs, there is some basic difference in the movement or disposition of the materials in the cells.

Analog	Analog taken up (cpm)	
	No glucose	Glucose
Pyrimethamine	51.6	0.7
Aminopterin	55	523

Effects on Synthetic Processes Mediated by Tetrahydrofolate

A block in the reduction of folate would be expected to depress the C_1 unit transfers and the synthesis of nucleic acids and proteins as long as there is no supply of tetrahydrofolate or folinate. The analogs in addition

might inhibit directly the reactions in which tetrahydrofolate functions. There is essentially no information on this second possibility. Cyclohydrolase is inhibited weakly by amethopterin (43% at 0.5 m*M*) and aminopterin (69% at 0.5 m*M*) (Tabor and Wyngarden, 1959), and formyltetrahydrofolate synthetase is even more weakly inhibited by several analogs (Jaenicke and Brode, 1961; Whiteley *et al.*, 1959). The many other reactions involved have never been examined for inhibition by analogs. All one can say at the present time is that the known inhibitions on folate reduction are sufficient to explain most or all of the effects of these analogs.

Some examples of the inhibition of syntheses will be mentioned to illustrate the nature of the metabolic actions of these analogs. Aminopterin and amethopterin invariably depress the incorporation of formate-C^{14} into purines and nucleic acids; this has been shown in rabbit bone marrow (Totter and Best, 1955), leukemic spleen extracts (Balis and Dancis, 1955), and the whole animal (Skipper *et al.*, 1950). It appears that thymine synthesis is more sensitive than purine synthesis to inhibition by these analogs. In bone marrow 0.0021 m*M* aminopterin inhibits incorporation into thymine 72% but into adenine or guanine only 22%. It would be interesting to know what the effect on adenine nucleotides is, but little is known. Aminopterin elevates ATP in liver, has no effect on spleen or muscle ATP, and reduces tumor ATP (Zahl and Albaum, 1955). Indeed, the total adenine nucleotides in the sarcoma decrease. One might expect the effects to depend on the relative rates of adenine synthesis and turnover in the tissues. Aminopterin lowers liver NAD levels — 39% fall at 60 μg/day and 56% fall at 100 μg/day — and if this occurs throughout the body it could be an important consequence of interference with folate metabolism (Strength *et al.*, 1954). The *in vitro* depression of respiration by aminopterin is not completely reversed by added NAD, and it is very possible that other coenzymes (e.g. NADP, coenzyme A, or FAD) are decreased.

The interconversion of glycine and serine is inhibited by aminopterin when only folate is supplied, but the activity is restored with folinate (Blakley, 1954). Tetrahydroaminopterin does not inhibit serine synthesis when folinate is provided, but 2-deaminofolate inhibits some 55% at 0.75 m*M* in rabbit liver extracts (Blakley, 1957). The incorporation of formate-C^{14} into lymphoma proteins is inhibited 72% by 0.0073 m*M* amethopterin, but little effect is observed in normal liver (Williams *et al.*, 1955). In general these analogs block nucleic acid synthesis more than protein synthesis, but this may vary a good deal from one tissue to another, or one organism to another. Most of the inhibitions in nucleic acid and protein synthesis have been attributed solely to defects in the formation of the constituent units, and little consideration has been given to other possible contributing factors, such as lowered levels of various coenzymes with impairment of oxidative and phosphorylative reactions.

Effects on Certain Enzymes Unrelated to C_1 Transfer

Brief mention should be made of the early demonstrations that 7-methylfolate is inhibitory to dopa decarboxylase and that this is reversible with folate at 10–100 times the analog concentration (Martin and Beiler, 1947). Pteroylaspartate and 7-methylpteroate are less potent inhibitors, but the difference is not great, so that the glutamate portion is not very important for the binding (Martin and Beiler, 1948). Tyrosine decarboxylase is not inhibited by 0.67 mM 7-methylfolate whereas 0.067 mM inhibits dopa decarboxylase 25%. The mechanism of this inhibition, the role of folate in decarboxylase activity, and the effects of the more commonly used folate analogs are all unknown. The decarboxylase inhibition prompted a study of the effects of 7-methylfolate on blood pressure (Martin *et al.*, 1947), and it was found that 5 mg/kg in the dog depresses the blood pressure quite significantly and for an extended period of time, but whether this is related to decarboxylase inhibition is problematical.

The inhibition of acetyl transfer by amethopterin ($K_i = 0.032$ mM) in pigeon liver extracts is interesting because it represents another possible site of action for this analog, even though it is obviously much less potent here than on folate reduction (Jacobson, 1960). The inhibition is not counteracted by folate, tetrahydrofolate, or folinate, and is competitive with the acetyl donor (*p*-nitroacetanilide) but noncompetitive with the acetyl acceptor (aniline or sulfanilamide). These results do not implicate a folate compound in the acetylation reaction — indeed, folate and folinate are weak inhibitors, and the mechanism is more likely a simple binding to the substrate site. The 10-methyl group is important since aminopterin is only one-tenth, or less, as inhibitory as amethopterin. Evidence for inhibition of acetylation *in vivo* is the higher sulfanilamide level and the lower acetylsulfanilamide level in rabbit plasma of animals treated with amethopterin (Johnson *et al.*, 1958).

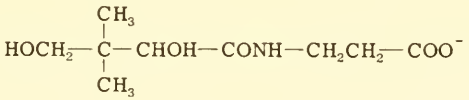
ANALOGS OF OTHER VITAMINS, COENZYMES, AND THEIR COMPONENTS

There has been a great deal of work on the growth inhibitions produced by numerous analogs of pantothenate, biotin, cobalamin, and other metabolically necessary cofactors, but relatively few reports on enzyme inhibitions are available. However, some of this isolated work on enzyme systems is interesting in itself and perhaps some reference to it will stimulate further study.

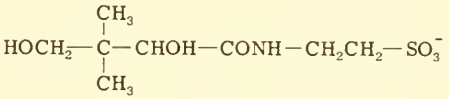
Pantothenate is required by many microorganisms and animals because it is a component of coenzyme A, the biosynthetic pathway being:

Pantothenate → pantothenylcysteine → pantetheine → CoA

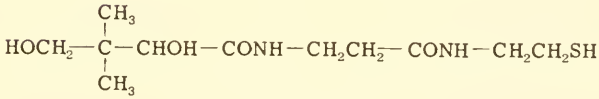
McIlwain (1945) found that pantothenate analogs, such as pantoyltaurine, do not displace bound pantothenate from bacterial cells, and concluded that these analogs are bacteriostatic because they block the formation of the metabolically active form of pantothenate (which was not known at that time). Furthermore, as Martin *et al.* (1950) showed, the analogs in general do not interfere in the reactions involving CoA. Pantoyltaurine and other analogs do not inhibit brain choline acetylase, even at concentrations around 5 m*M*. One analog, salicyloyl-β-alanide, does inhibit this enzyme (20% at 0.47 m*M* and 100% at 4.7 m*M*), but since neither pantothenate nor CoA reverses this inhibition it is doubtful if it is specific. Most effective analogs thus seem to block the pathway of pantothenate → CoA, and no known direct antagonists of CoA are known. Pantoylaminoethanethiol inhibits the synthesis of CoA from pantetheine (50% at a ratio of analog to pantetheine of 13) and thus inhibits sulfonamide acetylation in liver extracts provided with pantetheine (Boxer *et al.*, 1955). One cannot help but wonder in some of these instances if abnormal CoA analogs are formed,



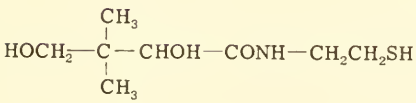
Pantothenate



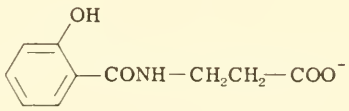
Pantoyltaurine



Pantetheine



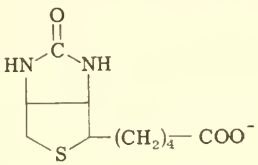
Pantoylaminoethanethiol



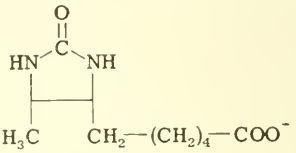
Salicyloyl-β-alanide

rather than a simple inhibition of the biosynthetic pathway. It is possible to go back farther and inhibit the synthesis of pantothenate by analogs of pantoate or β-alanine. For example, 2,3-dichloroisobutyrate blocks the coupling of these two components of pantothenate, competitive with pantoate and uncompetitive with β-alanine (K_i = 1.4–6.4 mM) (Hilton, 1958).

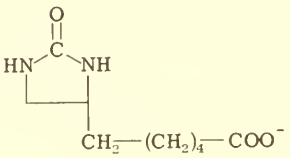
Biotin functions in several metabolic pathways (synthesis of aspartate and higher fatty acids, and CO_2 fixation) and in certain organisms can be formed from desthiobiotin. 2-Oxo-4-imidazolidinecaproate (desmethyldesthiobiotin) inhibits the growth of *E. coli* by competing with desthiobiotin for an enzyme involved in biotin synthesis, and this inhibition is competitive (Rogers and Shive, 1947). Biotin by an unknown mechanism stimulates fermentation in biotin-deficient yeast and this is inhibited by homooxybiotin, oxybiotinsulfonate (COO^- group replaced by SO_3^- group in oxybiotin), and γ-(3,4-ureylenecyclohexyl)butyrate when the analogs are added



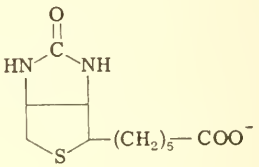
Biotin



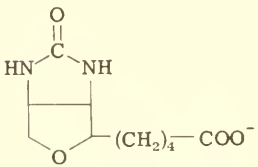
Desthiobiotin



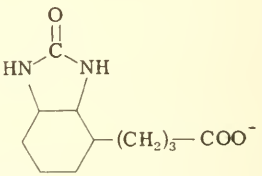
Desmethyldesthiobiotin



Homobiotin



Oxybiotin

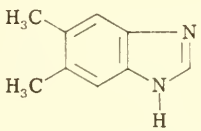


γ-(3, 4-Ureylenecyclohexyl)butyrate

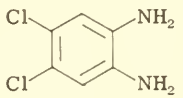
before the biotin, but not afterward (Axelrod *et al.*, 1948). This could mean that these analogs cannot displace biotin once it is bound or that they inhibit in some way the formation of an active form of biotin. Biotin is inactivated by kidney slices, possibly by removal of fragments from the side chain, by biotin oxidase, and this enzyme is inhibited by several analogs competitively (see accompanying tabulation) (Baxter and Quastel, 1953). There is thus the possibility that some analogs can conserve biotin in the tissues as well as inhibit its synthesis or function.

Analog	Concentration (m*M*)	(Analog)/(biotin)	% Inhibition
DL-Homobiotin	1.65	10	96
DL-Desthiobiotin	1.63	10	96
L-Biotin	0.4	10	82
DL-Biotindiaminecarboxylate	0.4	10	82
D-Biotinoldiamine	0.4	10	45
D-Biotinol	0.41	10	24
	2.1	50	61
D-Biotinsulfone	0.41	10	16

Analogs of cyanocobalamin (vitamin B_{12}) have not been extensively studied because of the complexity of the structure. 5,6-Dimethylbenzimidazole is a component of cyanocobalamin and 1,2-dimethyl-4,5-diaminobenzene is a precursor in the synthesis. Analogs of these substances are often inhibitory to bacterial growth and the biosynthesis of vitamin B_{12}. 1,2-Dichloro-4,5-diaminobenzene inhibits the synthesis of vitamin B_{12} in bacteria and the



5, 6-Dimethylbenzimidazole



1, 2-Dichloro-4, 5-diaminobenzene

growth of those bacteria requiring vitamin B_{12} (Woolley and Pringle, 1951). Vitamin B_{12} is unable to counteract these inhibitions. Although 5,6-dimethylbenzimidazole can be used by the rat to form vitamin B_{12}, this substance is inhibitory to *Lactobacillus lactis*, and like a number of analogs, is able to inhibit the synthesis of vitamin B_{12} in these bacteria (Hendlin and Soars, 1951). These inhibitions do not appear to be competitive with vi-

tamin B_{12}, but the inhibition by 1,2-diamino-4,5-dimethylbenzene is competitive. The only instance of the inhibition of vitamin B_{12} function is in the synthesis of methionine from serine in *E. coli* where the methylamide, ethylamide, and anilide analogs of cyanocobalamin inhibit competitively with respect to vitamin B_{12} (Guest, 1960). These analogs also inhibit the growth of organisms that require methionine or vitamin B_{12}, but do not when methionine is supplied. The inhibitions are reasonably potent, 61% depression being given by 0.029 m*M* of the anilide derivative when cyanocobalamin is 0.000032 m*M*, but the analogs are obviously bound less tightly than the cyanocobalamin to the enzyme involved. Hydroxocobalamin and cyanocobalamin, which are analogs of cobalamin coenzyme, inhibit potently and competitively the diol dehydrase from *Aerobacter*, but once inhibition occurs it cannot be reversed by either dialysis or the coenzyme (Lee and Abeles, 1963).

Lipoate functions in acyl transfer during the oxidation of keto acids and this is inhibited by 6-ethyl-8-mercaptooctanoate, an analog of 6-acetyl-6,8-dimercaptooctanoate (a functional form of lipoate) (Albrecht, 1957). This analog does not inhibit the anaerobic decarboxylation of pyruvate in extracts from *E. coli* but inhibits pyruvate oxidation. The phosphotransacetylase reaction and the formation of acetyllipoate are inhibited.

MISCELLANEOUS ANALOG INHIBITIONS

There are a number of reports of inhibitions by analogs that do not readily fall into any general classification. Some of these have been put into Table 2-38 in order to illustrate further the various types of analog, although in some cases it is not quite certain whether the inhibitor should be considered as an analog or not. In most instances the inhibitions are competitive, but in others it may be noncompetitive or mixed, indicating that the inhibitions do not all involve a simple competition between the analog and the substrate for an enzyme site. Unfortunately, most of these inhibitions have not been adequately studied with respect to mechanism. Some more important examples that could not be easily summarized in the table will be discussed briefly.

Inhibition of Morphine *N*-Demethylase

The antagonistic actions of nalorphine (*N*-allylnormorphine) to the pharmacological responses to morphine have been extended to the enzyme system for morphine inactivation, and it appears that the configurations of the tissue receptor groups and the enzyme active site are very similar (Axelrod and Cochin, 1957). Several normorphine analogs were tested on the *N*-demethylation of morphine by rat liver enzyme (see accompanying tabulation) and the role of the alkyl substituent in the binding is evident.

TABLE 2-38

MISCELLANEOUS INHIBITIONS BY ANALOGS OF SUBSTRATES OR COFACTORS

Enzyme	Source	Substrate[a]	Inhibitor	Concentration (mM)	% Inhibition[b]	K_i (mM)	Reference
Acetoacetate decarboxylase	*Clostridium acetobutylicum*	Acetoacetate	Acetopyruvate	0.001	65		Seeley (1955)
Acetylindoxyl oxidase	Corn seedlings	*N*-acetylindoxyl (5)	Catechol	0.1	92	0.003[c]	Beevers and French (1954)
			Dihydroxyphenylalanine	0.1	100	<0.0003	
			Indoxyl sulfate	2.5	27	2.0	
			Indole	2.5	45	0.92	
			Indole-3-aldehyde	5	51	1.44	
			N-acetylisatin	2.5	13	5.0	
			Isatin	2.5	18	3.4	
			Caffeate	4	88	0.16	
			Dioxindole	5	22	5.3	
Acyl-CoA dehydrogenase	Pig liver	Butyryl-CoA	Crotonyl-CoA	—	—	0.014	Hauge (1956)
Aminolevulinate dehydrase	Mouse liver	δ-Aminolevulinate(1.33)	δ-Oximinolevulinate	0.0133	27		Tschudy *et al.* (1960)
				0.133	75		
				1.33	96		
			δ-Chlorolevulinate	1.33	96		
			β-Ketoadipate	1.33	79		

TABLE 2-38 (*continued*)

Enzyme	Source	Substrate[a]	Inhibitor	Concentration (m*M*)	% Inhibition[b]	K_i (m*M*)	Reference
			δ-Acetamidolevulinate	1.33	0		
Butynamine demethylase	Rat liver	Butynamine(1)	2,4-Dichloro-6-phenylphenoxyethylamine	0.0014	50		McMahon and Mills (1961)
Carbamyl-P: ornithine transcarbamylase	Rat liver	Ornithine(×)	Citrulline	10×	25		Burnett and Cohen (1957)
			D-ornithine	10×	0		
Catalase	Horse liver	Hydrogen peroxide	Monoethyl peroxide	0.6	40		Blaschko (1935)
				6	73		
Catechol-*O*-methyl transferase	Rat liver	Epinephrine (0.01)	Pyrogallol	0.01	50		Axelrod and Laroche (1959)
	Rat liver	Norepinephrine	Pyrogallol	—	—	0.008	Crout (1961)
Cholesterol esterase	Pleuropneumonialike organisms	Cholesteryl valerate(4)	Estradiol	0.1	99		Smith (1959)
			Cholestane	0.1	98		
			Cholestan-3-one	0.1	76		
Cytochrome c	Heart	Cytochrome c(×)	Xanthyl-cytochrome c	3×	40		Dickman and Westcott (1953)

Dehydroshikimate reductase	Pea seedlings	Shikimate(0.3)	Vanillin	0.5	100	0.093	Balinsky and Davies (1961 b)
			Gallate	5	100	0.38	
			p-Hydroxybenzoate	5	100	0.74	
			Protocatechuate	1	23	0.75	
			Catechol	5	100	1.9	
			Guaiacol	5	91	11.0	
		Shikimate(1)	Pyrogallol	10	100		
Diaminopimelate decarboxylase	*E. coli*	Diaminopimelate (×)	Pyridine-2,6-dicarboxylate	1×	45		Dewey *et al.* (1954)
			α, *ε*-diamino-*β*-hydroxypimelate	1×	15		
Formate dehydrogenase	*E. coli*	Formate(200)	Hypophosphite	0.008	36		Crewther (1956)
				0.015	45		
				0.03	86		
Formate hydrogenylase	*E. coli*	Formate(200)	Hypophosphite	0.008	19		Crewther (1956)
				0.015	25		
				0.03	62		
	Zymosarcine ventriculi	Formate(7.15)	Formaldehyde	1	14		Bauchop and Dawes (1959)
				80	86		
D-Glucosamine phosphokinase	Beef brain	D-Glucosamine	*N*-Acetylglucosamine	1	10		Harpur and Quastel (1949)
				2	70		
Glutathione oxidase	Mouse kidney	Glutathione	Thioglycolate	2	30		Ames and Elvehjem (1945)
Glycolate oxidase	Rat liver	Glycolate(10)	Diphenylglycolate	52	50	9.0[c]	Kun *et al.* (1954)
Glyoxalase	Beef liver	Methylglyoxal	*S*-(*N*-ethylsuccinimido)-GSH	—	—	0.64	Kermack and Matheson (1957)

TABLE 2-38 (*continued*)

Enzyme	Source	Substrate[a]	Inhibitor	Concentration (m*M*)	% Inhibition[b]	K_i (m*M*)	Reference
			S-Methyl-GSH	—	—	2.3	
			γ-Glutamylalanylglycine	—	—	15.1	
			γ-Glutamyl-β-sulfoalanylglycine	—	—	18.7	
			S-Methylcysteine	—	—	22.5	
	Dog leucocytes	Methylglyoxal (2.13)	Dimethylacetone	10	>50		McKinney and Gemma (1958)
	Yeast	Methylglyoxal Glutathione	Ophthalmate	—	—	0.95	Cliffe and Waley (1961)
Glyoxylate transacetase (malate synthetase)	Yeast	Glyoxylate	Oxalate	—	—	0.019	G. H. Dixon *et al.* (1960)
			Glycolate	—	—	0.308	
			Acetate	—	(ni)		
			Oxalacetate	—	(ni)		
3-Hydroxyanthranilate oxidase	Calf liver	3-Hydroxyanthranilate	Anthranilate	—	—	0.04	Vescia and di Prisco (1962)
β-Hydroxybutyrate dehydrogenase	Pig heart	β-Hydroxybutyrate	Oxalacetate	6	42		Green *et al.* (1937 a)
				30	71		
			Acetoacetate	12	55		

p-Hydroxyphenyl-pyruvate oxidase	Dog liver	*p*-Hydroxyphenyl-pyruvate(1.2)	*m*-Hydroxyphenyl-pyruvate	0.2 0.5	50 >90	La Du and Zannoni (1955)
Indoleacetate oxidase	Pea epicotyls	Indoleacetate (0.11)	Chlorogenate Caffeate Chlorogenate	0.0022 0.0025 0.0028	50 50 100	Rabin and Klein (1957) Sondheimer and Griffin (1960)
Kynureninase	*Neurospora*	Kynurenine	DL-Dihydroxy-phenylalanine Putrescine Cadaverine DL-Ornithine	0.5 5 10 10 10	61 92 65 62 60	Jakoby and Bonner (1953)
Kynurenine formamidase	*Neurospora*	Formylkynure-nine(1) Formylanthra-nilate (6.7)	Kynurenine Anthranilate	0.4 0.2	0 0	Jakoby (1954)
Kynurenine transaminase	Rat kidney	α-Ketoglutarate (6) Kynurenine (3.7)	Adipate 3-Methylglutarate Caprate β-Ketoadipate Pimelate	6 6 6 6 6	65 47 41 33 30	Mason (1959)
Lecithinase A	Water moccasin venom	Lecithin(×)	(α, β-Distearoyl-oxypropyl) di-methyl(β'-hy-droxyethyl)am-monium acetate	0.06× 1.2	(i) >90	Rosenthal and Geyer (1959)
Lipase	Pancreas	Methylbutyrate	Isoamylbutyrate		(i)	Fodor (1952)

TABLE 2-38 (*continued*)

Enzyme	Source	Substrate [a]	Inhibitor	Concentration (m*M*)	% Inhibition [b]	K_i (m*M*)	Reference
	Rat liver	Methylthioacetate(10)	Methylacetate	10	0		Suzuoki and Suzuoki (1954)
				100	8		
		O,S-Diacetylthiamine(8)	Methylacetate	10	11		
				100	47		
Malate dehydrogenase	*Avena* coleptile	Malate	Oxalacetate	0.1	32		Berger and Avery (1943 b)
				1	87		
			Pyruvate	10	23		
	Pigeon liver	Malate(20)	*meso*-Tartrate	—	—	3.2	Scholefield (1955)
			Tartronate	—	—	0.09	
			Citrate	—	—	2.65	
	Mycobacterium tuberculosis	Malate(3)	Oxalacetate	0.06	64		Goldman (1956 b)
				0.15	85		
		Malate(8)	Oxalacetate	0.06	36		
				0.15	67		
		Malate(20)	Oxalacetate	0.06	26		
				0.15	65		
	Rat liver	Malate	β-Fluorooxalacetate	—	—	0.028	Kun *et al.* (1958)
Malate dehydrogenase	Beef lens	Pyruvate NADPH	Fumarate	16 33	30 50		van Heyningen and Pirie (1953)

(decarboxylating)		CO_2	Ethyloxalacetate	2	90		
(malic enzyme)	Pigeon liver	Malate(0.03–0.1)	Tartronate	0.03	15	0.1	Stickland (1959 b)
		NADP		0.06	31		
			Mesoxalate	—	—	0.1	
			meso-Tartrate	—	—	2	
		Malate(1)	Oxalacetate	0.067	49		
				0.167	66		
	Pigeon liver	Pyruvate	Tartronate	0.01–0.1	50		Stickland (1959 a)
		NADPH	Oxalacetate	1–10	50		
		CO_2	Mesoxalate	20	50		
			Succinate	50	50		
	Flax rust uredospores	Malate(5)	Oxalacetate	1	55		Johnson and Frear (1963)
Morphine *N*-demethylase	Microsomes (liver)	Morphine	*N*-Allylnormorphine	0.1	50		Axelrod and Cochin (1956)
	Mouse liver	Dromoran(0.4)	*l*-3-Hydroxy-*N*-allylnormorphinan	0.1	38		Takemori and Mannering (1958)
Oxalacetate decarboxylase	*Micrococcus* lysodeikticus	Oxalacetate(10)	Fumarate	20	77		Ochoa and Weisz-Tabori (1948)
			Malate	20	93		
Oxalosuccinate decarboxylase	Pig heart	Oxalosuccinate (7.5)	Oxalacetate	10	8		Ochoa and Weisz-Tabori (1948)
			cis-Aconitate	5	24		
			Isocitrate	6.6	88		
L-Pantoate: β-alanine ligase (AMP)	*E. coli*	β-Alanine	Acetate	—	—	110	van Oorschot and Hilton (1963)
			Chloroacetate	—	—	170	
			Dichloroacetate	—	—	33	

TABLE 2-38 (*continued*)

Enzyme	Source	Substrate[a]	Inhibitor	Concentration (m*M*)	% Inhibition[b]	K_i (m*M*)	Reference
			Trichloroacetate	—	—	1	
			Propionate	—	—	28	
			α-Chloropropionate	—	—	70	
			β-Chloropropionate	—	—	430	
			α, α-Dichloropropionate	—	—	5	
			α, α, β-Trichloropropionate	—	—	1	
Penicillinase	*Proteus morgani*	Benzylpenicillin (2.7)	Cloxacillin	0.0002	50		Hamilton-Miller and Smith (1964)
			Oxacillin	0.0007	50		
			Propicillin	0.0048	50		
			Methicillin	0.0083	50		
			Phenethicillin	0.0112	50		
			Phenbenicillin	0.0389	50		
	E. coli	Benzylpenicillin (2.7)	Cloxacillin	0.0008	50		Hamilton-Miller and Smith (1964)
			Oxacillin	0.0011	50		
			Phenbenicillin	0.017	50		
			Methicillin	0.044	50		
			Phenethicillin	0.065	50		
			Propicillin	0.082	50		

	Bacillus cereus	Benzylpenicillin	6-(2,6-Dimethoxybenzamido)-penicillanate	0.034	50	1.8	Citri and Garber (1961); Garber and Citri (1962)
	Bacillus cereus	Penicillin	Cephalosporin C	—	—	<0.3	Abraham and Newton (1956)
	Staphylococcus aureus	Penicillin(2.8)	Valylvaline				
			D, D-	25	50		Saz *et al.* (1961)
			D, L-	25	15		
			L, L-	25	18		
			L, D-	25	0		
			Valylleucine				
			D, D-	25	42		
			D, L-	25	38		
			L, L-	25	54		
			L, D-	25	31		
Peroxidase	Horseradish	*p*-Coumarate(0.5) NADH(0.075)	Ferulate	0.005	28		Gamborg *et al.* (1961)
				0.01	40		
				0.025	61		
			Sinapate	0.0025	53		
			Caffeate	0.001	50		
			Chlorogenate	0.0025	60		
			p-Hydroxyphenylpyruvate	0.5	46		
Phenylamine-β-hydroxylase	Beef adrenal	Dopamine(2)	*p*-Tyramine	2	55		Goldstein and Contrera (1962)
			p-Hydroxyamphetamine	2	31		

TABLE 2-38 (*continued*)

Enzyme	Source	Substrate[a]	Inhibitor	Concentration (mM)	% Inhibition[b]	K_i (mM)	Reference
			α-Methyldopamine	2	30		
			Phenylethylamine	2	27		
			Amphetamine	2	18		
			3-Methoxydopamine	2	9		
			Epinine	2	8		
Porphobilinogen deaminase	Spinach leaves	Porphobilinogen	Isoporphobilinogen	—	—	0.57	Carpenter and Scott (1961)
			Opsopyrroledicarboxylate	—	—	0.28	
Pyridoxamine: oxalacetate transaminase	Rat kidney	Oxalacetate(0.4)	Ketomalonate	0.4	14		Wu and Mason (1964)
		Oxalacetate(3.33)	L-Glutamate	3.33	84		
			L-Aspartate	3.33	56		
			α-Ketoglutarate	3.33	13		
			β-Ketoadipate	3.33	11		
			Glutarate	3.33	0		
			Adipate	3.33	0		
Pyruvate decarboxylase	*Zymosarcina ventriculi*	Pyruvate	Acetaldehyde	20	48		Bauchop and Dawes (1959)
			Formaldehyde	80	100		

Sarcosine oxidase	Rat liver	Sarcosine	Methoxyacetate	—	—	1.8	Frisell and Mackenzie (1955)
Succinate dehydrogenase	Rat heart	Succinate(15)	Itaconate	32	50		Booth *et al.* (1952)
	Rat muscle	Succinate(3.3)	Itaconate	3.3	61		Lang and Bässler (1953)
				6.7	94		
Succinic semialdehyde dehydrogenase	Monkey brain	Succinic aldehyde (0.02)	*p*-Hydroxybenzaldehyde	0.0125	75		Albers and Koval (1961)
Sulfanilamide acetylase	Pigeon liver	Sulfanilamide (0.8)	2-Hydroxy-5-bromobenzamide	0.1	61		Johnson (1955)
			o-Cresotamide	1	58		
			Salicylamide	1	56		
			6-Aminonicotinamide	1	50		
			p-Hydroxybenzamide	1	10		
			p-Aminosalicylate	2	72		
D-Tartrate dehydrase	*Pseudomonas*	D-Tartrate(4)	*meso*-Tartrate	4	54		Shilo (1957)
			Tartronate	4	66		
			D-Malate	4	72		
			L-Malate	4	0		
			L-Tartrate	4	0		
L-Tartrate dehydrase	*Pseudomonas*	L-Tartrate(4)	*meso*-Tartrate	4	93		Shilo (1957)
			D-Tartrate	4	35		
meso-Tartrate dehydrase	*Pseudomonas*	*meso*-Tartrate (4)	D-Tartrate	4	74		Shilo (1957)
			D-Malate	4	74		
			Tartronate	4	42		

TABLE 2-38 (*continued*)

Enzyme	Source	Substrate[a]	Inhibitor	Concentration (m*M*)	% Inhibition[b]	K_i (m*M*)	Reference
Tartronic semialdehyde reductase	*Pseudomonas ovalis*	Tartronic semialdehyde(0.5)	Glycolate	10	86		Gotto and Kornberg (1961)
			Glyoxylate	10	48		
			Tartronate	10	26		
			Oxalate	10	22		
			Pyruvate	10	15		
			L-Malate	10	15		
			Glyoxal	10	14		
L-Thyroxine deiodinase	Rat kidney	L-Thyroxine (0.0001)	3,5,3′,5′-Tetraiodothyropropionate	0.001	76		Larson and Albright (1961)
			D-Thyroxine	0.001	45		
			D-Methyl-DL-thyroxine	0.001	30		
	Rat liver	L-Thyroxine	D-Thyroxine	—	—	0.0024	Wynn and Gibbs (1963)
			N-Acetylthyroxine	—	—	0.0026	
			Tetraiodothyroacetate	—	—	0.0018	
			Tetraiodothyropropionate	—	—	0.0015	
			3,3′,5′-Triiodothyronine	—	—	0.0059	

			3,5,3′-Triiodothyronine	—	—	0.0153	
			3′-5′-Diiodothyronine	—	—	0.0106	
			3.5-Diodothyronine	—	—	0.069	
			3′-Iodothyronine	—	—	0.052	
			Thyronine	—	(ni)		
Tryptophan pyrrolase	Rat liver	Tryptophan and hematin	Mesoporphyrin	—	—	0.0005	Greengard and Feigelson (1962)
			Protoporphyrin	—	—	0.0005	
Urease	Jack beans	Urea(83)	Methylurea	83	5		Takeuchi (1933)
			Oxyurea	83	80		
	Jack beans	Urea	Acetamide	500	14		Kistiakowsky and Shaw (1953)
			Thiourea	500	35		
	Jack beans	Urea	Methylurea	—	—	5.6	Shaw and Raval (1961)
	Corynebacterium renale	Urea(25)	Thiourea	200	35		Lister (1956)
				400	70		
UDP-glucose pyrophosphorylase	Rat liver	UDP-glucose	Galactose-1-P	—	—	8.9	Oliver (1961)
	Guinea pig brain	UDP-glucose	Galactose-1-P	—	—	15.5	Oliver (1961)
Xanthine: cytochrome c oxidoreductase	Milk	Xanthine	Myoglobin I_A	—	—	0.00042	Quinn and Pearson (1964)
			Myoglobin I_B	—	—	0.00024	
			Myoglobin II	—	—	0.0064	

[a] Substrate concentration given in mM in parentheses where necessary or available; (×) is undesignated concentration.

[b] The designations (*i*) and (*ni*) refer to inhibition and no inhibition, respectively.

[c] K_i calculated from data assuming competitive inhibition.

Analog (0.2 mM)[a]	% Inhibition	Relative $-\Delta F$ of binding (kcal/mole)
N-Methallylnormorphine	74	4.90
N-Isobutylnormorphine	69	4.75
N-Allylnormorphine	64	4.61
N-Hexylnormorphine	44	4.13
N-Butylnormorphine	35	3.88
N-Propylnormorphine	20	3.40
N-Ethylnormorphine	13	3.09
N-Isopropylnormorphine	11	2.97
Normorphine	0	<2.45

[a] Morphine = 1 mM.

Some generalizations may be made: (1) increase in chain length increases the binding energy, (2) unsaturation increases the binding by 1.0–1.2 kcal/mole, (3) each additional methylene group augments binding by approximately 0.3 kcal/mole, and (4) the inhibition by nalorphine is noncompetitive (it was stated that this may be a slow pseudoirreversible inhibition but incubation with morphine and inhibitors was for 2 hr.

Dehydroshikimate Reductase

The inhibition of this enzyme by various phenolic compounds points to the manner in which the substrate is bound and the configuration of the active site. Relative binding energies are given in Table 2-39. These inhibitions are all strictly competitive. It is seen that all effective inhibitors have a *p*-OH group, and Balinsky and Davies (1961 b) postulated from the possible ring configurations of shikimate that this group must lie approximately in the equatorial plane. Additional OH groups increase the binding slightly or not at all, so that *m*-OH groups seem to participate little in the binding. One might expect the carboxylate group to be bound to an enzyme cationic group, but this does not appear likely; e.g., the addition of a COO^- group to catechol increases the binding very little, and the benzoates without a *p*-OH are bound very poorly. The stronger reaction of the aldehyde group in vanillin also indicates that the forces here are not merely electrostatic. Substitution of benzoate in the *o*-position is detrimental to the binding and this may be due to steric hindrance, as shown in the diagram of the active site presented by Balinsky and Davies (Fig. 2-21). The energy of binding of the *p*-OH group is greater than 2.3 kcal/mole and thus hydrogen bonding may be involved. It is worth noting that the experiments were run at

TABLE 2-39

ANALOG INHIBITION OF DEHYDROSHIKIMATE REDUCTASE[a]

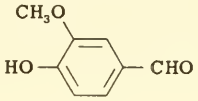
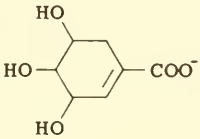
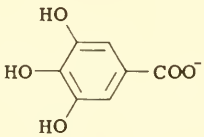
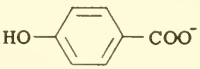
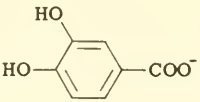
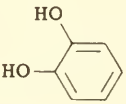
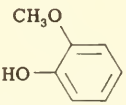
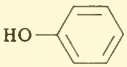
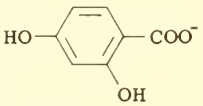
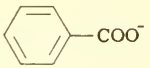
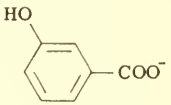
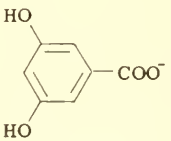
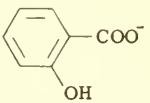
Analog	Structure	Relative - ΔF of binding (kcal/mole)
Vanillin		5.72
(Shikimate)		(5.29)[b]
Gallate		4.85
p-Hydroxybenzoate		4.13
Protocatechuate		4.12
Catechol		3.86
Guaiacol		2.78

TABLE 2-39 (*continued*)

Analog	Structure	Relative - ΔF of binding (kcal/mole)
Phenol		1.84[c]
2, 4-Dihydroxybenzoate		1.84[c]
Benzoate		< 1.84[c]
m-Hydroxybenzoate		< 1.84[c]
3, 5-Dihydroxybenzoate		< 1.84[c]
Salicylate		< 1.84[c]

[a] From Balinsky and Davies (1961b).
[b] The value for shikimate was obtained from K_m assuming that this is a true substrate constant.
[c] Values calculated on the basis of inhibitions reported and are very approximate.

pH 9 where the phenolic groups are partially ionized, and this may account for some of the differences in inhibitory activity between the compounds. Variation of the inhibition with pH might provide some interesting information.

Kynurenine Transaminase

The transamination between kynurenine and α-ketoglutarate catalyzed by an enzyme from rat kidney is inhibited by a variety of mono- and dicarboxylates (Mason, 1959). The inhibitions are competitive with respect to kynurenine and are reversible. The results are interpreted in terms of

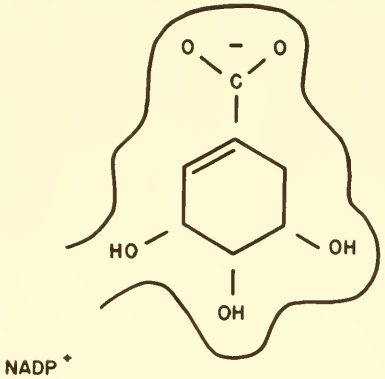


FIG. 2-21. Topographical scheme of the active site of dehydroshikimate reductase. (From Balinsky and Davies, 1961 b.)

two types of interaction: (1) electrostatic binding of carboxylate groups to cationic groups on the enzyme, and (2) van der Waals' forces between the hydrocarbon portions of the inhibitors and the enzyme surface. The variation of inhibition in the dicarboxylate series (Table 2-40) indicates that two cationic groups interact maximally with adipate. The distance between these groups was given by Mason as 11 Å on the basis of an extended adipate molecule; the intercarboxylate distance for adipate is given as 6.87 Å in Table 1-1. However, the cationic groups need not be the same distance apart as the carboxylate groups, and not only is the distance important but the allowed configuration of the methylene chain to interact maximally with the enzyme. As Mason points out, the data from the phthalates do not support this distance entirely, since terephthalate inhibits least of the three isomers and its intercarboxylate distance is the closest to that of adipate. The intercarboxylate distance in isophthalate is 5.84 Å (Table 1-1) and this might indicate that the cationic groups are closer than might be expected from the data on the flexible dicarboxylates, but again there is the problem of the orientation of the benzene ring. The importance of van der Waals' interactions is shown by the increasing inhibition given by the higher fatty acids, and the greater inhibition by the alkyl-substituted glu-

TABLE 2-40

INHIBITION OF KYNURENINE TRANSAMINASE BY MONO- AND DICARBOXYLATES[a]

Inhibitor	Concentration (mM)	% Inhibition	Relative $-\Delta F$ of binding (kcal/mole)
Alkyl monocarboxylates			
Formate	6	0	<1.43
Acetate	6	0	<1.43
Propionate	6	0	<1.43
Butyrate	6	0	<1.43
Valerate	6	0	<1.43
Caproate	6	1	1.43
Heptanoate	6	8	2.75
Caprylate	6	15	3.19
Nonanoate	6	24	3.55
Caprate	6	41	4.04
Straight-chain dicarboxylates			
Oxalate	6	0	<1.43
Malonate	6	0	<1.43
Succinate	6	0	<1.43
Glutarate	6	8	2.75
Adipate	6	65	4.64
Pimelate	6	30	3.74
Suberate	6	2	1.86
Azelaeate	6	35	3.88
Sebacate	6	65	4.64
1,10-Decanedicarboxylate	3	74	5.33
1,11-Undecanedicarboxylate	3	78	5.47
1,14-Tetradecanedicarboxylate	3	87	5.86
Cyclic monocarboxylates			
Benzoate	6	0	<1.43
γ-Phenylbutyrate	6	9	2.91
Cyclohexanecarboxylate	6	0	<1.43
γ-Cyclohexanebutyrate	6	64	4.62
Cyclic dicarboxylates			
o-Phthalate	12	14	2.72
Isophthalate	12	35	3.45
Terephthalate	12	9	2.48
Cyclohexane-1,2-dicarboxylate	6	9	2.91
Glutarate derivatives			
2-Methylglutarate	6	15	3.19
2,2-Dimethylglutarate	6	35	3.88
2,4-Dimethylglutarate	6	33	3.82
3-Methylglutarate	6	47	4.18
3,3-Dimethylglutarate	6	56	4.41
3-Methyl-3-ethylglutarate	6	39	3.98
3,3-Diethylglutarate	6	20	3.40
β-Ketoglutarate	6	0	<1.43
2,2-Dimethylsuccinate	6	2	1.86

[a] Kynurenine was 3.7 mM and α-ketoglutarate was 6 mM; pH = 6.3. (From Mason, 1959.)

tarates compared to glutarate. One of the cationic groups on the enzyme seems to have a pK_a around 6.7, since the inhibition by the dicarboxylates decreases from pH 5.5 to 8.5 and approaches that of the monocarboxylates (Fig. 2-22). The increase in inhibition of the dicarboxylates with longer chain lengths than suberate is explained by the ability of the flexible hydrocarbon portions to orient for effective interaction with the enzyme surface between or around the cationic groups. The contribution of a methylene group to the binding is around 0.2–0.4 kcal/mole. It is likely that a

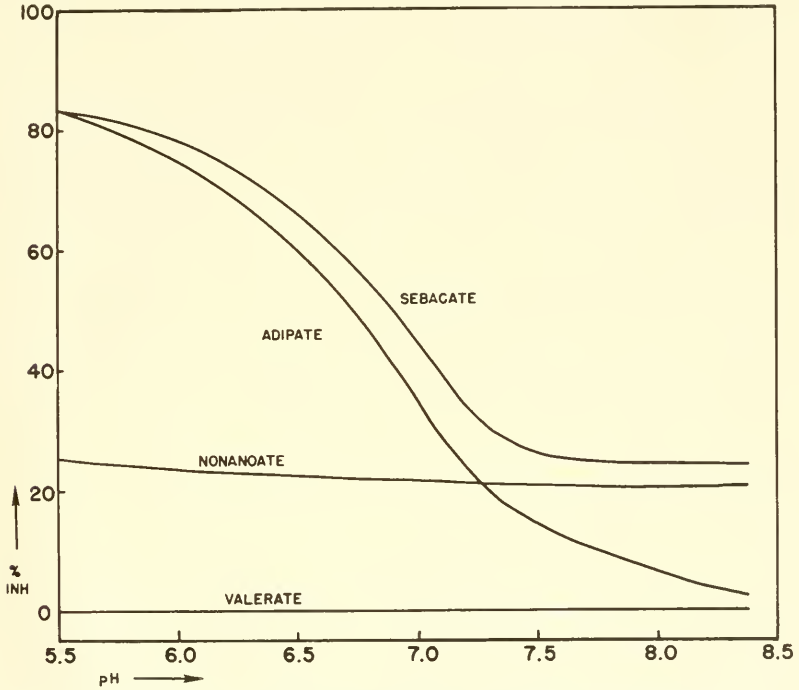


FIG. 2-22. Effects of pH on the inhibitions of kynurenine transaminase by various fatty acid anions at 6 mM. (From Mason, 1959.)

hydrophobic region of the enzyme lies at some distance from the cationic groups since the fatty acids do not begin to inhibit until the chain length reaches 5 or 6 carbon atoms, and the marked differences between the binding energies of benzoate and γ-phenylbutyrate, and cyclohexanecarboxylate and γ-cyclohexanebutyrate, indicate that the ring interaction is effective when the ring is separated from the carboxylate group by several angstroms. However, these differences may be due more to steric factors, a ring close to the carboxylate group interfering with its electrostatic interaction. Indeed, there seems to be a region for van der Waals' interactions between the

two cationic groups on the enzyme, since the 3-substituted glutarates are bound more tightly than the 2-substituted isomers.

The administration of nicotinylalanine to rats leads to a 4-fold increase in the urinary level of *N*-methylnicotinamide (Decker *et al.*, 1963). Nicotinylalanine is possibly formed from tryptophan through 3-hydroxykynurenine but studies with tryptophan-C^{14} indicate it not to be a metabolite but actually a strong inhibitor of kynureninase and kynurenine hydroxylase. This inhibition presumably occurs *in vivo*, resulting in a sequential block in the major route of kynurenine degradation. The effect of this analog on the transaminase is not known.

Inhibition of Urease by Methylurea and Thiourea

There is a good deal of disagreement on the analog inhibitions of urease and the kinetics are certainly not simple. Takeuchi (1933) originally found very little inhibition (perhaps 5%) by 83 m*M* methylurea (although marked inhibition by oxyurea — which may be $H_2N—CO—NH_2OH$ — occurs, this is probably not competitive). Sophianopoulos and Corley (1959) obtained competitive inhibition by methylurea at lower concentrations (unspecified), but higher concentrations (300–1000 m*M*) increase the substrate inhibition produced by urea; these latter effects may be related to enzyme denaturation. The inhibition was found to depend on the pH by Shaw and Raval (1961), it being noncompetitive between pH 7 and 8.9, and competitive below pH 7. Furthermore, the kinetics correspond to the reaction of 2 molecules of methylurea with the active center, which may relate this type of inhibition to substrate inhibition.

Thiourea was claimed to stimulate urease at 5 m*M* (Sizer and Tytell, 1941), to have no effect below 50 m*M*, and to inhibit 35% at 500 m*M* (Kistiakowsky and Shaw, 1953). This inhibition is completely reversible and occurs rapidly. At pH 6 the inhibition is competitive but as the pH is raised, deviation occurs. The kinetics again point to 2 molecules of thiourea reacting with the enzyme. The reaction

$$E + 2\,I \rightarrow EI_2$$

is not affected by change of pH, whereas the reaction

$$ES + 2\,I \rightarrow ESI_2$$

is sensitive to pH, which serves to explain the change in inhibition type with the pH. Lister (1956) reported that of the 17 urea analogs tested, only thiourea is inhibitory — 35% at 200 m*M*, 70% at 400 m*M*, and 85% at 1000 m*M*, when urea is 500 m*M*. The inhibition is prevented by cysteine, which brings up the possibility of disulfide bond formation by thiourea at high concentrations.

Inhibition of Catechol-*O*-methyltransferase by Pyrogallol

This inhibition is of interest because of the bearing it has on the metabolism of epinephrine and norepinephrine. Bacq (1936) observed that pyrogallol increases the responses of tissues to sympathetic nerve stimulation and to epinephrine. However, he then attributed this action to the antioxidant properties of pyrogallol. Lembeck and Resch (1960) and Vanov (1962) have recently confirmed this by showing that the pressor response to epinephrine is prolonged by pyrogallol. The inhibition of the catechol-*O*-methyltransferase was reported by Bacq *et al.* (1959), who believed that this could explain the sensitization of smooth muscles to the catecholamines by pyrogallol and other phenolic compounds. Axelrod and Laroche (1959) also found a potent inhibition of this enzyme (50% when pyrogallol = epinephrine = 0.01 m*M*), which decreases with increasing substrate concentration, indicating a competitive action. Furthermore, about 70% of intravenously injected epinephrine-H^3 is metabolized in 10 min in mice, but pretreatment with 100 mg/kg pyrogallol reduces the amount metabolized to 22%. The half-life of norepinephrine in mice is increased from 22 to 42 min by 10 mg pyrogallol, while at the same time *O*-methylation is inhibited 99%, indicating other pathways for norepinephrine metabolism (Udenfriend *et al.*, 1959). Probably the monoamine oxidase pathway is also important. The administration of pyrogallol to rats does not by itself increase brain norepinephrine levels, but in conjunction with iproniazid (which inhibits monoamine oxidase) it does, in this case the two major degradative pathways being blocked (Jäättelä and Paasonen, 1961). This is a good example of the action of two inhibitors on a divergent multienzyme system and, in addition, has interesting possibilities for clinical application.

Repeated administration of pyrogallol causes a rise in the blood pressure but this is soon followed by a loss of response or tachyphylaxis (Wylie *et al.*, 1960). The rate of urinary excretion of *O*-methylated derivatives of the catecholamines is briefly decreased by pyrogallol, but if the administration is continued the rate returns to normal (Nukada *et al.*, 1962). Long-term treatment with pyrogallol leads to an increase in *O*-methyltransferase and monoamine oxidase in the liver of rats, so it may well be that these enzymes are adaptively altered. The urinary excretion changes of the catecholamines and their *O*-methylated products are shown in Fig. 2-23.

The kinetics of the *in vivo* inhibition have been studied by Crout (1961). Inhibition of *O*-methyltransferase occurs very rapidly in liver, heart, and brain even when the pyrogallol is injected intraperitoneally, and by 30 min has developed appreciably. The inhibition of the enzyme obtained from rat tissues, however, is only partly competitive (actually the curves appear to indicate pure noncompetitive inhibition) despite the fact that pyrogallol is a substrate for the enzyme. The K_i of 0.008 m*M* for pyrogallol indicates the high potency of the inhibition (K_m for norepinephrine is 0.3 m*M*). Fur-

ther work on the exact mechanism of this inhibition might provide interesting information. Wylie *et al.* (1960) found pyrogallol to be the most potent inhibitor of a series of polyphenols and epinephrine analogs, inhibiting 50% at 0.03 mM when epinephrine is 0.3 mM. However, gallate, adrenalone, and arterenone are almost as active. A new inhibitor of *O*-methyltransferase was studied briefly by D'Iorio and Mavrides (1963). This is 3.5-diiodo-4-hydroxybenzoate and it inhibits the rat liver enzyme competitively with $K_i = 0.013$ mM.

Inhibition of the Oxidation of Aromatic Compounds by Bacteria

The metabolism of *o*-nitro- and *p*-nitrobenzoate by *Nocardia* is quite strongly inhibited by *m*-nitrobenzoate, *o*-nitrophenol, and *p*-nitrophenol (Cain, 1958). The competitive nature of this interference was demonstrated by reciprocal plotting. The enzymes involved here are not well characterized and, indeed, the action could be on a transport mechanism at the mem-

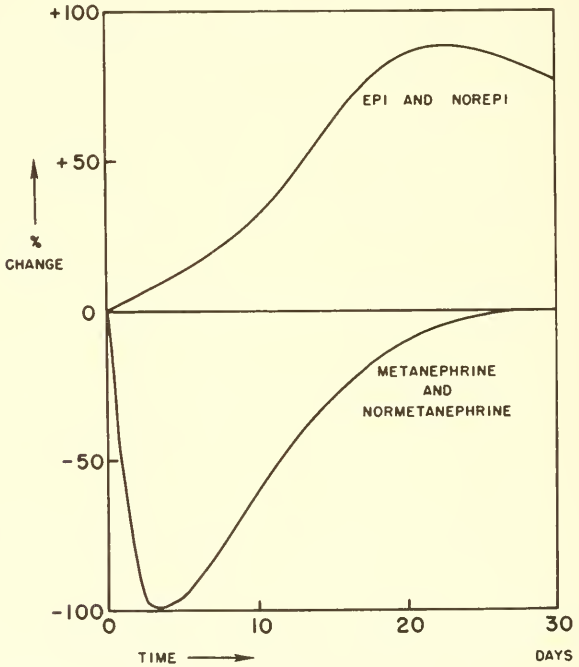


Fig. 2-23. Effects of pyrogallol injected subcutaneously at a dose of 20 mg/kg on the urinary excretion of free and methylated catecholamines by rabbits. (From Nukada *et al.*, 1962.)

brane. Durham and Hubbard (1959, 1960) favor competition for a transport system in the inhibition by *p*-aminosalicylate of the oxidative assimilation of *p*-aminobenzoate in *Flavobacterium*. In the presence of *p*-aminosalicylate there is more *p*-aminobenzoate remaining in the medium and almost complete inhibition of uptake is seen at a *p*-AS/*p*-AB ratio of 10. *p*-Aminobenzoate may be not only a necessary metabolite for folate synthesis, but also the principal source of energy for growth. It is very difficult in such cases to determine whether the inhibition is on a surface transport or an intracellular enzyme until the enzymes responsible for the metabolism have been isolated and examined.

Inhibition of Acetate and Fat Metabolism by Propionate

The original work in this field was done by Jowett and Quastel (1935 a,b). The transformation of butyrate into acetoacetate in guinea pig liver slices is inhibited strongly by benzoate, β-phenylpropionate, and cinnamate. Propionate also inhibits but more weakly (59% at 10 m*M*). Much later Felts *et al.* (1956) reported that 4 m*M* propionate almost completely depresses the incorporation of acetate-1-C^{14} into fatty acids in rat liver slices. The formation of $C^{14}O_2$ is also suppressed. Propionate is known to be inhibitory to the growth of many bacteria and fungi, so the question of the mechanism of its action is of some importance. It has often been attributed to a combination with and depletion of coenzyme A. This inhibition has been studied most thoroughly by Pennington (1956, 1957), who found that the reaction acetate-1-$C^{14} \rightarrow C^{14}O_2$ in rat liver can be inhibited readily and almost completely, while simultaneously the total amount of acetate disappearing is reduced. This also occurs in kidney, heart, and diaphragm, but to a lesser extent. Even concentrations as low as 0.5 m*M* are 40% inhibitory in the liver. It was felt that propionate blocks both the uptake of acetate and the formation of acetyl-CoA. The oxidation of pyruvate is inhibited much less and that of butyrate not at all. However, most of the action must be on the intracellular metabolism inasmuch as marked inhibition is seen in liver homogenates (Pennington and Appleton, 1958). Addition of coenzyme A in the presence of propionate increases the amount of CO_2 formed from acetate slightly but does not reverse the inhibition, indicating that a simple depletion of coenzyme A is not the mechanism. It was postulated that perhaps propionate inhibits after being metabolically altered, possibly to propionyl-CoA, or directly inhibits acetyl-CoA synthetase. This is an interesting and metabolically important inhibition so that one looks forward to studies on the enzymes involved in acetate metabolism.

A few instances of the inhibition of acyl-CoA metabolism by analogs have been reported. Tetrolyl-CoA and propiolyl-CoA, the acetylenic analogs of butyryl-CoA and propionyl-CoA, respectively, are potent noncompetitive inhibitors of fatty acid synthesis in brain and liver extracts (Brady, 1963;

Robinson *et al.*, 1963). The mechanism of inhibition here, however, appears to be by reaction with enzyme SH groups. The coenzyme A is necessary since free tetrolate does not inhibit, and is split off during the reaction. Palmityl-CoA noncompetitively inhibits the condensing enzyme with respect to acetyl-CoA, and perhaps competitively with respect to oxalacetate (Wieland and Weiss, 1963). The inhibition develops slowly and the authors suggest configurational changes in the enzyme, although there is no direct evidence for this. Such an inhibition might be regulatory with regard to the operation of the cycle and the formation of acetoacetate in the liver by controlling the rate of oxidation of acetyl-CoA through the cycle. Higher acyl-CoA's inhibit rat liver acetyl-CoA carboxylase very strongly, oleyl-CoA and stearyl-CoA being competitive with K_i's of 0.0013 mM and 0.00071 mM, respectively, this possibly playing an important role in the homeostatic control of fatty acid synthesis (Bortz and Lynen, 1963).

Inhibition of cholesterol biosynthesis will be discussed in a subsequent chapter, but it is worthwhile mentioning at this point that α-phenyl-n-butyrate not only lowers serum cholesterol but also inhibits the incorporation of acetate into fatty acids, whereas the oxidation of acetate is only weakly inhibited (Steinberg and Fredrickson, 1955). The evidence points to an action early in acetate metabolism, possibly the acetylation of coenzyme A or transacetylations from acetyl-CoA.

Antagonism between Tungstate and Molybdate

Molybdate is a necessary cofactor in the growth of many organisms and has been found to participate in certain enzyme reactions, such as those catalyzed by xanthine oxidase and nitrate reductase. If chicks are fed on a low-Mo diet containing 4.5-9.4% mg sodium tungstate, the growth rates are depressed and signs of molybdenum deficiency appear (Higgins *et al.*, 1956 a). The levels of molybdenum in the tissues fall to less than 10% of the normal and xanthine oxidase activity is severely depressed, leading to an alteration in the excretory pattern of purines. These changes are reversed by adding 2–6 mg% sodium molybdate to the diet. Similar falls in xanthine oxidase were observed in rats.

Aspergillus niger requires molybdate especially when nitrate is the sole source of nitrogen, since the enzymic reduction of nitrate by nitrate reductase involves molybdate as a prosthetic group (Higgins *et al.*, 1956 b). Tungstate is able to compete with molybdate and inhibition of growth occurs when the (tungstate)/(molybdate) ratio is 20. *Azotobacter vinelandii* is likewise inhibited by tungstate when nitrogen or nitrate is the source of amino acids and proteins, but not when ammonia is provided. The uptake of Mo^{99} by the cells is also depressed by tungstate. The ability of tungstate to inhibit growth is dependent on the level of molybdate in the medium and it requires rather high ratios of (tungstate)/(molybdate) to inhibit well (Bulen, 1961).

The question of the site, or sites, of tungstate inhibition is not yet settled. Bulen believes that the primary effect is a depression of molybdate uptake and has provided evidence that that there is no antagonism of the enzymically functioning molybdate. The growth of the crown-gall organism *Agrobacterium tumefaciens* is also inhibited in nitrate medium (about 50% at 0.05 m*M*), but when ammonia is added there is only slight inhibition at 1 m*M* (Kurup and Vaidyanathan, 1963). Molybdate antagonizes the growth depression. A decrease in nitrate reductase activity during the inhibition was observed. Before the problem of the site of inhibition can be finally settled, more work must be done on isolated molybdenum-dependent enzymes. The NADH-dependent nitrate reductase from wheat is not inhibited by 1 m*M* tungstate (Spencer, 1959). In any event, this represents a unique type of competitive inhibition which is basically due to the similar structures and properties of tungstate and molybdate.

Inhibition of Penicillinases

Some of the data are summarized in Table 2-38, and it is evident that the penicillinases from different bacteria exhibit various patterns of sensitivity. In particular, the gram-negative and gram-positive organisms possess different types of enzyme, the former usually being more sensitive to penicillin analogs. The inhibitions are generally competitive. The exact mechanism of the inhibition, however, is not clear, since there is some evidence that the analogs alter the configuration of the enzyme (Garber and Citri, 1962; Citri and Garber, 1963). In the first place, the analogs accelerate the temperature inactivation of penicillinase and, in the second place, the inhibition by 6-(2,6-dimethoxybenzamido)penicillanate is accompanied by the appearance of groups sensitive to iodine. These penicillinase inhibitors are of use in determining the mechanism of penicillin resistance in bacteria; if the resistance is due to the increased synthesis of penicillinase, the inhibitor will abolish the resistance. (Hamilton-Miller *et al.*, 1964).

Inhibition of Cycle Enzymes by γ-Hydroxy-α-ketoglutarate

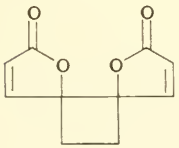
Glyoxylate and oxalacetate condense under physiological conditions to form a product which inhibits certain steps in the tricarboxylate cycle. The reaction is quite rapid at 20° and pH 7.4, and since both glyoxylate and oxalacetate are produced normally in cells, it was of some interest to study the properties of this condensation product, which was considered to be α-hydroxy-β-oxalosuccinate by Ruffo *et al.* (1962 a). It was found to be competitive with respect to citrate and *cis*-aconitate, and to inhibit 50% at concentrations around 0.12 m*M*. When glyoxylate is added to mitochondria there is respiratory inhibition and some accumulation of citrate if oxalacetate is present, which is due to the inhibition of aconitase and

isocitrate dehydrogenase (Ruffo *et al.*, 1962 b; Ruffo and Adinolfi, 1963). If no oxalacetate is present, glyoxylate directly inhibits the oxidations of α-ketoglutarate and succinate, so that two sites of cycle inhibition can occur, one by glyoxylate alone and one by its condensation product with oxalacetate. Payes and Laties (1963) have claimed that the α-hydroxy-β-oxalosuccinate (oxalomalate) initially formed in the condensation reaction is rapidly decarboxylated to γ-hydroxy-α-ketoglutarate, which is the actual inhibitor. γ-Hydroxy-α-ketoglutarate competitively inhibits yeast aconitase ($K_i = 0.14$ mM), potato α-ketoglutarate dehydrogenase ($K_i = 0.7$ mM), and isocitrate dehydrogenase. It is also moderately inhibitory to the respiration of potato slices, 35% depression being observed with 2 mM and 71% with 5 mM. It is rather surprising that it does not serve as a substrate for α-ketoglutarate dehydrogenase and enter into the sequence of reactions involving coenzyme A, as does γ-methyl-γ-hydroxy-α-ketoglutarate (parapyruvate).

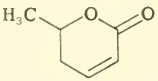
CHAPTER 3

DEHYDROACETATE

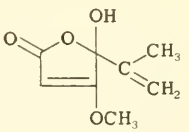
Since this substance inhibits succinate dehydrogenase and may exert some of its effects because of a structure analogous to certain cellular metabolites, it is appropriate to discuss it at this time, although little of its basic metabolic effects is understood. In a study of the formation of ethyl acetoacetate, Geuther (1866) found that distillation of this substance yielded a crystalline material, to which he gave the name dehydroacetic acid. It was soon shown to contain a pyran ring structure (Feist, 1890) and has been used extensively in organic preparative procedures. The effects on biological systems were not studied until Brodersen and Kjaer (1946) in Copenhagen investigated a series of unsaturated lactones for antibacterial activity. They reasoned that several such compounds are antimicrobial — anemonin from species of Ranunculaceae, parasorbic acid from the mountain ash (*Sorbus*), Dicumarol from sweet clover, penicillic acid from certain species of *Penicillium*, kojic acid from *Aspergillus*, patulin (clavacin) from various fungi, and others — and that a correlation between activity and the —O—CO—C=C— structure might exist:



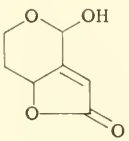
Anemonin



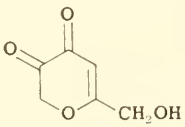
Parasorbic acid



Penicillic acid



Patulin

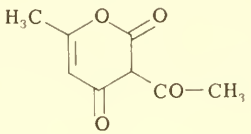


Kojic acid

Dehydroacetic acid was found to be rather inactive against most of the 25 types of bacteria studied and no further mention was made of it. McGowan *et al.* (1948) investigated 80 compounds with ethylenic linkages for the purpose of correlating fungistatic activity with the ability of the substituents to withdraw electrons from these double bonds, and found dehydroacetate to exert very little effect. An investigation stimulated by the antibacterial effects of usnic acid from lichens led Ukita *et al.* (1949) to examine dehydroacetate, which they found to inhibit staphylococci and mycobacteria slightly; however, other substances were more potent and of greater interest. The Dow Chemical Company meanwhile had been studying the antimicrobial action of dehydroacetate and on June 28, 1949 issued three patents for its use in food preservation.* These were based on work started in 1946 in cooperation with the Department of Pharmacology of the University of Michigan Medical School, the results of which were published in a series of papers in 1950. Almost all of our present basic knowledge of dehydroacetate stems from this work and essentially no fundamental biochemical reports have been made since, although a great many papers on its practical use appear annually.

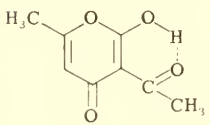
CHEMICAL PROPERTIES

The structure of dehydroacetic acid was debated for many years until Rassweiler and Adams (1924) proved that the formula suggested by Feist (1890) is basically correct. The dipole moment of 2.83 was claimed by Le-

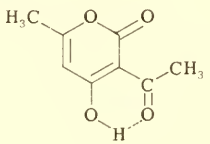


Dehydroacetic acid

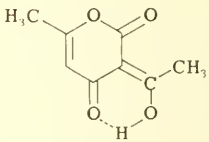
Fevre and LeFevre (1937) to be consistent with this structure if restrictions were imposed on the rotation of the acetyl group. The formula is commonly written in the keto form, but it is likely that a tautomeric equilibrium with the following enolic forms occurs:



(a)



(b)



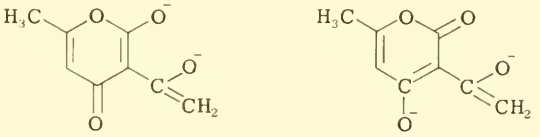
(c)

* Dehydroacetate is actually a rather weak inhibitor of microbial growth but can be used to preserve food because it is so little toxic to humans.

Hydrogen bonding of the type suggested by Forsén and Nilsson (1961) is represented; this undoubtedly occurs in the pure form but may not in aqueous solution. Proton magnetic resonance and infrared spectra suggest structure (b), but the other forms are not excluded. The restriction of the acetyl group rotation may derive from such hydrogen bonding.

Ionization

It is important with regard to the action of dehydroacetate on enzymes and its penetration into cells to determine the predominant forms in aqueous solution at physiological pH. It is usually stated that dehydroacetic acid is a very weak acid, and Wolf and Westveer (1950) remarked that it would exist primarily in the ionized state at pH 9. The acidic property is the result of enolization and each of the enolic forms shown above could lose a proton to form the corresponding anion. However, once this occurs, resonance between the three forms is possible, stabilizing the anion and increasing the acidity and, furthermore, producing a more diffuse negative charge. In order to determine the state of the inhibitor under physiological conditions, titrations were done, starting at pH 3.64 (the pH of a saturated solution). It was found that two equivalents of base are taken up between pH 4.2 and 6.4, with a mean pK_a of 5.20, so that around pH 7 dianions of the α-pyran and γ-pyrone type must be present.



General Properties

Dehydroacetic acid has an absorption peak around 313 mμ (Calvin *et al.*, 1941), sublimes at 109°, is fairly soluble in organic solvents but poorly soluble in water (around 0.25% at 37°). The sodium salt, however, is quite soluble in water (33%) (Wolf, 1950) and sufficiently stable in solution for most purposes. It may be catalytically hydrogenated with PtO_2 to give the corresponding 3-ethyl compound (Malachowski and Wanczura, 1933), or with Ni under pressure to yield more completely hydrogenated forms, corresponding to the uptake of 3–5 moles of H_2 (Adkins *et al.*, 1931).

Synthesis

Dehydroacetic acid was first obtained by Geuther (1866) by distilling the ethyl ester of acetoacetate, and Conrad (1874) found that a reasonable yield could be obtained by heating this substance under pressure. The present

method of synthesis involves heating ethyl acetoacetate with a small amount of $NaHCO_3$ at 200°–210° for 7–8 hr with subsequent distillation at 128°–140° *in vacuo*, the yield being over 50% (Arndt, 1955). It is also formed by the tetramerization of ketene, and by suitable catalytic means may be almost quantitatively obtained from diketene (Steele *et al.*, 1949), the reaction presumably proceeding through the enolized forms of the diketene. The simplest method of purification of dehydroacetic acid is probably recrystallization from ethanol.

Estimation

Two methods suitable for tissue analyses were developed by Woods *et al.* (1950). A colorimetric test, based on the reaction of the acetyl group with salicylaldehyde in alkaline solution to give a red-orange color, is sensitive in the range 10–200 μg. The spectrophotometric test, based on absorption at 312 mμ, is approximately 10-fold more sensitive. Both tests depend on the proper pretreatment and extraction of the tissue samples, since neither test is particularly specific. The spectrophotometric tests give the best recoveries and are preferable for most analyses.

INHIBITION OF ENZYMES

Dehydroacetate at concentrations between 2.3 and 93 mM progressively depresses the oxygen uptake of slices and minces of cerebral cortex and kidney respiring endogenously, but the excess oxygen uptake following addition of various substrates is inhibited only in the case of succinate (Seevers *et al.*, 1950). This suggested that dehydroacetate might inhibit succinate oxidase and thus this enzyme was examined in some detail.

Succinate Oxidase

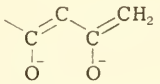
Inhibition of succinate oxidase is proportional to the logarithm of the dehydroacetate concentration from 27 to 77% inhibition (Seevers *et al.*, 1950). Although no data were given, it was stated that the substrate concentration has little or no effect on the degree of inhibition, indicating it is not competitive. If this is true (see page 621), K_i would be around 11.6 mM indicating much less affinity of the enzyme for dehydroacetate than for malonate. The site of inhibition in the succinate oxidase sequence was determined in two types of experiment. In the first, the cytochrome system was blocked by cyanide and cresyl blue added as a hydrogen carrier; dehydroacetate inhibited this system to the same degree as the normal one, indicating the action not to be on the cytochrome system (see accompanying tabulation). In the second, the cytochrome system was studied directly and no inhibition

Cyanide (mM)	Cresyl blue (mM)	Dehydroacetate (mM)	O_2 Uptake (μl/30 min)	% Inhibition by dehydroacetate
—	—	—	165	—
2	—	—	0	—
2	6	—	88.5	—
—	—	9.3	84	49
2	6	9.3	43.5	51

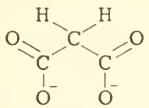
by dehydroacetate was observed. It was concluded that dehydroacetate acts on succinate dehydrogenase. Although this is probably true it is evident that, according to the modern elaboration of succinate oxidase, other sites are possible.

Evidence that dehydroacetate does not inhibit by reacting with the SH groups of succinate dehydrogenase was obtained. First, the inhibition is almost instantaneous and readily reversible, unlike inhibitions with most SH reagents. Second, cysteine, glutathione, and dimercaprol are unable to protect the enzyme against dehydroacetate.* Third, no inhibition of urease was observed, this enzyme being sensitive to most SH reagents; indeed stimulation was observed. All in all, one must conclude that the possibility of the reaction of dehydroacetate with SH groups has not been eliminated, although there is little positive evidence for such a mechanism.

The possibility of competitive inhibition of succinate dehydrogenase cannot be eliminated since no data were given for the statement, "Increasing the substrate concentration does not appreciably alter the degree of inhibition." If, for example, a succinate concentration of 50 mM was used (which is the only concentration mentioned in the paper) with dehydroacetate at 9.3 mM, increasing the succinate to 100 or 200 mM would not be expected to reduce the inhibition markedly. It is interesting to speculate that the dianionic forms of dehydroacetate have a basically similar charge distribution to malonate. However, due to resonance the charge magnitude



Dehydroacetate



Malonate

* Unfortunately the cysteine and glutathione were used at only about one fifth the dehydroacetate concentration, so that even total reaction of the inhibitor would have reduced the inhibition relatively little, actually about 5%. Cysteine reduced the inhibition around 5% from the predicted value but glutathione did not. Cavallito and Haskell (1945) mentioned that dehydroacetate does not react with cysteine.

on dehydroacetate might be lower than on malonate. The succinate dehydrogenase from calf thymus nuclei is inhibited somewhat more potently by dehydroacetate at pH 6.6 than at pH 7.6 (see accompanying tabulation)

Dehydroacetate (mM)	% Inhibition	
	pH 6.6	pH 7.6
1	33	25
5	56	42
10	67	47

(McEwen *et al.*, 1963 a). This does not fit the dianion inhibition theory very well, since at pH 7.6 there should be more of the dianion than at pH 6.6. One cannot attribute the change in inhibition to the ionization of enzyme groups because malonate inhibits better at the higher pH from the limited data provided. Unfortunately the succinate concentration was unvaried from 20 mM and the formal nature of the inhibition remains unknown. The mechanism of the inhibition of succinate dehydrogenase is thus at present unsolved.

One further experiment deserves brief mention. It was claimed that although malonate protects succinate oxidase from SH reagents, it does not protect against dehydroacetate (see accompanying tabulation). First, one

Dehydroacetate (mM)	Malonate (mM)	% Inhibition of O_2 uptake
9.3	—	49
—	0.33	33
9.3	0.33	65

would not expect malonate at a concentration inhibiting only 33% to protect very much. Second, the inhibition given by both inhibitors is exactly what would be predicted if both acted at the same site on the enzyme. No conclusions as to the mechanism of inhibition can be deduced from this experiment.

Other Enzymes

Dehydroacetate has no effect on cholinesterase up to 20 mM (Seevers *et al.*, 1950), or on pepsin, amylase, and trypsin at 8.5 mM (Bauer and La Sala, 1956), while urease is stimulated by concentrations up to 93 mM and pan-

creatic lipase is stimulated at 8.5 mM. The ATPase of pea mitochondria is also stimulated by dehydroacetate, phosphate splitting being increased around 200% by 1 mM (Forti, 1957), which might be related to the uncoupling action reported by Marré *et al.* (1956). Catalase is inhibited weakly ($K_i = 22$ mM) by dehydroacetate compared with other organic acids (Lück, 1957). The enzymes responsible for the destruction of mitomycin in *Streptomyces* mycelia are not affected by 4.8 mM dehydroacetate (Gourevitch *et al.*, 1961). It is difficult to explain some of the actions on metabolism with this limited amount of information.

EFFECTS ON RESPIRATION AND GLYCOLYSIS

The effects of dehydroacetate on the endogenous respiration of minces of various rat tissues are shown in Fig. 3-1. Brain and kidney respiration is progressively depressed but muscle is anomalous in that marked stimulation is observed at high dehydroacetate concentrations, while liver is stimulated

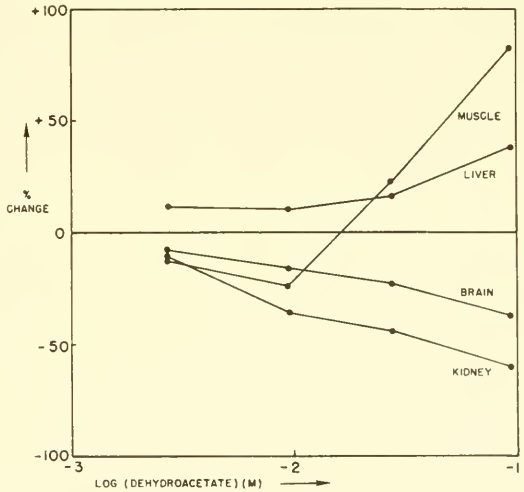


FIG. 3-1. Effects of dehydroacetate on the respiration of rat tissue minces measured over a period of 2 hr. (From Seevers *et al.*, 1950.)

moderately at all concentrations used. Brain slices respond as do the minces, but liver slices are unaffected by dehydroacetate up to 9.3 mM and are then depressed at higher concentrations. Mudge (1951) found 10 mM dehydroacetate to depress rabbit kidney slice respiration 25%, which is similar to the inhibition reported by Seevers *et al.* (1950) in rat kidney. The stimulation of respiration in muscle and liver might result from metabolism

of dehydroacetate; this will be discussed later (page 629), but Shideman *et al.* (1950 b) do not think it is likely. The endogenous respiration in such minces and slices is poorly understood, so that it is difficult to interpret these results, and the data when glucose is present are inconsistent. It is also difficult to relate these changes in respiration to inhibition of succinate oxidase (the effects of malonate and dehydroacetate are quite different), and such statements as "It appears probable that the manifestations of toxicity result largely, if not exclusively, from a specific type of chemical (or physicochemical) action involving interference with oxidative or other enzyme mechanisms which proceed by way of the Krebs cycle" (Shideman *et al.*, 1950 b) appear to have little basis, particularly since the effects of dehydroacetate on the operation of the cycle (as in mitochondrial preparations) have not been studied. It is significant that dehydroacetate at 4.7–9.3 m*M* stimulates the anaerobic glycolysis in rat brain mince 40–50% (Seevers *et al.*, 1950), an effect greater than any observed on respiration. On the other hand, 50 m*M* dehydroacetate inhibits the formation of $C^{14}O_2$ from glucose-6-C^{14} 87% in suspensions of isolated thymus nuclei, simultaneously the O_2 uptake being depressed only 14% and the ATP level falling 27% (McEwen *et al.*, 1963 b). Malonate at 10 m*M* has very little effect and this was attributed to a failure to penetrate into the nuclei; dehydroacetate either penetrates better than malonate or exerts an effect other than inhibition of succinate oxidation.

EFFECTS ON TISSUE FUNCTIONS

Dehydroacetate in the whole animal produces changes in central nervous system, cardiovascular, and renal functions. Only the renal effects have been investigated in detail. In addition, the actions on the isolated intestine have been studied relative to the metabolic disturbances produced.

Intestine

The contractile amplitude of isolated rabbit intestine is depressed slightly by 1 m*M* and markedly by 10 m*M* dehydroacetate (see tabulation below)

Dehydroacetate (m*M*)	Substrate	% Inhibition of amplitude
1	Acetate	2
1	Glucose	8
10	Acetate	72
10	Glucose	54

(Weeks *et al.*, 1950). When the intestine is allowed to contract for 30–60 min in the absence of substrate the amplitude is reduced to 15–35% of normal. The addition of glucose, acetate, or pyruvate allows recovery, essentially complete in the case of glucose. Dehydroacetate at 10 m*M* effectively prevents this recovery with acetate and pyruvate, but only partially counteracts the effect of glucose. This was taken to mean that dehydroacetate blocks the cycle preferentially. Malonate neither depresses the amplitude in the presence of substrates, nor prevents the recovery of substrate-depleted intestine upon addition of substrates. This could be related to the poor penetration of malonate into the cells. Dehydroacetate, even though it may have a double negative charge, might penetrate better than malonate because the lipophilic fraction of the molecule is greater (sodium dehydroacetate is fairly soluble in a number of organic solvents).

Heart

Dehydroacetate given intravenously to dogs causes some slowing of the heart rate at a dose of 300 mg/kg (Seevers *et al.*, 1950), but in general the effects on the cardiovascular system are minimal. Like many substances with the —CH=CH—CO— grouping, dehydroacetate exerts a positive inotropic action on hypodynamic cat papillary (Bennett *et al.*, 1958). Although not so potent as many other compounds, it is active at 5 m*M* and rated the same as β-angelicalactone. Isolated rat atria are depressed quite markedly by 5–10 m*M* dehydroacetate, and to some extent even by 1 m*M*, and this is accompanied by rather unique effects on the membrane potentials (Webb and Hollander, 1959). The resting and action potential magnitudes are reduced more strikingly than with most metabolic inhibitors. However, the action potential duration is actually increased, due to a slowing of repolarization, an effect observed with no other inhibitor. The depolarization rate is unaffected, so that the moderate slowing of conduction noted must be related to the reduced action potential. Malonate at higher concentration scarcely alters the properties of such atria, again indicating a difference either in penetration or in action.

Renal Transport

When dehydroacetate is injected into dogs in amounts sufficient to give plasma concentrations of 20–25 mg% (1.2–1.5 m*M*), the renal tubular transport of certain substances is markedly depressed — *p*-aminohippurate 81%, phenolsulfonphthalein 80%, penicillin-G 60%, and *N*-methylnicotinamide 77%, while the transport of glucose, creatinine, and phosphate is unaltered (Shideman *et al.*, 1950 b). Since diuresis is observed after dehydroacetate, it is likely that water and ion transport is also affected to some extent. It is thus clear that certain transport systems are inhibited and others are

untouched by dehydroacetate. The mechanism is entirely tubular and no changes in femoral flow, renal blood flow, or glomerular filtrate rate occur (Shideman and Rene, 1951 b). The action is similar to that of carinamide *p*-(benzylsulfonamido)benzoate, a previously used blocker of penicillin excretion. Indeed, dehydroacetate at 0.5 g every 6 hr prolongs penicillin blood levels in patients (Schimmel *et al.*, 1956).

The active accumulation of phenolsulfonphthalein (Rathbun and Shideman, 1951), phenol red (Shideman and Rene, 1951 b), and *p*-aminohippurate (Shideman and Rene, 1951 b; Farah and Rennick, 1956) in kidney slices is readily inhibited by dehydroacetate. The effect on *p*-aminohippurate uptake is shown in Fig. 1-18, from which it is seen that 50% inhibition is given by 0.14 m*M* in dog kidney slices. The accumulation of tetraethylammonium ion is completely resistant to dehydroacetate, and it is believed that the transport of this ion is not dependent on the cycle (Farah and Rennick, 1956; Farah, 1957).

It appears that certain renal transport mechanisms are more sensitive to dehydroacetate than any other cell functions examined. The question as to the relation of this inhibition to succinate oxidase or cycle depression is difficult to resolve. Dehydroacetate might block transport by acting directly on the carrier system or by reducing the energy available for the transport. Shideman and Rene (1951 b) incline to the latter view and attribute the inhibition to an action on the cycle. The evidence for this comes partially from the observation that high concentrations of acetate are able to counteract the effects of dehydroacetate, both *in vivo* and in slices (Stoneman *et al.*, 1951; Rathbun and Shideman, 1951; Shideman and Rene, 1951 a). However, the ability of acetate to reverse an inhibition is not evidence for an action on the cyle, much less on succinate oxidase; indeed, the opposite might be justifiably concluded. Furthermore, there is not a good correlation between the activity in depressing *p*-aminohippurate transport and the inhibitory potency on succinate oxidation, especially when carinamide is considered, this substance being a weak succinate oxidase inhibitor but a more potent transport inhibitor than dehydroacetate. Of course, different penetrabilities into the renal cells may account in part for this lack of correlation.

Ion transport in kidney slices is also inhibited by dehydroacetate (Mudge, 1951). Rabbit kidney slices were leached in 0.15 *M* NaCl to lower the intracellular K^+, and then incubated in 10 m*M* K^+ with 10 m*M* acetate as the substrate, during which period the lost K^+ is regained and the excess intracellular Na^+ is pumped out. Dehydroacetate at 10 m*M* inhibits this K^+–Na^+ exchange 81%, while simultaneously the respiration is inhibited only 25%. The action of dehydroacetate on the pH-regulating exchanges of the kidney is not known, but may be important in contributing to the acidosis observed in whole animals, and indirectly in the effects on certain tissues such as the central nervous system.

EFFECTS ON THE WHOLE ANIMAL

The potential use of dehydroacetate as a food preservative led Spencer *et al.* (1950 a,b) of the Dow Chemical Company, and Seevers *et al.* (1950) of the University of Michigan, to investigate the effects on various animals and man when administered by different routes for varying durations of time. These studies were very thorough and our knowledge at this level of action is better than for most inhibitors. We shall summarize only the more important aspects relative to the metabolic disturbances produced.

Acute Toxicity

The earliest evidence of toxicity in rats, dogs, and monkeys when dehydroacetate is given by any route is loss of appetite. As the dosage is increased, various symptoms related mainly to the central nervous system appear: ataxia, salivation, emesis, incoordination, weakness, and stupor, followed by muscle twitching and a gradual increase in muscle tone, passing into clonic and tonic convulsions which persist until death, which is attributed to respiratory paralysis. During the later phases, convulsions may be initiated by excess sensory stimulation, indicating a general increase in the reflex excitability. The effects are produced rather slowly and death occurs usually after 24–72 hr. The acute oral toxicity in rats is given as: $LD_{0.1}$ = 0.52 g/kg, LD_{16} = 0.80 g/kg, LD_{50} = 1.0 g/kg, LD_{84} = 1.23 g/kg, and $LD_{99.9}$ = 1.92 g/kg.

The initial effects of intravenous injection (0.3–0.4 g/kg) are probably related to the temporary disturbance in blood pH if the solutions are not neutralized. The alterations in nervous system function may not be specific and due to the direct action of dehydroacetate on the nerve cells, but dependent on the developing acidosis. An initial respiratory alkalosis in dogs is soon followed by a shift toward metabolic acidosis, compensated at first but later becoming uncompensated. As the plasma pH suddenly drops to levels approaching 7, with simultaneous decreases in plasma bicarbonate and P_{CO_2}, convulsions occur. We have noted that renal transport is more sensitive to dehydroacetate than is nerve or muscle respiration, especially in the presence of glucose. The plasma levels of dehydroacetate in acute poisoning are probably between 1 and 3 m*M* in most cases, which, coupled with the possible concentration in the kidney, could easily disturb renal function seriously. The concentration in the central nervous system during poisoning appears to be quite low (see page 631). All of this points to an indirect action on the nervous system and perhaps a primary renal site for the toxicity.

Chronic Toxicity

Rats fed diets containing 0.02–0.10% dehydroacetic acid for 2 years showed no obvious adverse effects on growth, mortality, hematology, organ

weights, or tissue cytology, with the possible exception of an increase in liver fat at the highest dose level. Monkeys maintained on oral doses of 50–100 mg/kg/day for a year likewise showed no evidence of a toxic effect. Even the maximum tolerated dose of 200 mg/kg/day, producing some toxic effects in the monkeys, produced no pathological changes in the tissues. One must conclude that dehydroacetate is a relatively nontoxic substance. Human subjects can tolerate 14–17 mg/kg/day for 26–48 days (approximately 1.2 g/day or a total of 30–60 g), maintaining a plasma level of 15–25 mg% (around 1 m*M*), and experience only occasional anorexia and nausea. Human subjects are more sensitive to dehydroacetate than are experimental animals on a dosage basis, but the plasma concentrations are approximately the same, indicating that man either metabolizes or excretes dehydroacetate less readily (see accompanying tabulation).

Species	Tolerated levels			Nontolerated levels		
	Dose (mg/kg)	Days	Plasma conc. (mg%)	Dose (mg/kg)	Days	Plasma conc. (mg%)
Rat	50	730	11–17			
Dog	50	199	12–13	60–80	73	20–25
Monkey	50	378	12–16	100	397	20–30
Man	6–13	173	12–17	14–17	26–48	15–25

Urinary Excretion of Succinate

If dehydroacetate is able to inhibit succinate oxidase in the whole animal, one would expect to find an accumulation of succinate in the body and an increased excretion. This was indeed found, providing the best evidence that such enzyme inhibition actually occurs *in vivo*. Rats given 600 mg/kg of dehydroacetic acid orally show an increase in urinary succinate during the first day from a control level of 3.2 mg/day to 24.8 mg/day (Seevers *et al.*, 1950). Dogs given 200 mg/kg orally for 3 days produce a maximal excretion of succinate on the second day (more than 175 mg/day compared to a control of 28 mg/day), and the rise is maintained after cessation of administration, being around 90 mg/day 4 days after the last dose. One would also expect other acids to accumulate if the cycle is depressed and the appearance of ketonemia, but this is not mentioned.

Antidotes

Seevers *et al.* (1950) attempted to combat the toxic effects of dehydroacetate in dogs by the administration of glucose, ammonium lactate, calcium

lactate, ammonium chloride, magnesium sulfate, sodium bicarbonate, and dimercaprol [!], but no protection or benefit was observed, which is not very surprising. It would be interesting to know if either fumarate or malate, the products of succinate oxidation and possible restorers of cycle activity, is effective. Barbital controls the convulsions produced by dehydroacetate and allows recovery from an otherwise fatal dose, indicating that the convulsions must contribute to the death of the animals.

DISTRIBUTION AND METABOLISM

Whether given orally or parenterally, during acute or chronic administration, most of the dehydroacetate seems to be metabolized in the body, since less that 25% is found in the urine (monkeys 10%, dogs 20%, man 22%) and only around 5% in the feces (Shideman *et al.*, 1950 a,b). Only insignificant amounts of conjugated dehydroacetate occur in the urine inasmuch as 2–4% more can be obtained on acid hydrolysis. It was not possible to demonstrate destruction of dehydroacetate by slices of rat liver, kidney, or brain, or in muscle mince in incubations up to 4 hr, although, as pointed out, the analyses may not have been specific enough to have detected certain chemical modifications. No evidence could be found for the appearance of 2,6-dimethyl-1,4-pyrone or 6-methyl-2*H*-pyran-2,4(3*H*)-dione, substances formed in the chemical degradation of dehydroacetate. Nor were positive tests for acetomalonate or acetoacetate, two possible metabolic products, obtained in dog or human urine. The stimulation of liver and muscle respiration by dehydroacetate might indicate metabolism of dehydroacetate in these tissues, but this is not at all certain. However, it has been shown that in animals with carbon tetrachloride liver damage the toxicity of dehydroacetate is increased 29%, pointing to the liver as at least one site for the metabolism.

The oral administration to rats of small doses (60 mg/kg) of dehydroacetate, labeled in four of its carbon atoms, leads after 5 days to the following distribution of the label: urine 23%, feces 19%, carcass 22%, and respiratory CO_2 12.4% (Barman *et al.*, 1961). The urine contains five labeled substances: unchanged dehydroacetate (4.7%), hydroxydehydroacetate (7%), triacetic acid lactone (1.2%), urea (0.2%), and an unknown pyrone metabolite (the figures in parentheses give the percentages of the dose). In addition, the imino derivatives of dehydroacetate and hydroxydehydroacetate are found, since reaction with ammonia occurs in the urine. The major metabolic pathway was postulated to be

Dehydroacetate → hydroxydehydroacetate → triacetic acid lactone → acetoacetate + acetate

The hydroxylation of the 3-$COCH_3$ group occurs in slices of liver, but not

in kidney, muscle, or testis (Barman *et al.*, 1963). The rabbit apparently metabolizes dehydroacetate more readily than the rat. The absence of glucuronides or ethereal sulfates in the urine was confirmed. The urinary pyrone metabolite is probably the 3-carboxylate of triacetic acid lactone.

Dehydroacetate is excreted by the kidney quite slowly. This could be due to the binding of a major fraction in the plasma to protein, or to active resorption by the tubules; both actually occur. The fraction bound to plasma proteins depends on the species and the dehydroacetate concentration (Woods *et al.*, 1950) (some averages are shown in the accompanying tabulation). However, even when the renal excretion is corrected for the amount

Species	Plasma protein (%)	Total plasma dehydroacetate (mg%)	Bound dehydroacetate	
			%	mg/g
Rat	5.58	5.7	90.5	0.93
Dog	4.28	4.0	58.5	0.62
Man	5.15	6.2	98.5	1.20

bound, it is evident that this is not the primary factor in the slow excretion. Dehydroacetate is resorbed through the tubules to about the same extent as water (98–99%). Since the ring structure of dehydroacetate is identical to the pyranose form of glucose, it was felt that transport by the glucose system might occur, but phlorizin, at a concentration that markedly inhibits glucose transport, does not alter dehydroacetate resorption. The relationship between the transport of dehydroacetate and the effects of dehydroacetate on other transport mechanisms, if any, is not clear.

Both dehydroacetic acid and its sodium salt are absorbed rapidly when given orally, peak plasma concentrations occurring in 90–120 min. It can be detected in the blood 3–4 days after single doses, and when administered chronically many days are required for the plasma level to drop to negligible concentrations. The distribution of dehydroacetate in the tissues (Table 3-1) and its variation with time illustrate the complexities of the factors governing penetration and binding. The low concentration in the central nervous system and the relatively high level in spinal fluid are surprising. The biliary circulation of dehydroacetate may play a minor role in retaining it in the body.

Dehydroacetate is secreted in the saliva at a reasonably high concentration, an injection of 5 mg intraperitoneally in the rat giving salivary levels of 0.25–0.33 m*M* at 2–6 hr (Zipkin and McClure, 1958), roughly about half the plasma concentration. Dehydroacetate has been incorporated into toothpastes to inhibit bacterial growth and caries. However, it has been found

TABLE 3-1

DISTRIBUTION OF DEHYDROACETATE IN TISSUES OF THE DOG[a]

Tissue	Dehydroacetate		
	Intravenously (160 mg/kg)		Orally (80 mg/kg/day) for 46 days (mg%)
	At 1 hr (mg%)	At 5 hr (mg%)	
Blood	25	21	18
Kidney	15	3.9	2.4
Intestine	14	11	2.0
Heart	11	0.8	3.1
Spleen	11	0	1.5
Muscle	9.7	2.4	0.8
Liver	6.7	5.5	1.8
Cerebrum	6.6	0	0.9
Lung	6.6	13	3.1
Cerebellum + medulla	1.6	0	0
Bile	0	—	27
Spinal fluid	—	19	16
Colon	—	4.4	4.5

[a] From Woods *et al.* (1950).

that dehydroacetate in the diet of rats markedly potentiates the development of caries (Zipkin and McClure, 1957, 1958). Dehydroacetate at 0.1% in a cariogenic diet or drinking water (corresponding to around 5 mg uptake per day) increases significantly the frequency of caries. It is suspected that the cariogenic action of dehydroacetate may be related to its secretion in the saliva, but the mechanism is unknown.

ANTIMICROBIAL ACTIVITY

Dehydroacetate has been used widely the past few years as a food preservative, especially against molds, and is certainly one of the safest and most effective. This has stimulated extensive work to determine the minimal growth inhibitory concentrations for various microorganisms, some of the results of which are summarized in Table 3-2. Two things are immediately evident from this table. Dehydroacetate is in general a rather weak antimicrobial agent; it is of practical value because of its low toxicity. It

TABLE 3-2

ANTIMICROBIAL ACTIVITY OF DEHYDROACETATE

Organism	Minimal inhibitory concentration (mM)	Reference[a]
Bacteria		
Aerobacter aerogenes	17.8	(6,7)
Alcaligenes faecalis	23.8	(6)
Bacillus anthracis	59.5	(1)
B. cereus	17.8	(6)
B. megaterium	17.8	(6)
B. mesentericus	17.8	(6)
B. subtilis	17.8	(6)
Corynebacterium diphtheriae	3.0	(1)
Escherichia coli	23.8	(6)
Lactobacillus acidophilus	59.5	(2)
L. brevis	12–60	(2)
L. casei	12–24	(2)
L. fermenti	12–60	(2)
L. plantarum	12	(2)
	5.9	(6)
Mycobacterium tuberculosis	5.9	(5)
Pseudomonas aeruginosa	23.8	(6)
Salmonella pullorum	17.8	(6)
Salmonella typhosa	12	(6)
Staphylococcus aureus	108	(4)
	10	(5)
	17.8	(7)
Vibrio cholerae	59.5	(1)
V. metchnikowii	5.9	(1)
Fungi		
Aspergillus niger	3.0	(6)
Botrytis allii	0.3	(3)
Fusarium graminearum	0.47	(3)
Penicillium digitalum	2.4	(3)
	1.8	(6,7)
P. expansum	0.6	(6)
Rhizopus nigricans	2.4	(6,7)
Trichophyton interdigitale	0.3	(6)
T. mentagrophytes	0.59	(4)
	0.3	(6)
Yeasts		
Candida albicans	1.9	(4)
Saccharomyces cerevisiae	5.9	(6,7)

[a] References: (1) Brodersen and Kjaer (1946); (2) Fitzgerald and Jordan (1953); (3) McGowan *et al.* (1948); (4) Stedman *et al.* (1954); (5) Ukita *et al.* (1949); (6) Wolf (1950); (7) Wolf and Westveer (1950).

is clear that fungi are more sensitive to dehydroacetate than are bacteria. The means of the concentrations in the table are, of course, not quantitatively significant, but show well the difference: 26 m*M* for bacteria and 1.1 m*M* for fungi. The mechanism of growth inhibition is completely unknown. There is no obvious correlation between cycle activity in these organisms and susceptibility to dehydroacetate. The fungi behave differently than bacteria with regard to so many drugs that one must assume basic differences in metabolism or permeabilities, and it would be impossible at this time to attribute the greater sensitivity to dehydroacetate to any one factor. Permeability seems to be of importance in the action of dehydroacetate, as indicated by the effects of pH. A decrease in activity with increasing pH has been generally noted (Shibasaki and Terui, 1953; Bandelin, 1958), with the exception of *Salmonella* and *Staphylococcus* (Wolf and Westveer, 1950). The results of Bandelin on several fungi are typical (see accompanying tabulation). A 100- to 200-fold increase in activity as the pH is raised from

Organism	Minimal inhibitory concentration (m*M*)			
	pH 3	pH 5	pH 7	pH 9
Alternaria solani	0.059	0.12	1.18	11.8
Aspergillus niger	0.12	0.30	2.36	11.8
Chaetomium globosum	0.059	0.30	2.36	5.9
Penicillium citrinum	0.059	0.30	2.36	11.8

3 to 9 is observed. The only obvious explanation is that the anionic forms of dehydroacetate do not penetrate well. The major decrease in activity occurs between pH 5 and 7, correlating with the pK_a's near 5.2.

CHAPTER 4

SULFHYDRYL REAGENTS

A substance which can react with sulfhydryl groups and thus alter enzymic, metabolic, or functional processes is generally called a *sulfhydryl reagent.* Such substances represent a very important group of inhibitors and have been used extensively to determine if enzymes or metabolic reactions depend in any way on intact sulfhydryl groups. In addition, they are often used to estimate the number and reactivity of sulfhydryl groups on proteins, or to histochemically localize the sulfhydryl groups in cells or tissues. The next few chapters will be concerned with sulfhydryl reagents, and in this chapter we shall discuss several general aspects of inhibition resulting from modifications of sulfhydryl groups and some of the problems encountered in work with these substances. This is one phase of inhibition that has recently received considerable attention, and several reviews covering certain aspects of the problem are available. The articles by Boyer (1959) and Putnam (1953), and the books "Glutathione" (1954) and "Sulfur in Proteins" (1959), are particularly recommended.

The terminology to be adopted attempts to follow the most recent usage. The sulfhydryl group (= mercapto group) will for brevity be designated as an *SH group.* Compounds containing SH groups will be designated as *thiols* (elsewhere occasionally called sulfhydryl compounds or mercaptans). A sulfhydryl reagent will be termed an *SH reagent.* The designation as a sulfhydryl enzyme has often been meant to imply that the catalytic activity of the enzyme is dependent on SH groups, i.e., that the SH groups actually participate in the enzyme reaction. As Boyer (1959) has pointed out, not a single enzyme has been definitely shown to involve protein SH groups in the catalysis, and we shall see that the inhibition of an enzyme by an SH reagent does not prove that the SH groups are functional. Hence, a more practical definition of a *sulfhydryl enzyme* at the present time is an enzyme that shows a loss of activity when some or all of its SH groups are modified.

ROLE OF SH GROUPS IN METABOLISM AND FUNCTION

Cellular components containing SH groups may be conveniently grouped in three categories: (1) *low molecular weight thiols*, such as the cofactors lipoate, coenzyme A, and glutathione, or various amino acids and related compounds, such as cysteine, homocysteine, 2-thiolhistidine, ergothioneine, and thioglycolate, (2) *nonenzyme proteins*, probably including most of the cytoplasmic proteins (e.g., those involved in movement, such as actomyosin, ciliary proteins, and proteins of the mitotic spindle), plasma membrane proteins, and structural proteins, and (3) *enzymes* of all types and catalyzing every variety of reaction. Modification of or reaction with any of these SH groups may directly or indirectly alter cellular metabolism and function. Even reaction with nonenzyme protein SH groups may disturb metabolism, because of the role such proteins may play in the structural organization of the metabolic units or in the permeabilities of cells. In addition to the free SH groups, many proteins and enzymes contain disulfide (S—S) groups that, in the case of enzymes, are probably not involved directly in the catalysis but in the structural stability. These disulfide groups can under certain circumstances be reductively cleaved to form free SH groups, with simultaneous loosening of the protein structure, or can perhaps react directly with certain agents to form mercaptides.

In the early days of interest in thiols, it was believed that SH reagents altered metabolism, and were sometimes lethal, as a result of reaction with glutathione or other low molecular weight thiols, but it was soon realized that enzyme SH groups are a much more important site of attack. Even today the role that such small thiols play in metabolism and the importance of their reaction with SH reagents are not well understood, except in the case of coenzyme A and lipoate. The ubiquitous glutathione plays at present an indeterminate role in metabolism, except for its likely participation in the reactions of phosphoglyceraldehyde dehydrogenase, glyoxalase, maleate isomerase, maleylacetoacetate isomerase, formaldehyde dehydrogenase, and indolylpyruvate tautomerase, and in transpeptidation and folate splitting. Low molecular weight thiols have also been supposed to regulate metabolism by redox equilibria with enzyme SH groups, maintaining a certain fraction of these in the reduced or active state.

The SH groups of enzymes have been considered to bind cofactors or coenzymes to the apoenzyme, or to form acyl or phosphoryl complexes with intermediates derived from substrates, or to function directly as redox couples in electron transfer, but there is little evidence for any of these, as likely as they may be. The SH group readily donates electron pairs and thus is one of the most reactive enzyme groups with regard to the formation of covalent bonds, so it would not be surprising if covalent intermediate complexes occur. Whatever the role SH groups play in enzyme catalysis,

their modification often abolishes activity and, since metabolism depends on sulfhydryl enzymes, it is evident that most important metabolic pathways would be sensitive to SH reagents. In addition, coenzyme A, lipoate, and glutathione function in key metabolic positions. Thus glycolysis, the tricarboxylate cycle, fatty acid oxidation, photosynthesis, phosphate transfer, and various synthetic pathways are inhibitable by SH reagents. Many effects of thiols on metabolism have been observed but no detailed mechanisms emerge. Brain respiration and glycolysis *in vivo* proceed at only a fraction of their maximal rates; it has long been known that glutathione stimulates aerobic glycolysis in the brain, and thus it has been implicated in the regulation of cerebral metabolism. McIlwain (1959) reported that the aerobic glycolysis of brain slices is stimulated by glutathione, cysteine, homocysteine, 2-mercaptoethanol, and other thiols, although the effects on respiration are rather slight. However, the respiratory stimulation by 50 m*M* KCl is depressed by these thiols, as is the excess respiration in the presence of dinitrophenol. The glycolytic stimulation is accompanied by a decrease in creatine-P and a rise in inorganic phosphate, these changes being correlated with the metabolic changes. If the respiratory augmentation produced by increased functional activity were mediated through glutathione or similar thiols, there would have to be a fairly large change in their concentrations, or in the ratios of the oxidized and reduced forms, which is not observed. The metabolic relations are clear but it is not known by what mechanism the thiols reduce brain creatine-P.

Studies of the effects of SH reagents on cell function are complicated by the fact that undoubtedly some of the proteins of the functional systems contain SH groups, and may even be dependent upon them. This has been investigated principally in the proteins involved in motility; for example, the polymerization of G- to F-actin, the interaction of actin and myosin, the ATP-induced contractions of glycerinated flagella, the round-up of cultured fibroblasts, the formation of the mitotic apparatus, and many other phenomena appear to be dependent on free SH groups. Cell excitability and impulse conduction, based on ionic fluxes and a specific membrane structure, must also involve SH groups in the membrane. Thus effects of SH reagents on cell function cannot be immediately interpreted in terms of an enzymic or metabolic site of action.

CHEMICAL PROPERTIES OF SH GROUPS

Only a few characteristics of the SH group that are particularly important in enzyme inhibition will be discussed. A brief and excellent summary of sulfur chemistry is that of Calvin (1954) and much of interest may be found in "Organic Sulfur Compounds" edited by Kharasch (1961), as well as in the general references given earlier in this chapter.

The reactions of most SH reagents with thiols depend on the pH and this undoubtedly relates to the ionizations of both SH reagent and the SH groups reacted. In most cases, as with the mercaptide-forming reagents and the alkylating agents, the rate and degree of reaction increase with increasing pH, and it is likely in these cases that the ionized mercapto anion, R—S^-, is more reactive than the un-ionized R—SH form. One may visualize some of these reactions as a competition between the SH reagent and a proton for the R—S^- group. The reaction with a heavy metal ion, for example, may be written as:

$$R—SH + Me^+ \leftrightarrows R—S—Me + H^+$$

and it is obvious that increase of the pH will favor the formation of the mercaptide complex, or, to put it in another way, that the reaction:

$$R—S^- + Me^+ \leftrightarrows R—S—Me$$

will proceed more readily. On the other hand, reactions with double bonds (as with maleate or quinones) or oxidations to the disulfide may occur more readily when the SH group is un-ionized. In any event, the state of ionization of the SH group is important in enzyme inhibition and may account partly for the different reactivities of protein SH groups. The ionization of SH groups is well discussed by Edsall and Wyman (1958), mainly on the basis of work by Benesch and Benesch (1955). The ionization microconstants for cysteine are given in Table I-14-4. It is clear that the pK_a of an SH group is markedly dependent on the electric field present, that is, on the vicinal ionic groups. One might roughly estimate the pK_a's for an SH group as shown in the following tabulation:

	pK_a
Near a + charged group	7.2–8.5
No electric field	8.5–9.2
Near a — charged group	9.2–10.2

On the surface of a protein the electric field will be the resultant of all the contributions of the ionic groups. Enzyme SH groups must therefore exhibit a wide range of ionizations at any designated pH, and in most cases will exist mainly in the un-ionized form at physiological pH. The pK_a of the SH groups on aldolase in 4 *M* urea is around 8.66, but the native enzyme has 6 exposed SH groups with pK_a values near 10.5 and buried SH groups with an apparent pK_a of 11.5 (Donovan, 1964).

Many of the atomic and bond properties will be found in the tables of Chapter I-6. The bond dipole moments are fairly high (C—S 1.73 and S—H 0.68, corresponding to fractional atomic charges of 0.20 and 0.11, respectively) and the bonds with sulfur are readily polarized (the molar refractions are C—S 4.43, S—H 4.62, and S—S 7.41) compared with most other bonds occurring in proteins. Furthermore, the bond energies are uniformly low compared with the corresponding oxygen bonds, except for the disulfide bond, which is a good deal stronger than the peroxide bond (see accompanying tabulation). These fundamental properties account for many of the

Bond energies (kcal/mole)

C—S 54	C—O 80
S—H 87	O—H 105
S—S 66	O—O 34

characteristic reactions of the SH group and the relatively unique role of these groups in enzyme activity and inhibitition. The inherent dipole moments and the high polarizability of sulfur bonds may play an important role in the interactions of enzymes with substrates and inhibitors, whereas the bond energies are involved in determining ionization tendencies, oxidation-reduction potentials, and the equilibria between SH groups and disulfide structures.

The thiol-disulfide equilibria are important for enzyme structure in all probability but, in addition, may well be determining factors in the states and reactivities of the SH groups. It has been shown recently that the reaction of a thiol with a disulfide is not a simple oxidation-reduction but an exchange reaction involving a two step ionic displacement (Eldjarn and Pihl, 1957 a, b; Parker and Kharasch, 1959; Foss, 1961), often with the formation of mixed disulfides:

$$\text{X—SH} + \text{Y—S—S—Y} \leftrightarrows \text{X—S—S—Y} + \text{Y—SH}$$

$$\text{X—S—S—Y} + \text{X—SH} \leftrightarrows \text{X—S—S—X} + \text{Y—SH}$$

Low molecular weight thiols, such as glutathione or cysteine, could thus interact with enzyme SH and disulfide groups to form mixed disulfides. Particularly in the cell, where such thiols occur, these interactions may be important in regulating enzyme activity, and could easily affect the reactivity of enzymes with SH reagents. That this can actually occur with proteins was shown by the use of a colored disulfide, with which seralbumin and β-lactoglobulin react to form mixed disulfides (Klotz *et al.*, 1958). If an SH enzyme and oxidized glutathione (GSSG) are allowed to react, one would

have ESH, ESSG, ESSE, GSSG, and GSH (where E represents the enzyme) present, perhaps only ESH being catalytically active and the forms ESSG and ESSE protected from SH reagents. Although it is usually thought that only the SH form can react with most SH reagents, it is possible that disulfides are occasionally reactive; aryl arsinites, for example, can exert a nucleophilic displacement on the disulfide bond:

$$\mathrm{RSSR} + \mathrm{R'AsO(OH)^-} \leftrightharpoons \mathrm{RS\underset{\displaystyle O}{\overset{\displaystyle OH}{AsR'}}} + \mathrm{RS^-}$$

However, such reactions are probably slower than with free SH groups. The new reagent, dithiothreitol ($HS—CH_2—CHOH—CHOH—CH_2—SH$), which has a low redox potential (−0.33 v at pH 7), is highly water-soluble and reduces protein disulfide groups (Cleland, 1964). It was suggested that it might be valuable in protecting enzyme SH groups, having several advantages over the ones commonly used, and could also be applied for the purpose of maintaining enzymes in the SH state for inhibition studies.

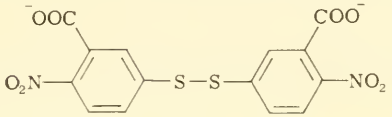
Hydrogen bonding by sulfur should be mentioned since it must play a role in both intra- and intermolecular interactions of the SH group, but most of the data we have derives from studies of the small thiols and there is very little information on hydrogen bonding of protein SH groups. Boyer (1959) has presented the evidence for the occurrence of hydrogen bonds to sulfur in a variety of compounds. Sulfur does not form hydrogen bonds as readily as oxygen or nitrogen, since it is less electronegative (as indicated by the smaller dipole moment of the S—H bond compared to the O—H and N—H bonds) (Table I-6-1). However, there is evidence for intramolecular S—H···O and S—H···N bonds in cysteine and its peptides, and Benesch *et al.* (1954) have advanced hydrogen bonding to explain some of the different reactivities of simple thiols. It is possible that hydrogen bonding of enzyme SH groups can modify their susceptibilities to various SH reagents.

Detection and Determination of Enzyme SH Groups

Valuable reviews of the general methods for the determination of SH groups in proteins and enzymes have been provided by Hellerman and Chinard (1955) and R. Benesch and R. E. Benesch (1962). Some of the most reliable methods involve the use of mercurials (to be discussed later, pages 762 and 766). Here we shall mention only a few of the more recently developed reagents which may be applicable in inhibition studies.

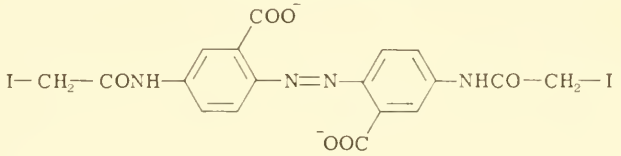
Bis(*p*-nitrophenyl)disulfide reacts with thiols at pH 8 to form 1 mole of *p*-nitrophenol per mole of thiol, and this anion, being highly colored, can be used to determine the thiol concentration (Ellman, 1959). However, this

reagent is poorly soluble in water, so carboxyl groups were introduced to solubilize it; this compound is 5,5′-dithiobis(2-nitrobenzoate) and reacts



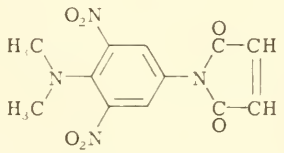
5,5′-Dithiobis (2-nitrobenzoate)

with thiols and SH groups in the blood and tissues. Another water-soluble reagent for free SH groups is 2,2′-dicarboxy-4,4′-diiodoaminoazobenzene, which was shown to react only with the SH groups on denatured meromyosin (Fasold *et al.*, 1964). The number of SH groups reacted can be determined



2,2′-Dicarboxy-4,4′-diiodoaminoazobenzene

spectrophotometrically because of the chromogenic azo link. A yellow SH reagent, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, was introduced by Witter and Tuppy (1960) and found to react with the free SH groups of seralbumin. The treated protein could be hydrolyzed with pepsin and the *N*-(4-dimethylamino-3,5-dinitrophenyl)succinimido-cysteine peptides isolated by means of their yellow color. This reagent was used by Gold and Segal (1964) to obtain information on the nature of the active site of 3-phosphoglyceraldehyde dehydrogenase. Following pepsin treatment the single hexapeptide – Ala-Ser-(DDPS-Cys)-Thr-Thr-AspNH_2 – was found to contain essentially all the color. This provides evidence that the three active sites on the enzyme are similar in structure, at least in part, and that the reactive SH groups are not those of glutathione, which occurs on the enzyme.



N-(4-Dimethylamino-3,5-dinitrophenyl) maleimide

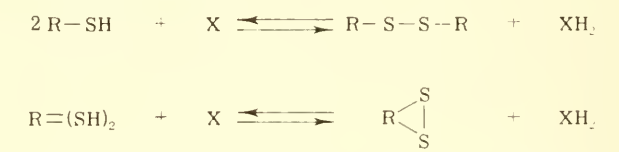
Such reagents would not be particularly useful for the inhibition of SH enzymes because of their bulky structure and the presence of a variety of

groups, but they could be applied to the determination of changes in the SH content after treatment of the enzymes with the usual inhibiting reagents.

TYPES OF SH REACTION IMPORTANT IN INHIBITION

The reactions of most of the important SH reagents have usually been classified into four types. The SH groups have been written as un-ionized in all cases, not implying that this is necessarily the only reactive form. The mechanisms of these reactions will be discussed in the chapters devoted to the individual inhibitors.

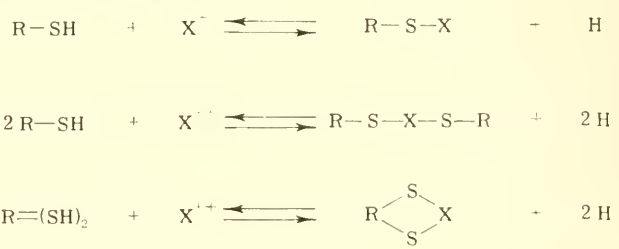
(I) *Oxidation of SH groups*



(X may be an acceptor of either hydrogen atoms or electrons.)

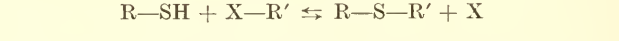
Examples: *o*-iodosobenzoate, porphyrindin, porphyrexide, iodine, alloxan (not the only mechanism), ferricyanide, oxidized glutathione, tetrathionate, sulfite, performic acid, and oxygen (catalyzed by metal ions).

(II) *Mercaptide formation*



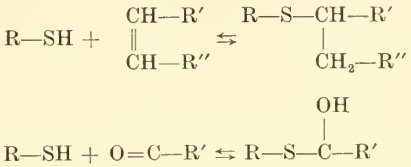
Examples: $HgCl_2$, organic mercurials, arsenite, organic arsenicals, and various heavy metal ions (Cu^{++}, Pb^{++}, Cd^{++}, Ag^{+}, etc.).

(III) *Alkylation of SH groups* (*alkyl transfer*)



Examples: iodoacetate, iodoacetamide, S- and N-mustards, chloracetophenone, chloropicrin, bromobenzylcyanide, and fluoropyruvate.

(IV) *Addition of SH groups to double bonds*



(This may also be considered as a type of alkylation reaction.)

Examples: maleate, *N*-ethylmaleimide, quinones, acrolein, acetoacetate, and methylglyoxal.

FACTORS DETERMINING THE REACTIVITIES OF SH GROUPS

The SH groups of various simple thiols, peptides, and proteins differ markedly in reactivity with SH reagents. Although this has been known for many years, the molecular basis for this differential reactivity is poorly understood. In general the reactivity is maximal in simple thiols and minimal in proteins, but in proteins there are usually reactive and unreactive SH groups. Barron (1951) classified SH groups as (a) freely reacting, (b) sluggish, and (c) masked, depending on whether they react readily, slowly, or not at all. Although such a division is often useful in discussing SH groups, there is actually a continuous sequence of groups from highly reactive to unreactive. If a protein is allowed to react with an SH reagent under approximately physiological conditions, one generally finds that the SH groups disappear at different rates, perhaps several reacting completely before others are affected. A graded response is clearly seen in the reaction of aldolase with *p*-chloromercuribenzoate (Swenson and Boyer, 1957). Ten SH groups react relatively rapidly, a few more slowly, and the rest not at all unless the enzyme is unfolded by high concentrations of urea. Furthermore, the reaction of the first 10 SH groups does not alter the enzyme activity, indicating that these groups are not part of, or even too near, the active center, whereas disappearance of the more slowly reacting groups abolishes the activity. A similar situation has been observed with urease, which has 5 cysteine residues per molecule, one freely reacting and 4 sluggish, the catalytic activity being affected only by modification of the latter (Hellerman, 1939; Hellerman *et al.*, 1943). Thus porphyrindin, iodoacetamide, and iodosobenzoate react with one SH group but do not inhibit (except at very high concentrations), whereas *p*-chloromercuribenzoate can combine with another SH group abolishing the activity. These examples — and we shall have occasion to discuss many others — illustrate four most important principles: (1) the differential reactivity of enzyme SH groups, (2) the increase in reactivity of many of the SH groups following denaturation, (3) the different reactivities of var-

ious SH reagents, and (4) the lack of correlation between the reactivity of SH groups and their relationship functionally or spatially to the active center. These principles are central to the problem of inhibition by SH reagents and require some general discussion relative to the possible mechanisms involved.

The various, and mostly obvious, hypotheses to explain the differential reactivity of SH groups have frequently been presented with a prolixity inversely proportional to the amount of evidence available. Indeed, at the present time there is little, if any, positive evidence for any explanation, but there are a number of factors that must be of some importance, and these can be enumerated. It should be emphasized that differential reactivity should be based on accurate spectrophotometric or argentimetric titrations of the enzyme SH groups under various conditions. The fundamental problem is to determine the cause for the slow reactions of all or a fraction of an enzyme's SH groups, the total number of such groups being determined by quantitative titrations of the enzyme after complete unfolding. The theories assume either that (I) free SH groups are present in the native catalytically active enzyme, but are for some reason unable to react readily with SH reagents, or that (II) the unreactive SH groups are so modified that they are no longer free.

(I) *Free SH groups present in native enzyme*

A. *Steric factors impede reaction*: the reagent is simply unable to approach the SH group because it is located in a pit or crevice of the enzyme, or actually within the protein structure.

B. *Electrostatic factors impede reaction*: the SH group is in the electric field of surrounding groups, this discouraging reactions with reagents of the same charge sign as these groups.

C. *Ionization state impedes reaction*: if either the SH or the S^- form reacts preferentially but is not significantly present at the experimental pH, the reaction will be appreciably slowed.

(II) *SH groups are not free in the native enzyme*

A. *Present as disulfide groups*

B. *Hydrogen bonded to adjacent groups*

C. *Present in thiazolidine or thiazoline rings*

D. *Reacted with some component of enzyme reaction*: for example, acylated, phosphorylated, or complexed with some metal ion.

The fact that complete opening up or unfolding of the protein structure invariably increases the susceptibility of certain SH groups to attack does not necessarily imply that the groups are in some way within the enzyme,

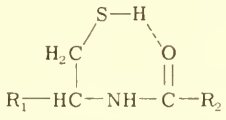
since denaturation can also dissolve disulfide bonds, hydrogen bonds, ring structures, and other possible chemical interactions of the SH groups. As long as one studies only a single SH reagent, it is easy to postulate a reasonable mechanism for the unreactivity of particular SH groups. For example, if one finds that iodoacetate does not alkylate an enzyme SH group, the group may be thought of as sequestered within the protein structure, but if subsequent work shows that *p*-chloromercuribenzoate reacts readily with this group, this hypothesis must be abandoned inasmuch as *p*-chloromercuribenzoate is a larger molecule than iodoacetate. Likewise, postulating that negative charges surround the SH group, preventing the approach of iodoacetate, will not be valid if *p*-chloromercuribenzoate is effective, since both of these reagents are negatively charged, as has been pointed out by Boyer (1959) in perhaps the best discussion of differential SH group reactivity. It is certainly likely that steric and electrostatic factors are occasionally important, but one must demonstrate some correlation of the unreactivity of the SH groups with the properties of a variety of SH reagents. Haurowitz and Tekman (1947) believed that protein SH groups are often inaccessible to reagents because of the tightly folded nature of the polypeptide chains, rather than chemically combined, because unfolding is accompanied by the appearance of reactivity in other than SH groups, e.g. phenolic groups. Although this is suggestive, it is not proof for the inaccessibility theory. Unreactivity due to an unfavorable ionization state has perhaps been insufficiently considered, particularly for inhibitors such as iodoacetate, and there is no question but that the very low concentration of S^- near neutrality for some SH groups must be important.

Turning to the second group of theories, no one denies that disulfide groups occur in some enzymes, but that this generally cannot explain the differential reactivity of SH groups is obvious. Indeed, one finds a wide range of reactivities in simple thiols where cryptic exclusion or disulfide bonding may be eliminated. Benesch *et al.* (1954) not only demonstrated different nitroprusside reaction rates with various biologically important thiols, but showed that urea increases the reactivity of the more slowly reacting SH groups, just as it does in proteins. This was interpreted in terms of the breaking of hydrogen bonds and thus the initial sluggishness of reaction as due to hydrogen bonding of the SH groups to adjacent amino or peptide groups. This may occur in a cysteine peptide in the following way:

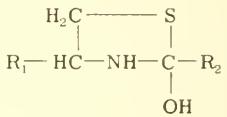
the hydrogen donator depending on the pH. We have seen that SH groups form only weak hydrogen bonds (page 640), so that it is likely that this

alone cannot depress the reactivity too greatly. However, in addition the hydrogen bond may bring an adjacent side chain into the region of the SH group and this second steric factor may further reduce the reactivity. Benesch and Benesch (1953) compared the peptides, phenacetyl-L-cysteinylglycine (PCG) and phenacetyl-L-cysteinyl-D-valine (PCV), with respect to the polarographic reduction of their mercaptides with mersalyl and Ag^+, and found that a relative suppression of the ability of the PCV SH group to react with these reagents is evident. This was interpreted as due to the steric interference of the isopropyl group of valine in PCV, brought into the proximity of the SH group by hydrogen bonding, whereas in PCG there is no such side chain. In proteins the interference may be even greater. This concept thus involves both a reaction of the SH group and steric factors.

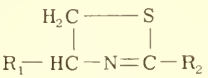
The hydrogen-bonded structures could further lose water to form more stable thiazolidine or thiazoline rings. Linderstrøm-Lang and Jacobsen (1941) found that 2-methylthiazoline can hydrolyze under certain conditions to release an SH group and a peptide linkage, such as occurs during protein denaturation. Thus in a cysteine peptide, where the hydrogen bonding is now to the keto oxygen, the following structures can be written:



H-bonded form



Thiazolidine form



Thiazoline form

Such transformations have been more recently discussed by Calvin (1954) in connection with the structure of glutathione and the reactivities of SH groups in enzymes. Indirect evidence for these rings was obtained by comparing the absorption spectra of thiols with 2-methylthiazoline (although at high acidity so that the situation near neutrality is still not clear). If such structures occur in enzymes, they could unquestionably account for unreactivity.

It is quite possible that many or all of these mechanisms contribute in various situations to the differential reactivity of SH groups, and that we should not be too eager to argue for a single dominant factor. After all, there is a graded scale of reactivity, which in itself implies multiple mechanisms. It would probably aid in the characterization of SH groups if some standard method for designating the reactivity could be used, rather than designating them by terms such as "sluggish," etc. The time for 50% reaction, where determinable, might be the simplest and most useful, although some form of reaction rate constant would be preferable.

INTERPRETATION OF INHIBITIONS BY SH REAGENTS

The SH reagents are used most commonly to determine whether a particular enzyme is an "SH enzyme" or not. What this means depends on one's definition of "SH enzyme." If we take the definition proposed earlier (page 635) that an SH enzyme is one that is inhibited by SH reagents, not a great deal has been achieved by proving that an enzyme belongs to this class. In the past, many workers have been satisfied to stop at this distinction, and the designation of an enzyme as an SH enzyme has been deemed sufficient without further discussion as to the significance of the observation. On the other hand, some have assumed immediately that inhibition by SH reagents indicates a catalytically functional role for an SH group at the active center, and this, as we have seen, is entirely unjustifiable. It is thus important to determine as far as possible what such inhibition means and what valid conclusions may be drawn from the use of SH reagents.

The various mechanisms by which SH reagents can inhibit pure enzymes may be classified in the following way.

(A) *The SH group reacted is at the active center and is functional.* The SH group may be involved in the binding of substrate, coenzyme, or activator to the apoenzyme, or it may participate in the transfer of groups of electrons.

(B) *The SH group reacted is at the active center but is nonfunctional.* It is possible that an SH group occurs at the active center but is unrelated to the catalytic process.

(C) *The SH group reacted is vicinal to the active center.* The SH reagent introduces a new structure on the enzyme, and if this is near enough to the active center it may either sterically or electrostatically modify the reactions proceeding at the active center.

(D) *Reaction of the SH groups alters the enzyme protein structure.* This could also apply to reaction with disulfide groups or other complexes formed by SH groups. The change in protein structure would then reduce the

rate of the enzyme reaction, particularly if it included the region of the active center.

(E) *The SH group reacted is on the substrate.* This is a possibility especially in the case of proteolytic enzymes, the modification of the peptide or protein substrate preventing normal reaction with the enzyme.

(F) *The SH reagent interferes in a manner unrelated to SH groups.* Many SH reagents are not entirely specific for SH groups; e.g., iodoacetate also reacts with amino groups and with heavy metal ions such as Cu^{++}, and can often form complexes with protein groups other than SH, especially amino and carboxylate groups. Also the SH reagent may inhibit because it is structurally similar to the substrate and can compete with it for the active site; e.g., *p*-chloromercuribenzoate may act like a substituted benzoate on certain enzymes rather than as a mercurial.

Other mechanisms can be visualized in special cases and particularly for those enzymes comprising several units and catalyzing complex reactions, since the SH reagents can conceivably dissociate the functionally related units, just as *p*-chloromercuribenzoate can split the relatively simple muscle phosphorylase *a* into four equivalent fractions (Madsen and Cori, 1956).

It is very difficult to distinguish between the first four possibilities. Indeed, proof of the functional role of SH groups usually must come from evidence other than inhibition. Protection of the enzyme against SH reagents by the substrate does not provide adequate evidence that the reacted SH group is part of the active center, since the substrate could also slow down or prevent reaction with vicinal groups as well, and could also stabilize the protein structure around the active center. The secondary alteration of protein structure brought about by reaction of SH groups cannot always be detected by reversal experiments because the changes may, like certain types of denaturation, be reversible. One must therefore conclude that the demonstration of inhibition by SH reagents indicates at best (assuming the mechanisms (E) and (F) have been eliminated) only that one or more SH groups are sufficiently near the active center to interfere with the catalysis, either directly or by structural changes, when they are reacted. It must be admitted that such a conclusion is not very informative, especially when it is considered that most enzymes contain 5 to 30 SH groups per molecule and that statistically one would expect one or more of these to be near the active center. Indeed, it is rather surprising that in some instances a fair number of SH groups can be reacted without altering the catalytic activity.

One characteristic of inhibition by SH reagents which has been often neglected is that the reaction with the enzyme SH groups in most cases introduces a new side chain onto the protein. The inhibition may be as much related, if not more, to the properties of this side chain as to the

disappearance of a free SH group. These new groupings have varying sizes and frequently electrical fields. It is quite possible that, all else being equal, a smaller reagent of a particular type may exert less inhibition, due simply to the fact that it exerts less steric hindrance to the catalytic process. Thus the inhibition by methylmercuric chloride may be different from that by *p*-chloromercuribenzoate for this reason. The groups introduced by iodoacetate and *p*-chloromercuribenzoate are negatively charged, whereas those from iodoacetamide and phenylmercuric acetate are uncharged, and this could well be responsible for some of the differences observed between these inhibitors. This is one reason why many studies with SH reagents would profit from a quantitative comparison of the effects of a large number of inhibitors of different types.

Another factor of some importance may be the influence of the reaction of certain SH groups on the reactions of other SH groups. Further reaction apparently may be either depressed or accelerated. In phosphorylase a the reaction of the first SH group seems to facilitate the combination of the remaining 18 groups with *p*-chloromercuribenzoate (Madsen and Gurd, 1956). On the other hand, reaction of one SH group on hemoglobin prevents the further reaction of one or two other groups with Ag^+, implying that the SH groups here occur in clusters (Ingram, 1955). The number of molecules of SH reagent bound to the protein may thus not be equivalent to the number of SH groups.

Boyer (1959) has emphasized that insufficient consideration has usually been given to the possible secondary structural changes induced in enzymes by reaction with SH reagents. If some SH groups are unreactive because of steric blocking or chemical combination, these hindrances must be overcome in order to react these groups, and this could imply a modification in the protein structure that in itself might be inhibitory. It has been observed frequently that the poorly reacting SH groups are more important in the enzyme structure than the free readily reactive ones. One of the first statements of the importance of structure in the inhibition by SH reagents resulted from work on urease by Desnuelle and Rovery (1949). Phenylisocyanate reacts with certain SH groups rapidly but this does not inhibit; inhibition begins when the unreactive SH groups are attacked, and this was attributed to a reversible change in the enzyme structure. Similarly, various properties of aldolase change as the SH groups are progressively reacted with *p*-chloromercuribenzoate: after 3–5 are reacted, the enzyme begins to be more readily attacked by trypsin; after 10 are reacted, the turbidity increases, denoting marked structural changes, and inhibition is observed (Szabolcsi and Biszku, 1961). There is a progressive labilization of the tertiary structure, accompanied by appearance of previously masked SH groups, with further reaction and eventual denaturation. In many cases, the initial reactions must reduce the protein stability, perhaps only locally,

and this spreads and progresses rapidly as further groups are attacked, just as in other types of denaturation. The blocking of the SH groups of phosphoglyceraldehyde dehydrogenase changes the optical rotation and the intrinsic viscosity, the latter increasing linearly with the equivalents of *p*-chloromercuribenzoate reacted (Elödi, 1960). Reversibility with cysteine varies with the time of exposure to the mercurial, at first the effects being completely reversible and eventually irreversible, again indicating a progressive breakdown of the protein structure. Elödi postulated three phases: (1) a reversible reaction with certain SH groups, (2) an unfolding of the polypeptide helices as a result of the alteration of the SH groups, and (3) precipitation due to intermolecular bridges formed between the new groups appearing on the protein surface. Ribonuclease is perhaps another example of structural changes resulting from the scission of disulfide bonds, of which there are 4 in the native enzyme: breaking 1 does not alter the activity, breaking 2 inhibits about 20%, breaking 3 inhibits about 40%, and then suddenly the activity drops to zero as the last disulfide is split (Resnick *et al.*, 1959). The —S—S— bonds were believed to be of importance in providing stability to the secondary structure of the enzyme, their breaking leading to progressive unfolding.

PROTECTION AND INHIBITION REVERSAL BY THIOLS

Some of the problems involved in protection and reversal experiments with SH reagents and thiols were discussed at some length in Volume I (pages 622–626). A few of the conclusions reached there will be briefly summarized. (1) Protection or reversal by a thiol depends on the relative affinities of the SH reagent for the enzyme SH groups and the thiol, and the relative concentrations of the components, and hence every degree of reversibility of SH-inhibited enzymes may be observed. (2) Protection or reversal by a thiol does not provide conclusive information on the mechanism of the inhibition or the enzyme groups attacked. (3) Irreversibility is brought about not only by very tightly bound reagents, but by progressive structural changes in the enzyme, as discussed above. This type of irreversibility thus increases with the concentration of the SH reagent and the time of exposure. (4) The amount of useful information relative to the mechanism of inhibition obtained from such studies is much less then commonly believed.

The stability of the product formed by reaction of an enzyme SH group with an SH reagent varies with many factors, most of which have been mentioned in connection with the differential reactivity of these groups. Sometimes the product is completely stable for all experimental purposes and the reacted enzyme is permanently altered; such would be the case with most of the alkylating agents. Then the mercaptide complexes vary

greatly in stability, so that in some instances they can be split at a rate too rapid to be technically measurable, while in others the rate is too slow to measure. It is not necessary that this stability be correlated with structural changes in the enzyme or irreversible inactivation. The inability to reactivate an enzyme inhibited by an SH reagent can be attributed to a variety of factors, some of which are listed below.

(A) The binding of the SH reagent to the enzyme is stronger than to the reversor; one must use the proper reversor and concentration (e.g., dimercaprol will reverse some inhibitions untouched by cysteine).

(B) The enzyme is chemically altered by the SH reagent so that it is not a question of a tightness of binding; reversal can occur only by a chemical transfer of the attached group to another radical.

(C) The enzyme is structurally altered irreversibly (denatured) by the blocking of the SH groups.

(D) The SH reagent has caused a splitting off of some coenzyme or cofactor, which must be added back following restoration of the SH group for activity to be evident.

(E) The reversor may in some manner inhibit the enzyme, even though restoring the free SH groups initially, as when the SH groups are oxidized by disulfides formed from the oxidation of the added thiols.

In order that irreversibility be correctly attributed to protein denaturation, these other possibilities must be ruled out. Complete reversibility is more easily interpretable, and one can quite confidently say that at least no permanent derangements in the enzyme structure have been induced by the SH reagent.

Protection experiments, in which some thiol is added previous to, or with, the SH reagent, are, as has been emphasized earlier, of little value, since all one is doing is reducing the concentration of the free SH reagent (assuming that it reacts with the thiol). Actually, it is a little difficult to speak of this as protection, inasmuch as one usually would not call a reduction in inhibitor concentration a type of protection. It is very unlikely that worthwhile information can be obtained from such experiments, aside from the practical determination of the ability of substances to reduce the toxic effects of SH reagents.

GENERAL CONSIDERATIONS OF THE USES OF SH REAGENTS

Numerous types of reagent are available for satisfactorily specific reaction with SH groups but no single one is adequate for all purposes. The most useful information on the nature of enzyme SH groups, their locations and

relation to the catalysis, can be obtained by the proper use of several types of SH reagent. It is also advisable to use different concentrations of the reagents (it is not very informative to report that 1 mM of some SH reagent inhibits 100%) and calculate a K_i that characterizes the potency of the inhibition and the affinity of the enzyme for the reagent. In order to present the kinetics properly, it is necessary to determine if the inhibitions under the experimental conditions used are reversible, and for this purpose it is best to perform the reversal study in nitrogen. It is also useful to determine the degree of inhibition and the number and type of SH groups reacted simultaneously in order to correlate reactivity and relationship to the active center. Finally, at least some simple rate studies should be done to determine if the inhibitions observed are for equilibrium conditions. In many reports one finds only that the enzyme was incubated with the SH reagent for a certain period (even this information is frequently omitted) and it is impossible to determine if the inhibition observed is maximal or not. SH reagents have perhaps been used during the past several years more commonly than any other type of enzyme inhibitor and yet they have been used with little concern for the many complexities involved in the interpretation of the results, with a few notable exceptions.

Despite the generally good specificity of these reagents for SH groups, they are not specific inhibitors from the metabolic standpoint in most cases. Since SH groups are present not only in many enzymes but in most other proteins of the cell, one must expect that in complex systems there will be many components reacted. Whether some of these reactions will be of importance in what is measured will depend on the nature of the work. It is probably justifiable to suggest that the use of most SH reagents be restricted at the present time to enzyme studies for the purpose of determining the nature of the enzyme SH groups. As the complexity of the system increases, the value of SH reagents diminishes, at least if one is trying to correlate some enzymic or metabolic process with over-all cellular metabolism or function. In this connection it is interesting to note that although the most reactive SH reagents are generally best for pure enzyme work, this is not necessarily true for more complex systems. What one usually requires in metabolic or functional investigations is specificity with respect to a particular enzyme or metabolic pathway. Thus iodoacetate, although it is a rather poor reagent for the detection of SH groups and has frequently been maligned for this purpose, is actually more valuable in cellular work than most of the others since it has the ability, if used properly, of inhibiting the phosphoglyceraldehyde dehydrogenase and glycolysis without affecting other systems significantly, whereas a more reactive inhibitor, such as *p*-chloromercuribenzoate, is valueless for producing specific metabolic blockade. The choice of SH reagent to be used should always be made on the basis of the type of work to be done. Another factor to be considered in work with cel-

lular preparations is the penetrability of the SH reagent, and those reagents should be chosen that have the most likelihood of reaching the systems to be attacked. Thus iodoacetamide is often a better choice than iodoacetate for intracellular inhibition because it is uncharged and probably enters cells more readily.

The treatment of the individual SH reagents in the following chapters must be eclectic in view of the immense amount of reported work, particularly during the past few years. The attempt will be made to select the results of those investigations done most carefully and thoroughly, and to include work on the most important or interesting facets of inhibition by SH reagents. A third aim is to present all the available accurate data that may aid in the assessment of the specificity of these inhibitors in order to use them more profitably in complex systems.

CHAPTER 5

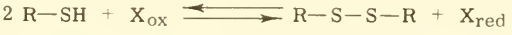
OXIDANTS

Many reagents have been used to oxidize protein and enzyme SH groups for the purpose of either estimating these groups or determining the relationship of the groups to the enzyme activity. Most of these oxidants at present are of little importance in the study of enzymes or metabolism, mainly because of their lack of specificity for SH groups. General over-all oxidation of an enzyme, involving several types of group and ending in partial or complete denaturation, provides no useful information. If oxidants are to be used for the specific modification of SH groups it is necessary that the choice of oxidant and the experimental conditions be made very carefully. The oxidizing activity of the reagent must be neither too high nor too low (i.e., its oxidation-reduction potential must be in the proper range relative to the SH groups under the selected conditions) and the ability of the substance to react in other ways with the enzyme must be minimal. Of the factors determining the rates of oxidation of SH groups and the specificity of an oxidant, the pH and the temperature are the most important. Some of the oxidants that have been abandoned in enzyme work might well be applicable in certain studies if the optimal conditions for their use were known.

The formation of enzyme disulfide groups during oxidation requires SH groups that are close enough to link together in S—S bonds, or are so located as to be able to approach each other readily. The SH groups may be on the same enzyme molecule or on different molecules:

Intramolecular oxidation:

Intermolecular oxidation:



The hydrogen atoms may be transferred directly to the oxidant, or may form H^+ ions, the oxidant accepting only electrons. A lone SH group on an enzyme, although fully exposed, may not be oxidized if sterically it cannot associate with another SH group. The formation of enzyme aggregates, or

actual precipitation, upon oxidation has occasionally been taken as evidence for intermolecular disulfide bonding, but this is perhaps not always valid, since the oxidation may bring about a dissolution of the protein structure leading to such intermolecular reactions as occur during any type of denaturation. If reversal of aggregation can be induced by reducing agents, it is more likely that disulfide bonds are responsible. One factor of primary importance in determining whether a disulfide bond can be formed is the steric relationship between the interacting groups. The C—S—S—C grouping is not linear, or even planar; the S—S—C bond angle is around 107° and the dihedral angle between the two C—S bonds is close to 90° (due to the electrostatic repulsion between unbonded electron pairs). Thus such bonds will be formed readily only when the residues to which the sulfur atoms are attached can assume the proper orientations.

The thiol-disulfide equilibrium:

$$\text{R—SH} \leftrightharpoons 1/2\ (\text{R—S—S—R}) + \text{H}^+ + e^-$$

has not been easy to determine, due to abnormal electrode reactions and the usual sluggishness of such systems, and hence values for the oxidation-reduction potential vary with the method used. It appears that E_0' at pH 7 for various low molecular weight thiols generally lies between -0.35 and 0.0 (Calvin, 1954; Clark, 1960, p. 486). The values of E_0' for protein SH groups are not known, but it is likely that they would lie mainly in this range also. It is possible, however, that some SH groups, due to their particular molecular environment, may have positive potentials, i.e., would be less easily oxidized than most SH groups of the smaller compounds. It is certainly true that certain SH groups on proteins, although readily accessible to alkylation or mercaptide formation, are not oxidized readily, but whether this is due to an especially high oxidation-reduction potential or steric factors, as discussed above, is not known. The values of E_0' depend strongly on the pH, which must be taken into account when experiments are run at pH's varying from neutrality. In any event, the oxidant should have a rather high potential (probably 0.2 or higher) in order to oxidize the susceptible SH groups to virtual completeness. On the other hand, it is usually desirable to oxidize the SH groups only to the disulfide stage. Strong oxidants can occasionally not only oxidize SH groups to sulfonate but attack enzyme groups other than SH so that specificity is lost. Thus iodate oxidizes gluten and thiolated gelatin mainly to the disulfide stage:

$$6\ \text{R—SH} + \text{IO}_3^- \rightarrow 3\ \text{R—S—S—R} + \text{I}^- + 3\ \text{H}_2\text{O}$$

but further oxidation also occurs simultaneously:

$$\text{R—SH} + \text{IO}_3^- \rightarrow \text{R—SO}_3^- + \text{H}^+ + \text{I}^-$$

(Hird and Yates, 1961). *o*-Iodosobenzoate usually oxidizes only to the disulfide stage at pH 7, but if too high a concentration is used, or the pH is much below 7, sulfinate or sulfonate groups are produced (Hellerman *et al.*, 1941). In addition, methionine residues may be oxidized to the sulfoxide stage. Sizer (1942 a,b, 1945) studied the effects on enzymes of many oxidation-reduction systems over a wide range of E_0' and found that as the E_0' is increased from around -0.5 there is little effect on activity until a critical value is reached, which is $+0.6$ for β-fructofuranosidase, $+0.35$ for intestinal phosphatase, and $+0.58$ for chymotrypsin, inactivation increasing rapidly above these values. Of course, it is not entirely a matter of the E_0', the nature of the oxidant being also very important. The enzymes used are not those containing SH groups most easily oxidized, but it does indicate that rather strong oxidants must be used with many enzymes.

Oxidants can inhibit enzymes by mechanisms other than oxidation of SH groups. They may (1) oxidize other enzyme groups, (2) be chemically incorporated into the enzyme (e.g., the iodination of tyrosine residues by iodine), or (3) inhibit reversibly by any of the mechanisms observed with nonoxidizing inhibitors. Other enzyme groups susceptible to oxidation are the hydroxyl groups of tyrosine and serine, the hydrocarbon chain of leucine, the indole ring of histidine, and perhaps the amino, guanidine, and peptide groups. A few examples will be mentioned and others will be discussed in the sections on the individual oxidants. Lieben and Bauminger (1933 a) showed that several amino acids are attacked by *permanganate.* During the oxidation of casein, the arginine content falls, urea and dixanthylurea appearing. Haas *et al.* (1951) emphasized the importance of tyrosine and tryptophan in the actions of permanganate on proteins. Although phenylalanine is quite refractory, tyrosine and tryptophan are oxidized, as shown by changes in the ultraviolet spectra. The spectra of insulin and pepsin treated with permanganate (0.1 mM at pH 2) change in a manner similar to that of the free amino acids, so it is likely that oxidation of these amino acids occurs when they are part of the protein structure. Oxidation of several proteins by *periodate* releases formaldehyde, which probably arises from hydroxylysine (Desnuelle and Antonin, 1946). One mole of ovalbumin reduces 30 moles of periodate to iodate, the protein losing all of its cysteine and cystine, one third of its tryptophan, and a small fraction of its tyrosine (Desnuelle *et al.*, 1947). Oxidation of seralbumin by periodate results in destruction of certain amino acids, producing changes in spectral and electrophoretic properties (Goebel and Perlmann, 1949). Periodate releases acetaldehyde from chymotrypsin, arising from terminal threonine, although this is not responsible for the inhibition of the enzyme inasmuch as it occurs before inactivation starts, and in this case no ultraviolet spectral changes are observed (Jansen *et al.*, 1950, 1951). *Nitrous acid* not only oxidizes certain enzyme groups, such as SH, but attacks free tyrosine and amino groups

(Philpot and Small, 1938; Weill and Caldwell, 1945 a). *Hypochlorite* oxidizes a number of amino acids, only glycine being resistant, and spectral changes occur with proteins indicating oxidation of tyrosine and tryptophan residues (Lieben and Bauminger, 1933 b); in addition it chlorinates amino groups (Wright, 1926). Sizer (1942 b) noted in his work with many oxidants that SH groups are by no means the only susceptible groups on enzymes, the tyrosine residues being particularly oxidizable. These results point to the importance of exercising great caution in the choice of oxidants and conditions for treatment of enzymes if specific oxidation of SH groups is desired. The need for characterizing well the enzyme changes — e.g., disappearance of SH groups as determined by the standard methods, or alterations in the ultraviolet spectrum — upon oxidation is also indicated.

Oxidation of Enzymes by Molecular Oxygen

Thiols and enzyme SH groups are not oxidized by O_2 unless certain metal ions are present. Thus papain is oxidized by O_2 in the presence of Cu^{++} or Fe^{+++} and the consequent inactivation of the enzyme is readily reversed by glutathione (Hellerman and Perkins, 1934). Enzymes such as papain and urease were the earliest studied with respect to the effect of oxidation on their catalytic activities, and this work led to the concept wherein the redox state of SH groups is an important regulating mechanism in cell metabolism (Hellerman, 1939). The initial over-all reaction may be written as:

$$2\ R{-}SH + O_2 \leftrightharpoons R{-}S{-}S{-}R + H_2O_2$$

but the hydrogen peroxide can produce further oxidation:

$$2\ R{-}SH + H_2O_2 \leftrightharpoons R{-}S{-}S{-}R + 2\ H_2O$$

or it can oxidize other components present. The kinetics of the Cu^{++}- and Fe^{+++}-catalyzed oxidations are complex and the mechanism is not completely understood. One theory involves the formation of a Fe^{++}-thiol radical from a Fe^{+++}-thiol complex; two such radicals would combine to form the disulfide and free Fe^{++}, which is reoxidized by O_2 (Williams, 1956). A second theory postulates a $Fe^{++}(thiol)_2$ chelate complex, which is oxidized by O_2 to the ferric complex, within which electron transfer occurs to form the disulfide and Fe^{++} (Martell and Calvin, 1952). Since some type of complex between metal ion and thiol must occur, it is evident that the susceptibility of various SH groups to this type of oxidation must vary greatly. It should be noted that the rates of such oxidations depend on the nature of the buffer used and the pH.

The toxic effects of high tensions of O_2 on cell metabolism may depend on the oxidation of enzyme SH groups. Brain respiration is slowly inhibited by O_2 and there is increasing inability of the tissue to oxidize glucose, pyru-

vate, lactate, fructose, and succinate (Dickens, 1946 a). It was suggested that the most sensitive system is perhaps the pyruvate oxidase due to the involvement of SH groups. This inactivation is not mediated through the H_2O_2 formed, inasmuch as the concentrations are never great enough due to the catalase present. The following enzymes are inactivated by high O_2 tensions: succinate dehydrogenase, phosphoglyceraldehyde dehydrogenase, choline oxidase, phosphoglucomutase, and other SH enzymes (Dickens, 1946 b). Lactate dehydrogenase, malate dehydrogenase, D-amino acid oxidase, and yeast hexokinase are resistant. It is interesting that malonate (1 mM) and Mn^{++} (0.25 mM) protect succinate dehydrogenase against oxidation by O_2, and that NAD protects phosphoglyceraldehyde dehydrogenase, indicating that either O_2 or some intermediate must react directly with the enzyme SH groups. Haugaard (1946) also found a good correlation between the SH nature of enzymes and their susceptibility to O_2, the following sensitive enzymes being added to the list above: α-ketoglutarate oxidase, pyruvate oxidase, glutamate dehydrogenase, and xanthine oxidase. Dickens stated that succinate dehydrogenase is irreversibly inactivated by O_2, but Haugaard found reactivation by cysteine or glutathione, indicating a simple disulfide formation. Inactivation of certain enzymes during extraction and purification is probably due to oxidation by O_2 since metal-chelating agents, such as ethylenediaminetetraacetate (EDTA), are able to protect against such inactivation.

The cytochrome system may be involved in the inactivation of enzymes by O_2. Since cysteine is oxidized by O_2 through the cytochrome system to form cystine, and since cystine will in turn oxidize certain enzyme SH groups (see page 661), enzyme extracts in which cysteine is present may be unstable. Thus cysteine inhibits succinate dehydrogenase (Potter and DuBois, 1943), but in mouse kidney homogenates the inhibition shows a lag period which is interpreted as due to the necessary oxidation of cysteine to cystine by the cytochrome system (Ames and Elvehjem, 1944 a, b).

Various Minor Oxidants

A number of strong oxidants, such as permanganate, perchlorate, dichromate, and related compounds, have been used in the past to oxidize enzyme groups. Most of these have dropped out of use because they were felt to lack specificity toward SH groups, or other groups. Actually none of these oxidants has been studied thoroughly with respect to what enzyme groups are oxidized, or to the optimal conditions for achieving specificity. It is quite possible that, at certain pH's and concentrations and temperatures, these reagents may be specific oxidants. Certainly some enzymes are quite susceptible and others very resistant, and it would be interesting to know the reasons. For example, permanganate at 10 mM inhibits α-amylase $> 75\%$ (Di Carlo and Redfern, 1947), at 0.05 mM inhibits β-amylase 85%

(Ghosh, 1958), at 1 m*M* inhibits green gram flavokinase 48% (Giri *et al.*, 1958), at 0.1 m*M* inhibits β-fructofuranosidase 94% (Sizer, 1942 a), at 10 m*M* inhibits β-glycerophosphatase 100% (Rao *et al.*, 1960), at 2 m*M* inhibits *Aerobacillus* hydrogenlyase 23% (Crewther, 1953), at 0.01 m*M* inhibits intestinal phosphatase 95% (Sizer, 1942 b), at 1 m*M* inhibits *Pseudomonas* proteinase 100% (Morihara, 1963), and at 0.1 and 1 m*M* inhibits beef liver urocanase 13% and 87%, respectively (Feinberg and Greenberg, 1959). Since these experiments were done at different pH's, temperatures, and incubation times, it is difficult to compare the results accurately. Indeed, no thorough investigation of the effects of pH or temperature on such oxidations has been made. The moderate inhibition (12%) of liver arginase by 5 m*M* permanganate was believed due to an effect on the Mn^{++} cofactor rather than on the enzyme (Greenberg *et al.*, 1956). Although yeast β-fructofuranosidase is so sensitive to permanganate, it is inhibited only 16% by 10 m*M* dichromate (Sizer, 1942 a) and only 43% after 90 min incubation with 123 m*M* periodate (Myrbäck, 1957 b). Dichromate is also less effective than permanganate on β-glycerophosphatase (Rao *et al.*, 1960) and β-amylase (Ghosh, 1958). In this connection, it must be remembered that the products of the reduction of the oxidant may also be inhibitory, e.g., the MnO_2 or Mn^{++} from permanganate. The results of Taylor and Gale (1945) on *E. coli* amino acid decarboxylases are interesting in that the effects of permanganate were found to depend on the substrate used. For example, 0.1 m*M* permanganate inhibits the decarboxylation of histidine 15%, arginine 17%, glutamate 41%, ornithine 98%, lysine 100%, and tyrosine 100%. Whether there are different enzymes or different effects with the various substrates is not known. Permanganate and other strong oxidants can occasionally act on substrates or other components of the reaction. This is illustrated in the effects of permanganate and *p*-benzoquinone on the growth of *Fusarium* conidia (Braune, 1963). Both are inhibitory alone but when present together nullify each other and may actually stimulate growth. This was shown not to be due to some oxidation product of *p*-benzoquinone or reduction product of permanganate. The formation was postulated of a substance X which protects against *p*-benzoquinone and heavy metal ions. Indeed, treatment of maleate or tartrate with permanganate gives rise to substance X. Although such effects in cellular systems are complex, related actions must be expected in certain enzyme systems.

Results with nitrous acid are difficult to interpret, but in all cases the inhibition progresses very slowly (Myrbäck, 1926). Whereas the α-amylase from *B. subtilis* cannot be reactivated by H_2S after inhibition by nitrite (Di Carlo and Redfern, 1947), the β-amylase of barley is completely reactivated (Weill and Caldwell, 1945 a). In the former case it was concluded that SH groups are not involved in the inhibition, and in the latter case that they are. Similarly, inhibitions by redox dyes may or may not be at-

tributed to SH oxidation, but because of the molecular complexity of most dyes it would be very unlikely that specific effects on SH groups could be obtained. Inhibitions by dyes will be discussed in a separate chapter.

DISULFIDES

Cystine oxidizes certain protein SH groups and was first used for the determination of these groups by Mirsky and Anson (1935). They found that those protein SH groups reacting with nitroprusside are completely oxidized by cystine. It was stated by Mirsky and Anson, and has been restated by others, that cystine is one of the most specific oxidants of protein SH groups. One disadvantage of cystine is its low solubility; dithioglycolate was suggested as superior in this regard but has been used very little. Another disadvantage is perhaps that the oxidation-reduction potential of the cystine-cysteine couple is not high enough to oxidize all the SH groups, which, if it is assumed that the mean potential of protein SH groups is similar to free cysteine, must be true. A further complication is the formation of mixed disulfides (see page 639):

$$E\text{—}SH + R\text{—}S\text{—}S\text{—}R \rightleftarrows E\text{—}S\text{—}S\text{—}R + R\text{—}SH$$

which is not the simple oxidation of enzyme SH groups previously supposed, a portion of the disulfide being bound to the enzyme.

A few results on enzyme inhibition are summarized in Table 5-1. The inhibitions are not to be taken quantitatively because the conditions and the incubation times vary greatly. Particularly important is the duration of contact between the enzyme and the disulfide, since in almost all instances the reaction has been found to proceed very slowly. Rapkine (1938) found that phosphoglyceraldehyde dehydrogenase requires up to 5 hr for maximal inhibition by GSSG, and Hopkins *et al.* (1938) showed that the inhibition of succinate dehydrogenase develops steadily over 2 hr, and probably continues after that time. Whether this is due to the slowness of the oxidation of enzyme SH groups or to secondary factors is not known. One reason for the lack of inhibition of certain enzymes by disulfides may well be that sufficient time was not allowed for reaction. Such slow reactions limit the use of the disulfides for either SH group determination or enzyme studies. Insufficient examination of the reversibility of disulfide inhibitions by reduction also makes it difficult to evaluate the mechanism. Rapkine (1938) found that both GSH and cysteine reactivate GSSG-inhibited phosphoglyceraldehyde dehydrogenase, and Hopkins and Morgan (1938) obtained similar results with succinate dehydrogenase, but reversibility has not been attempted in most work. Many enzymes require SH groups for activity but others are active only when disulfide bonds are formed. Cytochrome oxidase

TABLE 5-1
INHIBITION OF ENZYMES BY DISULFIDES

Enzyme	Source	Disulfide	Concentration (m*M*)	% Inhibition	Reference
Aldehyde: NAD oxidoreductase	Beef liver	GSSG	0.33	0	Deitrich and Hellerman (1963)
Aminopeptidase	Calf lens	GSSG	10	0	Spector (1963)
α-Amylase	*Bacillus subtilis*	Cystine	10	0	Di Carlo and Redfern (1947)
β-Amylase	Barley	Cystine	—	0	Weill and Caldwell (1945 b)
	Wheat	GSSG	10	0	Ghosh (1958)
Catalase	Beef liver	Cystine	0.037	17	Pihl *et al.* (1961)
Cholinesterase	Human plasma	GSSG	10	0	Mounter and Whittaker (1953)
	Human erythrocytes	GSSG	10	0	Mounter and Whittaker (1953)
Glutamine: pyruvate transaminase	Rat liver	GSSG	1	12	T'ing-sêng (1961 a)
Glutamine synthetase	Peas	GSSG	10	0	Varner and Webster (1955)
Phosphatidate phosphatase	Pig kidney	Cystine	5	7	Coleman and Hübscher (1962)
Phosphoglucomutase	Rabbit muscle	GSSG	20	25	Milstein (1961)
3-Phosphoglyceraldehyde dehydrogenase	Rabbit muscle	GSSG	10	33	Rapkine (1938)
			16	90	
	Rabbit muscle	GSSG	0.5	0	Pihl and Lange (1962)
Proteinase	*Aspergillus saitoi*	Cystine	10	10	Yoshida and Nagasawa (1958)
	Beef lens	GSSG	2.5	50	van Heyningen and Waley (1963)
Ribose-P isomerase	Rabbit muscle	Cystine	0.2	0	Dickens and Williamson (1956)
Succinate dehydrogenase	Mouse kidney	GSSG	0.5	40	Ames and Elvehjem (1944 a)
	Rabbit muscle	GSSG	10	41	Hopkins *et al.* (1938)
			20	60	
			100	93	
UDP-glucose dehydrogenase	Pea seedlings	GSSG	10	42	Strominger and Mapson (1957)
Urease	Jack beans	Cystine	—	0	Hellerman *et al.* (1943)

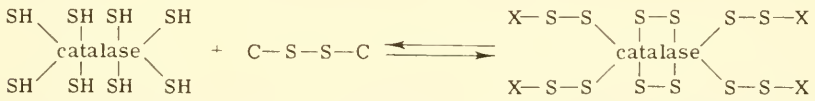
normally contains disulfide groups and can be inactivated by cysteine, GSH and other thiols; reactivation occurs with cystine or GSSG (Cooperstein, 1963). This may also be the case with lens aminopeptidase, which is inhibited rather strongly by cysteine and GSH but not at all by GSSG (Spector, 1963). Another reason for the lack of response to a disulfide is that the environment of the enzyme SH group may be unfavorable for the approach of the disulfide or may affect the redox potential in such a manner as to deter the interaction. If seralbumins are incubated with excess cystine, the SH content as determined by *p*-mercuribenzoate titration drops to zero; half the SH groups are lost from bovine hemoglobin (Isles and Jocelyn, 1963). GSSG has no effect on either type of protein. Incidentally, the reaction of cystine with bovine seralbumin leads to the disappearance of 1 molecule of cystine for each pair of SH groups lost, so it is likely that the cysteine formed is reoxidized and mixed disulfides are formed according to:

$$2\ \text{Cy—S—S—Cy} + 2\ \text{Prot—SH} \rightleftarrows 2\ \text{Cy—SH} + 2\ \text{Prot—S—S—Cy}$$

$$2\ \text{Cy—SH} \rightleftarrows \text{Cy—S—S—Cy} + 2\ \text{H}^+ + 2\ e^-$$

$$\text{Cy—S—S—Cy} + 2\ \text{Prot—SH} \rightleftarrows 2\ \text{Prot—S—S—Cy} + 2\ \text{H}^+ + 2\ e^-$$

Some evidence for the formation of mixed disulfides during the inhibition of enzymes has been reported. Beef liver catalase contains 8 SH groups titratable with *p*-chloromercuribenzoate. Reaction with cystine-S^{35} results in the oxidation of 4 of these groups to disulfides and the formation of 4 mixed disulfides (Pihl *et al.*, 1961):



This inhibition is spontaneously reversible; cystine at 0.037 m*M*, pH 7, and 37° inhibits maximally 17% around 5 min and then the inhibition decreases. Cystamine monosulfoxide likewise appears to form mixed disulfides with phosphoglyceraldehyde dehydrogenase, and this is reversible by thiols (Pihl and Lange, 1962). Cystamine itself inhibits glucose utilization in erythrocytes and this block is believed to be at hexokinase (Eldjarn and Bremer, 1962). The inhibition is reversible by thiols, and mixed disulfide formation was postulated; however, there was no direct evidence for this as opposed to simple oxidation. Dithioglycolate and cystine were reported to form mixed disulfides with myosin ATPase, but inhibition of the enzyme activity does not occur until the last 2 or 3 SH groups are altered (Bárány, 1959).

The optimal temperatures and pH's for reaction of enzyme SH groups with disulfides are difficult to determine in our present state of knowledge. Apparently the temperature coefficient is quite high; succinate dehydrogen-

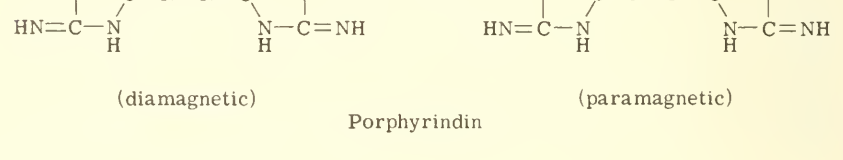
ase is inhibited 20% at 20°, 78% at 30°, and 93% at 40° by 100 m*M* GSSG after 4 hr incubation (Hopkins *et al.*, 1938). Thus one might expect inconveniently slow reactions at low temperatures. Yet Bárány (1959) ran his experiments with ATPase at 0°, although up to 15 hr was sometimes required for satisfactory reaction. Increase in the pH favors the oxidation of enzyme SH groups to disulfides, since the $-S^-$ form presumably reacts more readily. Hence, incubation of the enzyme with the disulfide at pH's around 9 may be useful where possible.

PORPHYREXIDE AND PORPHYRINDIN

Porphyrindin was originally synthesized by Piloty and Schwerin (1901 a, b, c; Piloty and Vogel, 1903) but only much later attracted attention, when it was studied by Kuhn *et al.* (1934) as the first clearly demonstrated double free radical, and shown by Kuhn and Franke (1935) to have one of the highest oxidation-reduction potentials among organic substances. It was introduced as a reagent for the determination of protein SH groups by Kuhn and Desnuelle (1938) because of its high potential and applied particularly by Greenstein (1938; Greenstein and Edsall, 1940; Greenstein and Jenrette, 1942) for this purpose. Meanwhile its synthesis was improved by Porter and Hellerman (1939). For the past 20 years it has been used sporadically to inactivate enzymes by oxidation of the SH groups. Possibly it has been neglected in enzyme studies since, although it is probably not as specific for SH groups as is *o*-iodosobenzoate, it is certainly more selective than most oxidants and, furthermore, reacts more rapidly and more completely.

Chemistry

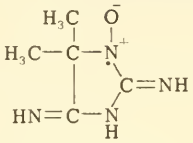
The structures of porphyrindin and porphyrexide may be written in several different ways because of resonance. Both in the crystalline state are paramagnetic, the values indicating one unpaired electron in porphyrexide and two in porphyrindin (Kuhn *et al.*, 1934). The paramagnetism, however, increases with temperature (Müller and Müller-Rodloff, 1935), suggesting equilibria between diamagnetic and paramagnetic forms. Thus the resonance structures for porphyrindin may be written as:



(diamagnetic) (paramagnetic)

Porphyrindin

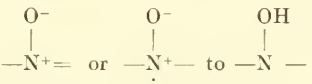
while for porphyrexide only the free radical form is possible:



Porphyrexide

The diamagnetic form is more stable than the paramagnetic by about 0.56 kcal/mole. Magnetic studies have indicated the free electrons to be fairly well localized and not diffusely distributed over the molecule. Possibly these free electrons contribute to the color of these substances: porphyrexide is red and porphyrindin a deep blue. Upon reduction the color disappears (leucoporphyrindin may be slightly yellow), this being the basis for the colorimetric titration of SH groups. Porphyrexide has an absorption maximum at 460 mμ and porphyrindin at 653 mμ (Kuhn and Franke, 1935).

Reduction involves the change from



and a disappearance of free radicals. The oxidation-reduction potentials at pH 7 and 18° are:

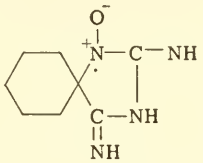
Porphyrexide: $E_0' = +0.725$ v

Porphyrindin: $E_0' = +0.565$ v

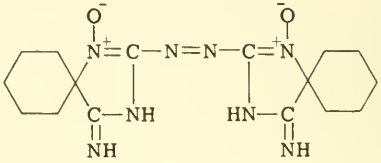
so that porphyrexide approaches the oxygen potential and both are well above most systems commonly seen in biological work. The oxidation of simple thiols is very rapid, cysteine and glutathione reacting almost instantaneously.

Porphyrindin is not very stable and the solid crystalline dye should be stored at low temperatures and desiccated. Greenstein (1938) pointed out that porphyrindin solutions are stable enough for an hour but the activity then decreases. Brand and Kassell (1940) reported that solutions at 0° show 3% deterioration in 1 hr, 5% in 2 hr, and 9% in 4 hr. At 25° deterioration occurs at a rate of about 0.5% per minute. Reactions of enzyme SH groups can usually be carried out at 0°, but in work with tissues at physiological temperatures this spontaneous decomposition must be borne in mind. Porphyrindin should be quantitatively determined in all accurate work since it is seldom pure; this may be done by titrating with asorbic acid (Chinard and Hellerman, 1954).

Spiro analogs of both porphyrexide and porphyrindin were synthesized by Porter and Hellerman (1944) and found to have high oxidation-reduc-



Spiroporphyrexide



Spiroporphyrindin

tion potentials. The E_h at pH 7 for spiroporphyrexide is + 0.69 v, the potential increasing at lower pH's. The spiroporphyrindin is quite insoluble and may be more polymerized than indicated. An interesting aspect of spiroporphyrexide is that it does not inhibit urease, despite its high potential, indicating possible steric effects of the cyclohexyl ring. This is a good example that not only is oxidation-reduction potential important in the oxidation of SH groups on enzymes but that structural configuration is a factor, as in any inhibition.

Oxidation of Thiols and Amino Acids

Porphyrexide and porphyrindin react very rapidly with thiols at neutrality and the end-point is generally quite sharp; cysteine, glutathione, and cysteinylcysteine are titrated quite comparably (Greenstein, 1938). The oxidation of cysteine at pH 7.2 is complete within 3–60 sec and glutathione is oxidized only slightly more slowly (Brand and Kassell, 1940). No reaction under ordinary conditions is seen with cystine, cysteate, tryptophan, hydroxyproline, histidine, methionine, serine, phenylalanine, or threonine. Tyrosine, however, is oxidized slowly with the formation of a pink-orange color, the reaction taking around 30 min for completion (2 equivalents of porphyrindin per mole of tyrosine) at pH 7.2 and 0°. Tyrosine and other phenols are oxidized more rapidly in alkaline solutions and at higher temperatures, but in most cases the reaction is much slower than the oxidation of SH groups (Greenstein and Edsall, 1940). At pH 7.33 and 25° the oxidation of tyrosine may be fairly rapid, and even tryptophan may be slowly reacted (half-reaction time around 2 hr) (Barron *et al.*, 1941). Porphyrindin can also oxidize a variety of other substances, such as ascorbate or thiamine (Kuhn and Desnuelle, 1938), and in complex systems or cellular preparations the effects may not be due entirely to SH group oxidation.

Oxidation of Proteins

Kuhn and Desnuelle (1938) showed that native ovalbumin does not react with porphyrindin (in common with other SH reagents) but that following

heat denaturation, titration gives results comparable to other methods for total cysteine. This was confirmed by Greenstein (1938), who used urea and guanidine for denaturation. The rapidity of SH oxidation was noted. Brand and Kassell (1940), on the other hand, did not find good end-points with denatured ovalbumin and, because of the pink color developed, felt that tyrosine groups are also oxidized. This was criticized by Greenstein *et al.* (1940) on the basis that far too much porphyrindin was used, and they emphasized that such high concentrations are probably not specific and should be avoided. The method was somewhat improved (Greenstein and Edsall, 1940; Greenstein and Jenrette, 1942) by reducing the reaction time and lowering the pH to 6.4–6.8 and, using myosin, seralbumin, ovalbumin, and tobacco mosaic virus, it was believed that accurate titration of the SH groups could be achieved with little interference from tyrosine oxidation. Barron *et al.* (1941) treated scarlet fever toxin with 1 m*M* porphyrindin for 1 hr at pH 7 and found that the activity of the toxin, as determined by skin tests, is abolished. Since other SH reagents do not inactivate the toxin, it was felt that oxidation of groups other than SH is involved. However, the SH reagents used (iodoacetate, iodoacetamide, hydrogen peroxide, alloxan, and Cu^{++}) are not the most satisfactory for the demonstration of SH groups, so this evidence is not conclusive. In order to achieve specificity toward SH groups it is advisable to (1) avoid alkaline conditions, (2) reduce the reaction time with porphyrindin as much as possible, (3) use as low concentrations of porphyrindin as possible, and (4) determine the disappearance of SH groups by some secondary titration.

Inhibition of Enzymes

Results of treating enzymes with these oxidants are shown in Table 5-2; one cannot help but be surprised that so little use has been made of these substances, especially during the past few years. It is evident that quite low concentrations are needed for those enzymes which have susceptible SH groups and that porphyrindin and porphyrexide are among the most potent oxidant inhibitors.

Balls and Lineweaver (1939 b) attempted to titrate papain with porphyrindin but found that no clear end-point could be obtained at room temperature, and at 2–3° no bleaching of the dye occurred during several minutes when dilute concentrations were used. Higher concentrations produce a pink coloration, even at pH 4.6. The native papain SH groups are thus not reactive with porphyrindin; neither are they reactive with nitroprusside. On the other hand, iodoacetate and iodoacetamide react and inhibit; papain treated with these alkylating agents still gives rise to the pink coloration, indicating that tyrosine is oxidized, although not necessary for enzyme activity. E. L. Smith (1958) concluded that the SH group which is

TABLE 5-2

INHIBITION OF ENZYMES BY PORPHYRINDIN AND PORPHYREXIDE

Enzyme	Source	Inhibitor[a]	Concentration (mM)	% Inhibition	Reference
Adenosinetriphosphatase	Rabbit muscle	P–n	0.013	32	Singer and Barron (1944)
Alcohol dehydrogenase	Yeast	P–x	1.66	45	Cedrangolo and Adler (1939)
α-Glycerophosphate dehydrogenase	Rabbit muscle	P–x	1.86	55	Cedrangolo and Adler (1939)
Lactate dehydrogenase	Rabbit muscle	P–x	1.86	20	Cedrangolo and Adler (1939)
Lipase	Pig pancreas	P–x	—	0	Cedrangolo (1941)
Malate dehydrogenase	Rabbit muscle	P–x	1.86	0	Cedrangolo and Adler (1939)
NADPH diaphorase	Yeast	P–n	—	0	Kuhn and Desnuelle (1938)
Papain	Papaya	P–n	—	0	E. L. Smith (1958); Balls and Lineweaver (1939 b)
Phosphoglyceraldehyde dehydrogenase	Rabbit muscle	P–x	0.9	75	Cedrangolo and Adler (1939)
Pyruvate decarboxylase	Yeast	P–x	0.7	60	Kuhn and Beinert (1943)
		P–n	2.1	0	Kuhn and Beinert (1943)
		P–n	0.019	6	Kuhn and Beinert (1947)
			0.075	21	
Succinate dehydrogenase	Pigeon breast	P–n	0.5	90	Barron and Singer (1945)
Xanthine oxidase	Milk	P–n	0.046	100	Harris and Hellerman (1956)

[a] Inhibitor is designated P–n for porphyrindin and P–x for porphyrexide.

at or near the active center of papain is in a high energy state, perhaps as a thiol ester, and this may explain why it is resistant to porphyrindin.

The reactive SH groups of urease are rapidly oxidized by porphyrindin and the nitroprusside test becomes negative (Hellerman, 1939). However, no inhibition occurs. Porphyrindin-treated enzyme is inhibited by *p*-chloromercuribenzoate, so that certain SH groups required for activity are resistant to porphyrindin. If an excess of porphyrindin is used, inactivation occurs slowly and is irreversible. Oxidation past the disulfide stage or oxidation of other groups is possible. Since, *p*-chloromercuribenzoate protects the enzyme from high concentrations of porphyrindin, it appears that SH groups are indeed involved. It was established later that there are two types of SH group in urease: reactive *a* groups not necessary for enzyme activity, and less reactive *b* groups at the active center. Porphyrindin reacts with the former but only slowly with the latter at higher concentrations (Hellerman *et al.*, 1943). If urease is denatured with guanidine, many more SH groups appear and react with porphyrindin.

Xanthine oxidase is inhibited readily by porphyrindin but this is not reversible with cysteine (Harris and Hellerman, 1956). The inhibition by *o*-iodosobenzoate is also irreversible. This problem comes up repeatedly with inhibitions by oxidants and seems on the surface to indicate that a simple oxidation to the disulfide stage does not occur. However, it is also possible that (1) the oxidation-reduction potential of the groups involved is such that cysteine will not reduce them, (2) the enzyme structure is altered by the formation of disulfide bonds, (3) oxidation past the disulfide stage has occurred, or (4) the formation of intermolecular disulfide linkages prevents the access of cysteine to the group. It is impossible to distinguish at this time between these different possibilities.

Effects on Tissue Function

Porphyrindin at 0.37–0.75 m*M* produces an increase in the contractile amplitude of the frog heart and this effect can last for as long as 90 min (Méndez, 1946; Méndez and Peralta, 1947). Higher concentrations of 3.7–7.5 m*M* bring about a progressive contracture, the heart stopping in systole in around 30 min. The rate is simultaneously slowed. The atria continue to beat after the ventricles have stopped. These effects can be prevented by glutathione but not reversed, as expected. In these respects the heart responds to porphyrindin much as it does to other SH reagents. The site or sites of action are not known, and it is useless to speculate since the relative sensitivities of the possible enzymes involved are undetermined.

A few miscellaneous and unrelated observations will be mentioned. The short-circuit current and potential of frog skin are altered by oxidants and reductants, such as quinones, dyes, and iodine, but there is little effect of porphyrindin at 1 m*M*; the potential may drop temporarily but the cur-

rent is unaffected (Eubank *et al.*, 1962). Porphyrindin injected at a dose of 200 mg in a pregnant mouse produced neuroblastic necrosis in the fetus, but not as much as *p*-chloromercuribenzoate, oxophenarsine, or *o*-iodosobenzoate, these damaging neuroblasts in a pattern similar to radiation (Hicks, 1953). SH reagents usually cause blebbing of Sarcoma 37 ascites cells. Porphyrindin has no effect at 2 m*M* but produces symmetrical blebs at 8 m*M* (Belkin and Hardy, 1961). Such blebbing involves a raising of the entire cell membrane and presumably is due to some disturbance in water transport.

FERRICYANIDE

Ferricyanide has been used widely as a fairly specific oxidant for the determination of protein SH groups. Furthermore, it is commonly used as an electron acceptor in various dehydrogenase systems, since it is reduced by some of the components in electron transport before cytochrome c, and ferrocyanide has often served as an electron donor in studying the cytochrome system. When ferricyanide or ferrocyanide is used for such purposes, it is important to consider the possibility of inhibition of the enzymes involved, particularly the dehydrogenases, and to use as low concentrations as possible. The first use of ferricyanide for the determination of SH groups was by Flatow (1928) and this was simplified by Mason (1930) so that the ferrocyanide formed could be colorimetrically estimated after transformation to Prussian blue, this being suggested by Folin's ferricyanide method for blood glucose. This reaction has been used for the histochemical localization of SH groups but is not very satisfactory. Anson and Mirsky (1931) noted that hemoglobin treated with ferricyanide yields a globin that no longer reacts with nitroprusside, but it remained for Schüler (1932) to show that ferricyanide oxidizes more than the heme group and that globin itself reacts after separation from heme. Mirsky and Anson during the next 10 years elucidated the nature of the reactions between ferricyanide and proteins, and applied their results to determination of protein SH groups.

Chemistry

The oxidation of thiols may be written as:

$$2\ Fe(CN)_6^{3-} + 2\ R{-}SH \leftrightarrows 2\ Fe(CN)_6^{4-} + R{-}S{-}S{-}R + 2\ H^+$$

When the SH groups are on different molecules, the kinetics are complex and the detailed mechanism of the reaction is not understood. The ferrocyanide formed is usually determined by addition of Fe^{+++}, forming Prussian blue, but in work with proteins it is advisable to determine also the disappearance of SH groups with nitroprusside or *p*-chloromercuribenzoate, since groups other than SH may be oxidized. The oxidation-reduction po-

tential of the ferricyanide-ferrocyanide couple is + 0.36 v and does not change from pH 4 to 10. The potential is, however, rather strongly dependent on ionic strength. The potential is thus sufficiently high for SH groups to be oxidized completely if they are available to the ferricyanide, and the reaction is generally quite rapid.

Most commercial preparations of ferricyanide contain ferrocyanide, which may be detected by the Prussian blue method, and the latter should be removed by addition of a little bromine water if SH determinations are done by the colorimetric technique. Solutions of ferricyanide should be stored in the cold and dark to avoid changes.

Oxidation of Thiols and Proteins

The reactions of ferricyanide with proteins have direct bearing on the effects of ferricyanide on enzymes so that it is necessary to discuss the results in some detail. Although titration of cysteine and glutathione with ferricyanide is rapid and provides good end-points, reactions with proteins may not be so clear-cut. The conditions for the reaction are very important. There is a marked effect of pH, as shown originally by Mirsky and Anson (1936 a) for hemoglobin (see tabulation). Indeed, at pH 6.8 one may specifically oxidize the heme iron to form methemoglobin without affecting SH groups. The conditions for reaction were: 83 m*M* ferricyanide incubated with the protein for 30 min at room temperature — all reactive SH goups are oxidized, as shown by titration of SH groups in denatured globin. Kolthoff and Anastasi (1958) have also observed that oxidation of the SH groups of denatured seralbumin is faster at pH 9 than 7. They noted that the reaction is accelerated by Cu^{++}, and Katyal and Gorin (1959) found in a study of ovalbumin that iodide also catalyzes the oxidation by ferricyanide.

pH	Total SH groups oxidized (%)
6.8	0
7.3	28
9.0	44
9.5	65

Ferricyanide is not specific for SH groups, however, unless the conditions are rigorously controlled, as shown early by Mirsky and Anson (1936 b) in proteins not containing cysteine (zein and serum globulin) but nevertheless reducing ferricyanide, or in proteins previously treated with cystine to oxidize all the available SH groups. These groups are oxidized more slowly than the SH groups and are more difficult to oxidize (e.g., milder oxidants than

ferricyanide will not oxidize them), but their total reducing capacity (the amount of ferricyanide they can reduce) is often greater than for the SH groups. Furthermore, the rate and degree of oxidation of these non-SH groups depend on the same factors as reaction with SH groups; thus, the rate is accelerated by rise in pH, rise in temperature, and denaturation, Native ovalbumin is not oxidized at all by ferricyanide, but denatured ovalbumin treated with cystine to remove SH groups reduces ferricyanide. In other words, these other groups become available during unfolding of the protein. β-Lactoglobulin, which contains 2 SH groups per molecule (molecular weight of 37,000), reacts very slowly with ferricyanide in the native state but rapidly in the presence of urea or guanidine (Leslie *et al.*, 1962 a). The stoichiometry indicates that the SH groups are oxidized beyond the disulfide stage. Since the reaction was carried out under fairly mild conditions (0.1–0.8 m*M* ferricyanide, pH 7, 37°, and 30–45-min incubation), it is evident that one cannot generally assume the simple formation of disulfides from the actions of ferricyanide on enzymes. Ferricyanide can oxidize tyrosine and tryptophan, but not histidine, and the characteristics of the oxidation parallel oxidations of proteins. Mirsky and Anson suggested that tyrosine and tryptophan are the residues responsible for ferricyanide reduction. Gelatin, which contains no (or very little) tyrosine and no tryptophan, scarcely reacts with ferricyanide, supporting this view. Anson (1939 b) observed that at pH 9.6, where much previous work had been done, the oxidation is nonspecific, but that at pH 6.8 combined with the treatment of the protein with Duponol PC the SH groups react rapidly and specifically if not too much ferricyanide is used (2–5 m*M* is best); under these conditions there is no reaction with cystine, tyrosine, tryptophan, or proteins that do not contain cysteine. The specificity of SH group oxidation could also be shown by pretreatment of denatured ovalbumin with iodoacetamide, following which ferricyanide is no longer reduced by the protein. Katyal and Gorin (1959) also demonstrated such specificity by blocking SH groups with *p*-chloromercuribenzoate. Mirsky (1941) discussed the method in detail and showed that when properly run the oxidation occurs within 1 min. Barron (1951) has also reported in detail his modification of the method. Various oxidations by ferricyanide have been reviewed by Thyagarajan (1958).

Inhibition of Enzymes

One must conclude from the results with proteins that application of ferricyanide to enzymes cannot be done haphazardly if specific effects on SH groups are to be anticipated. Unfortunately most studies have used ferricyanide along with numerous other inhibitors under the same conditions of pH, temperature, and incubation time, without considering that rather stringent conditions have been proposed for the use of ferricyanide. Some

inhibitions are summarized in Table 5-3. Certain enzymes which possess SH groups reactive with other reagents, e.g. urease, are not inhibited by even high concentrations of ferricyanide. One might imagine ferricyanide to be unable to gain access to the SH groups. Ferricyanide is not only a fairly large ion but has a strong negative charge. If the enzyme SH group occupied a region of high negative charge, this might repel the ferricyanide and reduce the reaction. Indeed, one must always consider the possibility that ferricyanide inhibits certain enzymes by mechanisms other than oxidation, and related more to its charge and structure. For example, it would not be so surprising if ferricyanide inhibits succinate dehydrogenase to some extent because it can interact with the cationic groups normally binding succinate. One notes also that ferrocyanide generally inhibits aconitase more strongly than does ferricyanide, and here redox reactions may be of no significance (Rahatekar and Rao, 1963). On the other hand, some enzymes are inhibited just as rapidly and completely by ferricyanide as by the more commonly used SH reagents; myosin ATPase is one example (Singer and Barron, 1944). The inhibitions of papain and aldolase are quite reversible with cysteine, indicating that reversible oxidation is the mechanism of the inhibitions. Oxidation of coenzymes or cofactors can also occur. Ferricyanide can directly oxidize NADH but the rate is slow (Schellenberg and Hellerman, 1958). In the case of homogentisicase it may well be the Fe^{++} that is oxidized but, on the other hand, there appears to be a tyrosine phenolic group at the active site (Tokuyama, 1959).

It is interesting that Weill and Caldwell (1945 b) report β-amylase to be not readily inhibited by either ferricyanide or Cu^{++} alone but strongly inhibited when both are present, even when the ferricyanide is at a concentration noninhibitory by itself (see accompanying tabulation). Could this

Ferricyanide (mM)	Cu^{++} (mM)	% Inhibition
0.2	—	12
—	0.32	4
0.02	0.32	93

relate to the observation of Katyal and Gorin (1959) that Cu^{++} accelerates the action of ferricyanide? Or does the Cu^{++} in some manner alter the enzyme structure so that ferricyanide can attack the SH groups more easily?

Effects on Cellular Metabolism and Function

Mendel (1937) reported that Balogh mouse tumor glycolysis is markedly depressed by 10 mM ferricyanide and that the inhibition is maintained

TABLE 5-3

INHIBITION OF ENZYMES BY FERRICYANIDE

Enzyme	Source	Ferricyanide (m*M*)	% Inhibition	Reference
Acetanilide deacetylase	Pig liver	1	Stim 8	Krisch (1963)
Aconitase	*Penicillium purpurogenum*	0.5	32	Rahatekar and Rao (1963)
	Aspergillus niger	0.5	13	Rahatekar and Rao (1963)
	E. coli	0.5	8	Rahatekar and Rao (1963)
	Micrococcus lysodeikticus	0.5	5	Rahatekar and Rao (1963)
	Bacillus subtilis	0.5	0	Rahatekar and Rao (1963)
	Pseudomonas fluorescens	0.5	0	Rahatekar and Rao (1963)
	Streptomyces griseus	0.5	0	Rahatekar and Rao (1963)
	Yeast	0.5	0	Rahatekar and Rao (1963)
	Peas	0.5	2	Rahatekar and Rao (1963)
	Vigna catjang (cholai)	0.5	0	Rahatekar and Rao (1963)
Adenylate deaminase	Rabbit muscle	1.33	0	Lee (1957)
Alanine dehydrogenase	*Bacillus cereus*	1	0	O'Connor and Halvorson (1960)
Aldolase	Rabbit muscle	0.5	88	Birkenhäger (1960)
Aminoacetone synthetase	Guinea pig liver	0.5	33	Urata and Granick (1963)
Aminopeptidase	Beef lens	0.3	50	Wolff and Resnik (1963)
α-Amylase	*Bacillus subtilis*	1	0	Di Carlo and Redfern (1947)
		10	10	
		100	34	
β-Amylase	Barley	0.2	12	Weill and Caldwell (1945 b)

	Sweet potato	1	0	Englard *et al.* (1951)
	Wheat	3	0	Ghosh (1958)
L-Arabinose dehydrogenase	*Pseudomonas saccharophila*	10	50	Doudoroff *et al.* (1958)
Arylsulfatase	*Charonia lampas* liver	2	41	Takahashi (1960 a)
Aspartate: α-ketoglutarate transaminase	Pig heart	0.1	17	Polyanovskii (1962)
Carbonic anhydrase	Spinach leaves	1	0	Chiba *et al.* (1954 a)
Cellulose polysulfatase	*Charonia lampas* liver	2	0	Takahashi (1960 b)
Chlorophyllase	Rye seedlings	2	17	Klein and Vishniac (1961)
Cholinesterase	*Electrophorus electricus*	0.5	10	Hargreaves (1955)
	Human plasma	10	0	Mounter and Whittaker (1953)
	Human erythrocytes	10	0	Mounter and Whittaker (1953)
		10	0	Markwardt (1953 a)
DDT dehydrochlorinase	Flies	1	0	Lipke and Kearns (1959)
Deoxyribonuclease	*Bacillus subtilis*	0.01	Stim 4	Watanabe and Yamafuji (1961)
		0.1	20	
		1	66	
2′-Deoxyribosyl-4-amino-pyrimidone-2,5′-P deaminase	Monkey liver	0.5	10	Scarano *et al.* (1962)
Formate transacetylase	*Micrococcus lactilyticus*	3.8	27	McCormick *et al.* (1962)
β-Fructofuranosidase	Yeast	10	16	Sizer (1942 a)
	Yeast (pH 5.3)	0.12	4	Myrbäck (1957 b)
	Yeast (pH 8.3)	0.12	34	
Galactose dehydrogenase	*Pseudomonas saccharophila*	10	100	Doudoroff *et al.* (1958)
Homogentisicase	Beef liver	0.25	76	Tokuyama (1959)
		0.5	85	
		1	90	
		2	100	

TABLE 5-3 (*continued*)

Enzyme	Source	Ferricyanide (m*M*)	% Inhibition	Reference
Hydrogenase	*Desulfovibrio desulfuricans*	1	7	Krasna *et al.* (1960)
Lipase	Wheat germ	2	43	Singer and Hofstee (1948 a)
	Lingeod muscle	2	1	Wood (1959)
	Pancreas	2	0	Wills (1960)
Luciferase	*Odontosyllis phosphorea*	1	65	Shimomura *et al.* (1963)
NADH oxidase	*Streptococcus faecalis*	0.2	15	Dolin and Wood (1960)
NADH: quinone oxidoreductase	Pig liver	1.3	50	Frimmer (1960)
Papain	Papaya	0.4	70	Hellerman and Perkins (1934)
Penicillinase	*Aerobacter cloacae*	2	13	Smith (1963 b)
Phosphatase	Beef intestine	1	44	Sizer (1942 b)
3-Phosphoglyceraldehyde dehydrogenase	Rabbit muscle	1	0	Birkenhäger (1960)
Proteinase	*Rastrelliger kanagurta* (mackerel)	10	8	Sundaram and Sarma (1960 a)
Pyruvate carboxylase	Chicken liver	6.7	0	Keech and Utter (1963)
Pyruvate oxidase	Dog heart	33	80	Kuratomi (1959)
Succinate dehydrogenase	*Mycobacterium tuberculosis*	10	2	Tanaka (1960)
	Rat liver	1	89	Potter and DuBois (1943)
	Horse leucocytes	0.25	100	Vercauteren (1957)
Tryptophan synthetase	*E. coli*	1	66	Wada *et al.* (1958)
β-Tyrosinase	*E. coli*	1	56	Ichihara *et al.* (1956)
Urease	Jack beans	—	0	Hellerman (1939)

when the ferricyanide is washed out. Ferricyanide slowly injected intravenously into mice (1.5 g/kg of sodium salt) produces no disturbance of tumor metabolism, but when the various tissues are treated with Fe^{+++}, only the tumor turns blue. The anaerobic glycolysis of no other tissue is depressed by ferricyanide, which in this respect differs from other glycolytic inhibitors (Mendel and Strelitz, 1937). Renal medulla was studied particularly because it has a significant rate of aerobic glycolysis and ferricyanide was found to have no effect (and in some cases even stimulated somewhat). This action was not investigated further until Birkenhäger (1959, 1960) attempted to locate the site of inhibition. He confirmed that 10 m*M* ferricyanide does indeed inhibit both aerobic and anaerobic glycolysis in Walker and Crocker tumors, but not in Ehrlich ascites cells, and further showed that it inhibits the extra glycolysis brought about by dinitrophenol. The respiration in the presence of glucose rises 30–60% in the presence of ferricyanide in the two former tumors, but not in the ascites cells. The problem of what happens to the glucose taken up, since this is not depressed as much as lactate formation, remains unsolved. A small accumulation of pyruvate was found under aerobic conditions but not anaerobically, and no other glycolytic intermediates could be detected. Use of glucose-1-C^{14} and glucose-6-C^{14} pointed to the conclusion that ferricyanide either directly or indirectly inhibits glycolysis at the level of phosphohexose isomerase or phosphohexokinase; this would make more hexose phosphate available for the pentose phosphate shunt. However, aldolase was found to be very sensitive to ferricyanide (88% inhibition at 0.5 m*M*) and addition of aldolase to a tumor extract in which glycolysis has been abolished by ferricyanide leads to recovery. Phosphoglyceraldehyde dehydrogenase is not sensitive to ferricyanide nor does its addition reverse the glycolytic inhibition. Birkenhäger ascribed the difference between cells and extracts in susceptibility to ferricyanide as due to permeability factors. Certainly one might expect an ion such as ferricyanide to enter cells with difficulty. However, the initial observation of Mendel that tumor tissue is specifically sensitive to ferricyanide remains to be explained. If such a difference exists, it must be due to ferricyanide penetrating into tumor cells more readily, since none of the enzymes considered to be the point of attack differs markedly in tumor cells compared with normal tissues.

Inasmuch as ferrocyanide is presumably formed in tissues during the reduction of ferricyanide, the effects of ferrocyanide on the tricarboxylate cycle may play a role in any over-all action. Martin (1955) noted that growth of *Aspergillus niger* is inhibited by ferrocyanide at concentrations below 0.002 m*M*. However, acid production may not be simultaneously inhibited, and is depressed 50% only at 0.4 m*M*. An accumulation of citrate is actually observed at 1 m*M* ferrocyanide and, at this concentration, isocitrate dehydrogenase is inhibited 100% (Ramakrishnan *et al.*, 1955). In

A. tereus 0.1 m*M* ferrocyanide has no effect on glucose utilization but increases the yield of itaconate, due presumably to the piling up of citrate (Bentley and Thiessen, 1957). The uptake and metabolism of itaconate are inhibited by ferrocyanide, which is reasonable on the basis of the inhibition of isocitrate dehydrogenase, and the assumption that itaconate feeds into the cycle (Shimi and Nour El Dein, 1962). There has been very little work on the effects of either ferro- or ferricyanide on cycle enzymes, but it appears likely that ferrocyanide blocks tricarboxylate steps selectively, whereas ferricyanide less potently inhibits pyruvate oxidation. More work should be done on these effects since no other specific inhibitor of isocitrate dehydrogenase is known.

A few miscellaneous observations on ferricyanide may be interpreted when more is known of the basic actions. Thus, 10 m*M* ferricyanide inhibits P^{32} incorporation into phospholipids 19% while stimulating respiration 17% in *M. tuberculosis*, in this way acting more like the uncoupling agents (azide, dinitrophenol, and arsenate) than the common SH reagents (Tanaka, 1960). Whereas other oxidants and SH reagents frequently cause mitochondrial swelling, ferricyanide is without effect, which could scarcely be due to permeability factors (Rall *et al.*, 1962). Porphyrin synthesis from porphobilinogen is strongly inhibited by 1 m*M* ferricyanide; this may be partly the result of direct oxidation of the porphyrins (Rimington and Tooth, 1961). The eggs of *Urechis* and *Hemicentrotus* are very sensitive to ferricyanide, 0.02 m*M* elevating the fertilization membrane in the former and causing cytolysis in the latter (Isaka and Aikawa, 1963). The dorsal-ventral modification produced by ferricyanide in *Dendraster* eggs, whereby either dorsal induction or ventral inhibition is manifest, is similar to that produced by iodoacetate or iodine, but is unexplainable since the factors involved in bilaterality are completely unknown (Pease, 1941). The effects of ferricyanide on the naturally occurring quinones must occasionally be important. For example, ferricyanide potentiates very markedly the growth-inhibiting activity of menadione on yeast, due to the fact it reoxidizes the reduced menadione and hence maintains the naphthoquinone in the active form (Kiesow, 1960 b).

IODINE

Iodine has been used more frequently than the oxidants previously discussed for the oxidation of protein SH groups and in enzyme studies, and yet it seems under most conditions to be less specific than the others. Although it is quite a potent inhibitor of many enzymes, unless one can determine if a particular protein group is oxidized, or otherwise attacked, the information obtained is negligible. Another complication in the use of iodine is the multiplicity of forms in solution and the difficulty in characterizing the nature of the oxidation reaction.

Chemistry

Iodine is soluble to the extent of 1.33 mM in water at 20°, which is much less than the other halogens. There is interaction with the water, which initially was written as a hydration:

$$I_2 + H_2O \leftrightarrows I_2 \cdot H_2O$$

but evidence pointing to the highly polarized state of iodine in the complex has suggested the following reaction:

$$I_2 + H_2O \leftrightarrows I^+ \cdot H_2O + I^-$$

The equilibrium constant for this reaction has been estimated as roughly 10^{-10}. Iodine may also undergo hydrolysis:

$$I_2 + H_2O \leftrightarrows I^- + HOI + H^+$$

the equilibrium constant being 3×10^{-13}. The hypoiodous acid formed has a pK_a of 12.3 so is little ionized at physiological pH's. The hypoiodous acid can also go to iodate, especially in alkaline solution:

$$3\ HOI \rightarrow IO_3^- + 2\ I^- + 3\ H^+$$

A third reaction of iodine is with the iodide ion:

$$I_2 + I^- \leftrightarrows I_3^-$$

to form the triiodide, which is the principal reason for the greater solubility of iodine in KI solutions. The equilibrium is given by:

$$\frac{(I_2)\ (I^-)}{(I_3^-)} = 1.38 \times 10^{-3}$$

Thus iodine would be soluble in 50 mM KI solution to the extent of 46 mM, an appreciable increase over the 1.33 mM in water. The production of nascent oxygen by the reaction:

$$I_2 + H_2O \rightarrow 2\ H^+ + 2\ I^- + O$$

which has been believed to be involved in protein oxidations, does not occur with iodine, although the other halogens react to some extent in this way. In most biological work, iodine is dissolved in fairly strong KI or NaI solution. This not only serves to increase the solubility, but limits the fraction of the iodine in other forms (HOI, IO_3^-, and $I^+ \cdot H_2O$); the principal form here is presumably the I_3^- anion. However, there will always be significant concentrations of iodine present. The relative importance of the I_2 and I_3^-

forms in the oxidation of SH groups is not known. The oxidation-reduction potential for iodine varies with the type of reaction in which it participates and the pH, but is usually sufficiently high to oxidize any accessible SH groups. It is important in certain enzyme studies to realize that iodine may disappear fairly rapidly from solution, independently of reaction with organic materials; such is favored by lack of iodide and high pH.

Reaction of Iodine with Thiols

Iodine is able to oxidize SH groups to four different states: the disulfide (S—S), the sulfenate (SO^-), the sulfinate (SO_2^-), and the sulfonate (SO_3^-). Apparently it is quite easy to oxidize beyond the disulfide state with iodine. The stoichiometry of a particular reaction will depend not only on the state of oxidation of the SH groups, but also on the degree of reduction of the iodine, this varying with the pH. For example, the following reactions can be written for oxidation to the disulfide state:

$$I_2 + 2\ R{-}SH \rightarrow R{-}S{-}S{-}R + 2\ I^- + 2\ H^+$$

$$2\ I_2 + 2\ R{-}SH + H_2O \rightarrow R{-}S{-}S{-}R + HOI + 3\ I^- + 3\ H^+$$

$$4\ I_2 + 2\ R{-}SH + 3\ H_2O \rightarrow R{-}S{-}S{-}R + IO_3^- + 7\ I^- + 8\ H^+$$

However, it is likely that near neutrality the first reaction is dominant. In the oxidation of cysteine, 3 equivalents of iodine are taken up to form cysteate:

$$3\ I_2 + R{-}SH + 3\ H_2O \rightarrow R{-}SO_3^- + 6\ I^- + 7\ H^+$$

It is interesting that, at pH 3.2, iodine oxidizes cysteine well, but does not react with cystine, tyrosine, or histidine. This indicates that the first product in the oxidation of cysteine is not cystine but free radicals, which can either combine to form disulfides or be further oxidized to sulfonate groups (Anson, 1940). At pH 6.8, cystine is the major product. In most instances, especially with proteins, several reactions will occur and mixed products will be found. In addition to these straightforward oxidations, we shall see that there is now evidence for the formation of sulfenyl iodide groups (SI), so that a certain fraction of the iodine can be incorporated into the thiol molecule.

Reactions of Iodine with Proteins

The SH groups of denatured ovalbumin are oxidized by iodine within 5 min at pH 3.2 and 37° (Anson, 1940). The rate of the reaction decreases as the pH is raised to 6.8. In acid media iodine does not react with tyrosine or proteins containing tyrosine (e.g. pepsin), whereas at neutrality it readily iodinates tyrosine. By proper choice of pH and iodine concentration it is possible to oxidize the SH groups of denatured ovalbumin without appre-

ciably altering tyrosine residues (Anson, 1941). Iodine reacts only with the tyrosine residues of seralbumin, and denaturation accelerates the formation of diiodotyrosine (Li, 1945). The rate of the reaction is, however, quite slow in native seralbumin (half-reaction time around 100 min). Human seralbumin iodinated at low temperature takes up 36 atoms of iodine per mole of albumin, but only 12 diiodotyrosyl groups are found (Hughes and Straessle, 1950). The remainder was believed to be incorporated into histidyl residues. Some oxidation of cysteinyl residues also occurs and this presumably is beyond the disulfide stage, since 2.2 moles of iodine are taken up per SH group. In any particular case, the amount of disulfide formed will depend to a large extent on steric factors, i.e., how readily the sulfhydryl radicals can combine; the seralbumin molecule is fairly large and, not surprisingly, disulfide groups are not found after oxidation. Although no degradative changes in seralbumin are observed, protein structure is certainly modified by treatment with iodine, since the water binding capacity is increased (Jensen *et al.*, 1950) and the rates of pepsin and trypsin digestion are decreased (Raghupathy *et al.*, 1958).

We have noted that 2 to 3 atoms of iodine are occasionally utilized for each protein SH group. This might indicate (1) oxidation of SH beyond the disulfide state, (2) reduction of the iodine beyond the iodide state, or (3) some substitution of iodine in the cysteinyl residue. This problem was studied by Fraenkel-Conrat (1955) with tobacco mosaic virus protein. It is possible that sulfenate or sulfenyl iodide groups might be produced, but it has always been thought that such groups are quite unstable and cannot exist for appreciable time. However, the virus protein SH groups react with 2 atoms of iodine fairly rapidly, and this was shown to be accompanied by the formation of sulfenyl iodide groups:

$$\text{V—SH} + \text{I}_2 \rightarrow \text{V—SI} + \text{I}^- + \text{H}^+$$

This group appears to be stable in this particular protein. Fraenkel-Conrat pointed out that further reaction with thiols can form mixed disulfides:

$$\text{V—SH} + \text{R—SH} \leftrightarrows \text{V—S—S—R} + \text{H}^+ + \text{I}^-$$

Such reactions have been studied further in β-lactoglobulin by Cunningham and Nuenke (1959, 1960, 1961), using a spectrophotometric method. This protein reacts with 4 equivalents of iodine to form 2 sulfenyl iodide groups per mole of protein:

$$\text{P(—SH)}_2 + 2\ \text{I}_2 \rightarrow \text{P(—SI)}_2 + 2\ \text{I}^- + 2\ \text{H}^+$$

Ovalbumin reacts similarly but 6 equivalents of iodine are taken up. The sulfenyl iodide groups are quite stable in these proteins, but can react with simple thiols (e.g., glutathione, cysteine, and others) to form mixed disul-

fides. Intermolecular disulfide formation was ruled out for these proteins. Ovalbumin has 4 SH groups and 2 S—S groups; the protein treated with iodine has 2 SH groups and 2 S—S groups, and has incorporated 1 iodine atom (Winzor and Creeth, 1962). Since 5 atoms of iodine are taken up, it is not a simple oxidation to disulfide. It was suggested that the following reactions occur:

$$2\ P(-SH)_2$$
$$\downarrow + I_2$$
$$HS-P-S-S-P-SH$$
$$\downarrow + 2\ I_2$$
$$IS-P-S-S-P-SI$$
$$\downarrow + 2\ I_2 + 3\ H_2O$$
$$IS-P-S\bar{O} \quad + \quad \bar{O}_2S-P-SI$$

where P represents that portion of the protein not reacting with iodine. Further oxidation of the sulfenate group to sulfinate may occur to give a homogeneous product. Therefore the formation of sulfenyl iodide groups and oxidation of SH groups to sulfenate and sulfinate must be considered as likely possibilities in enzymes treated with iodine.

Inhibition of Enzymes

Many enzymes have been found to be readily inhibited, often by low concentrations of iodine (Table 5-4). It is impossible to know in most cases whether the inhibition is due to reaction with SH groups or to iodination of tyrosine. The fact that most studies have been done at pH's around neutrality implies that both SH and tyrosyl groups could be reacted, so that the relative importance would depend on the accessibility of the groups and their location with respect to the active center. Fixation of iodine into an enzyme does not imply inhibition; an example is *Aspergillus* protease (Dhar and Bose, 1962). The inhibition of certain enzymes by iodine is probably related to oxidation of SH groups: papain, creatine kinase, urease, aldolase, lactate dehydrogenase, succinate dehydrogenase, pyruvate decarboxylase, and adenosinetriphosphatase. Other enzymes, such as pepsin or peroxidase, are inhibited through tyrosine iodination, and in some instances a mixed mechanism is probable.

One way of determining if SH group oxidation is responsible for enzyme inhibition is to attempt reversal with thiols. Complete reversal certainly implies such a mechanism, but negative results can be interpreted in various ways. Even oxidation to disulfide groups is not necessarily reversed by thiols if steric factors prevent reaction, and oxidation past the disulfide

stage would not be expected to be reversed. No reactivation of β-galactosidase (Knopfmacher and Salle, 1941) or α-amylase (Di Carlo and Redfern, 1947) is observed; however, in both cases there is some reason for believing that SH groups are involved. Partial reactivation of β-amylase (Weill and Caldwell, 1945 b) and phosphoglyceraldehyde dehydrogenase (Rapkine, 1938) was taken to mean that at least SH group oxidation is responsible for the inhibition. Essentially complete reactivation with thiols has been found for urease (Hellerman, 1939), papain (Hellerman and Perkins, 1934), cholinesterase (Nachmansohn and Lederer, 1939), and lactate dehydrogenase (Nygaard, 1955) so that an SH mechanism seems assured for these. The mechanism of the inhibition of β-fructofuranosidase is still unknown, although it is the first enzyme studied with iodine. The enzyme is inhibited fairly rapidly to the extent of 45–50%, but further inactivation proceeds very slowly (Myrbäck, 1926). A "Jodsaccharase" was assumed, but the iodine must not be fixed at the active center since there is no decrease in the affinity for the substrate. There is no reactivation by reduction (Myrbäck, 1957 a) so there is little evidence for SH group oxidation. Sulfenyl iodide groups may be involved.

There has been little study of the disappearance of SH groups during inhibition by iodine. Cardiac lactate dehydrogenase SH groups are rapidly oxidized by iodine, as determined with Ag^+ and spectrophotometrically with *p*-mercuribenzoate, and the inhibition develops in parallel fashion (Nygaard, 1956). The lactate dehydrogenase from rabbit muscle, on the other hand, incorporates iodine at 0° and pH 8 over many hours; when 1 atom of iodine is incorporated per molecule of enzyme, the inhibition is 30%, and the inhibition increases until 21 atoms of iodine are incorporated. Both NAD and oxalate protect the enzyme against iodination. Although the results with iodoacetamide indicate an SH group at the active site, one cannot be certain if this is the initial point of attack for the inhibition (Dube *et al.*, 1963). It is likely in situations like this that both SH group oxidation and iodination of tyrosine occur.

Pepsin is not an SH enzyme but is inhibited by iodine, and here it is highly probable that tyrosine iodination occurs. The activity of pepsin decreases with the amount of iodine incorporated; it is inactive when 35–40 atoms of iodine are bound (Herriott, 1937). 3-Iodotyrosine has been isolated from inhibited pepsin (Herriott, 1947), confirming the importance of tyrosine for the enzyme activity. Of the 6 tyrosyl groups in ribonuclease, 3 are unreactive, and the problem of where these are in the polypeptide chain was studied with iodine (Cha and Scheraga, 1961 a,b). At pH 9.4 and 10° — conditions favorable for tyrosine iodination with minimal effects on other groups — 3 tyrosyl residues are iodinated; the others can be iodinated only very slowly. The iodinated tyrosyl residues were located in the amino acid sequence. Such techniques will undoubtedly become more common

TABLE 5-4

INHIBITION OF ENZYMES BY IODINE

Enzyme	Source	Iodine (m*M*)	% Inhibition	Reference
Adenosine-5′-phosphosulfate reductase	*Desulfovibrio desulfuricans*	1	80	Ishimoto and Fujimoto (1961)
Adenosinetriphosphatase	Rabbit muscle	0.2	71	Ziff (1944)
	Rabbit muscle	0.01	Inh	Bailey and Perry (1947)
A-esterase (DFPase?)	Rabbit serum	0.1	5	Mounter (1954)
Aldolase	Rabbit muscle	0.002	18	Herbert *et al.* (1940)
		0.007	46	
		0.02	100	
	Peas	0.1	0	Stumpf (1948)
α-Amylase	*Bacillus subtilis*	0.1	8	Di Carlo and Redfern (1947)
		1	48	
		10	100	
β-Amylase	Barley	0.078	Inh	Weill and Caldwell (1945 b)
Arylsulfatase	*Alcaligenes metalcaligenes*	5	91	Dodgson *et al.* (1955)
Asclepain *m*	*Asclepias mexicana*	0.5	94	Greenberg and Winnick (1940)

Aspartase	*Propionibacterium peterssonii*	0.1	25	Ellfolk (1953)
		1	75	
Bromelin	Pineapple fruit	1	100	Greenberg and Winnick (1940)
Carbonic anhydrase	Spinach leaves	5	0	Chiba *et al.* (1954 a)
Cathepsin		0.2	73	Maschmann and Helmert (1933 b)
Choline acetylase	Rat brain	0.1	75	Nachmansohn and Machado (1943)
Cholinesterase	*Torpedo* electric organ	0.018	85	Nachmansohn and Lederer (1939)
	Human plasma	0.4	50	Mounter and Whittaker (1953)
Chymotrypsin	Pancreas	0.5	65	Sizer (1945)
Creatine kinase	Sheep muscle	0.013	30	Ennor and Rosenberg (1954)
Cytochrome b_5 reductase	Calf liver	0.01	100	Strittmatter (1961 a)
β-Fructofuranosidase	Yeast	0.05 mg/g	50	von Euler and Josephson (1923)
	Yeast (pH 4.15)	0.72	50	Myrbäck (1926)
	Yeast (pH 7)	0.72	100	
	Yeast	0.1	100	Sizer (1942 a)
β-Galactosidase	*E. coli*	0.05	0	Knopfmacher and Salle (1941)
		0.5	47	
		5	98	
β-Glycerophosphatase	*Phaseolus radiatus*	10	50	Rao *et al.* (1960)
Hydrogenase	*E. coli*	1	93	Joklik (1950 b)

TABLE 5-4 (*continued*)

Enzyme	Source	Iodine (m*M*)	% Inhibition	Reference
Indolylpyruvate keto-enol tautomerase	Rat liver	0.7	100	Spencer and Knox (1962)
Lactate dehydrogenase	Chick embryo	0.3	100	Solomon (1958)
	Beef heart	1	100	Neilands (1954)
Lipase	Pancreas	0.01	100	Wills (1960)
Malate dehydrogenase	Chick embryo	0.3	60	Solomon (1958)
	Chicken liver	0.3	100	Solomon (1958)
Mucinase	*Clostridium perfringens*	20	93	Robertson *et al.* (1940)
Papain	Papaya	0.2	66	Bersin and Logemann (1933)
	Papaya	2.3	70	Hellerman and Perkins (1934)
		5	90	
Peroxidase	Turnips	I/E = 3	0	Hosoya (1960)
		I/E = 15	45	
Phosphatase	Beef intestine	0.01	50	Sizer (1942 b)
	Calf intestine	0.1	Stim 20	Morton (1955)
		1	84	
		10	100	

3-Phosphoglyceraldehyde dehydrogenase	Rabbit muscle	0.9 3 9	46 61 80	Rapkine (1938)
Protease I	*Aspergillus oryzae*	0.1 1	3 50	Bergkvist (1963)
Protease II	*Aspergillus oryzae*	0.1 1	18 100	Bergkvist (1963)
Protease III	*Aspergillus oryzae*	0.1 1	65 100	Bergkvist (1963)
Proteinase	*Aspergillus parasiticus*	25	0	Dhar and Bose (1962)
	Aspergillus saitoi	10	100	Yoshida and Nagasawa (1958)
	Clostridium histolyticum	4.9	47	Kocholaty and Krejci (1948)
Pyruvate decarboxylase	Yeast	0.037 0.075	78 96	Kuhn and Beinert (1947)
	Yeast	0.012	63	Stoppani *et al.* (1953)
Solanain	Horse nettle	10	93	Greenberg and Winnick (1940)
Succinate dehydrogenase	Rat liver	0.33	52	Potter and DuBois (1943)
Urease	Jack beans	0.003	40	Grant and Kinsey (1946)
Xanthine oxidase	Milk	2 4 8	19 47 88	Gray and Felsher (1945)

when more enzymes are susceptible to sequential analysis. Another interesting approach to elucidating enzyme binding groups with iodine is illustrated by the study of the old yellow enzyme by Theorell (1956). Flavin is bound to the apoenzyme through its imino group and fluorescence is quenched; Weber had suggested that a tyrosine hydroxyl group might bind this imino group. This was examined by reaction of the apoenzyme with iodine; since no SH groups are present, this is relatively easy. It was found that very low concentrations of iodine decrease the coupling rate of FMN to the apoenzyme, and 90% of the iodine which disappears is recovered as diiodotyrosine.

Some results on the variation of inhibition with pH appear to point to the importance of tyrosine iodination. The iodination of pepsin is very slow below pH 4.5 and rises suddenly as the pH is increased to become maximal around pH 5.5 (Herriott, 1937). This is essentially the same pH dependence as found for glycyltyrosine. The inhibition of β-fructofuranosidase by iodine is minimal at pH 5.14 and much faster at pH's above 6 (Myrbäck, 1926), which might support the importance of tyrosine iodination in the inhibition. Although one might expect the effect of iodine on cathepsins to be mainly through reaction with SH groups, Maver and Thompson (1946) found greater inhibition by 0.25 mM iodine at pH 7 (71%) than at pH 3.5 (20%). Results with various SH reagents do not favor the importance of SH groups. This may well be a case where there is a mixed mechanism for the inhibition.

An interesting situation occurs in the reaction of the exopenicillinase of *B. cereus* with iodine (Citri and Garber, 1960, 1961). This enzyme can exist in two antigenically different states — α and γ — and these differ in response to iodine, although the enzyme activity is the same for both. α-Penicillinase is quite resistant to iodine whereas γ-penicillinase is inhibited by 0.5–1 mM iodine. The enzyme must be flexible since in the presence of the competitive inhibitor, 6-(2,6-dimethoxybenzamido)penicillanic acid, it becomes sensitive to iodine. Pretreatment with this inhibitor, followed by its removal, does not alter hydrolysis of benzylpenicillin, so that any structural change that occurs is not permanent. It is very difficult to understand how a rather large competitive inhibitor, which must cover the active center, could allow reaction of any group at the active center with iodine, unless the reacted group is just vicinal to the active center and either SH oxidation or iodination alters the structure.

Effects on Cellular Metabolism and Function

The uncoupling of oxidative phosphorylation by iodine has been claimed to relate to the effects of thyroxine. Klemperer (1955) reported that although iodide exhibits no uncoupling activity, iodine is quite effective in rat liver mitochondria with β-hydroxybutyrate as substrate (see accom-

panying tabulation). Iodine appears to fulfill the requirements of an uncoupler, in that it can reduce the P:O ratio significantly without depressing the respiration, although, to completely uncouple, the O_2 uptake must be

KI (m*M*)	I_2 (m*M*)	O_2 uptake	P : O
0	0	2.3	1.7
20	0	2.1	1.8
20	0.02	2.3	1.5
8	0	2.0	1.9
8	0.05	2.1	1.0
8	0.2	0.8	0

inhibited. Middlebrook and Szent-Györgyi (1955) found an uncoupling in mitochondria when Cl^- is partially replaced with I^-; at 25 m*M* I^-, phosphorylation is almost abolished without depression of respiration. It is not known if this is due to I^- itself, to reduction in Cl^-, or to iodine formed from I^-. Iodine causes mitochondrial swelling at a concentration as low as 0.005 m*M*, and in this it resembles thyroxine (Rall *et al.*, 1962). Iodine is able to oxidize NADH but addition of NADH does not reverse the swelling. Other oxidizing agents do not duplicate this effect. Furthermore, agents that inhibit thyroxine-induced swelling also inhibit that caused by iodine. Despite the superficial similarities in the actions of iodine and thyroxine, it is difficult to understand the nature of any relationship. It is very unlikely that thyroxine releases its iodine, and since thyroxine is always more potent than iodine, not enough iodine could be released in any event. It is possible that iodine does not act directly, but iodinates tyrosine or some protein, and that this product is the active uncoupler.

One might expect iodine to be an effective inhibitor of glycolysis, inasmuch as this pathway involves a number of SH-dependent steps. Yeast fermentation is indeed quite sensitive to iodine, 0.017 m*M* inhibiting 22% and 0.085 m*M* inhibiting 100% (Schroeder *et al.*, 1933 b). There is simultaneously a loss of GSH and probably other SH groups. This does not prove that the glycolytic inhibition is related to SH groups, but is suggestive. The locus of action is not known; one thinks of aldolase, because of its great sensitivity to iodine, but most of the glycolytic enzymes have not been tested. That iodine can oxidize SH groups in cells was shown by Cafruny *et al.* (1955 a). Kidney sections incubated with iodine exhibit 85% loss in SH groups in the proximal and distal tubules. However, so little work has been done on cell metabolism with iodine that it is impossible to predict if any pathways are inhibited selectively; it would appear to be unlikely, unless glycolysis proves to be more susceptible than other systems.

Iodine can interfere with the transport of substances across cell membranes. Hemolysis by glycerol and other nonelectrolytes is quite potently inhibited by iodine at 0.08 m*M* (LeFevre, 1947, 1948) as it is by various SH reagents. The effects are readily reversed by thiols. This was taken to indicate that SH groups are in some manner involved in the transport of these substances into the erythrocytes. However, it is not necessarily evidence for an active transport, since membrane permeability could be affected directly or indirectly. Iodine also inhibits the transport of phosphate into staphylococci and it was claimed that this process involves SH groups (Mitchell, 1954). Finally, iodine at 0.1 m*M* reduces the short-circuit current and electrical potential of frog skin (Eubank *et al.*, 1962), but there is no evidence as to the site or mechanism of this action.

Iodine has been studied a great deal in connection with its germicidal activity (Gershenfeld and Witlin, 1950) but not a great deal has been done from the metabolic standpoint. The effects of pH on the ability of iodine to kill bacteria, fungi, or spores are, however, of interest, since they would presumably apply to work with any cells. It has generally been considered that at lower pH's there is more free iodine, and hence greater penetrability into cells and greater activity. It is true that more iodate would be formed in alkaline solutions and, in the absence of much iodide, more hypoiodous acid. Wyss and Strandskov (1945) found the bactericidal activity to decrease at higher pH's and attributed this to a greater formation of HOI and IO_3^-. When iodide is present, the formation of HOI is suppressed, and the pH does not affect the activity. It was also observed, as would be expected, that the action of iodine is strongly dependent on temperature, requiring about 4 times as long to kill *Bacillus metiens* spores for each 10° drop in temperature.

PEROXIDES

Hydrogen peroxide and other peroxides occasionally depress enzymes and metabolism potently but little is known about the specificity with respect to SH groups. In comparison with other oxidants, no thorough studies of the effects of hydrogen peroxide on proteins have been made. Mirsky and Anson (1935) mention that hydrogen peroxide is convenient to use in the oxidation of SH groups, but it has never been widely applied for this purpose. The interesting effects of hydrogen peroxide on glycolysis and a few enzymes justify a brief discussion.

Chemistry

Hydrogen peroxide is a nonlinear molecule that is quite miscible with water:

$$H_2O_2 + H_2O \leftrightharpoons H_3O^+ + OOH^- \qquad K = 2.4 \times 10^{-12}$$

The ion product (H^+) (OOH^-) is 1.55×10^{-12} at 20°. Thus it is a very weak acid and the ion OOH^- is probably unimportant in its reactions. Hydrogen peroxide can function as both oxidant and reductant. It is a strong oxidizing agent in both acid and alkaline media, but a relatively poor reductant. Although the oxidation-reduction potential would be more favorable for oxidation in acid medium, the rate of oxidation is often greater in alkaline conditions. Hydrogen peroxide, of course, is an unstable substance, especially in the presence of organic material, and this must be considered in its use. Despite its instability ($-\Delta F = 23.4$ kcal/mole), it is rather stable in pure solution, but its decomposition is catalyzed by heavy metal ions, and is more rapid in alkaline than acid media.

Inhibition of Enzymes

The few results summarized in Table 5-5 are not comparable with each other because the conditions were quite different in the various studies. However, there is no doubt that some enzymes are very sensitive to hydrogen peroxide. The inhibition develops very slowly in some cases; with yeast β-fructofuranosidase the inhibition by 2.9 M hydrogen peroxide is 0% at 1 min, 10% at 30 min, 47% at 3 hr, and 100% at 21 hr (Myrbäck, 1957 b). Of course, at this very high concentration one has no idea of the mechanism of the inhibition, and can only marvel at the resistance of this enzyme. The inhibition of ATPase depends on the pH at which the reaction is run: Thus the enzyme was incubated with hydrogen peroxide at pH 7 for 15 min, and the inhibition was found to be 51% when the ATPase reaction was tested at pH 6.3 and 95% when tested at 9.2 (Mehl, 1944). The reason for this strange behavior is unknown. One factor that has not been generally considered is the possible presence of heavy metal ions in the hydrogen peroxide. Holmberg (1939) believed that the inhibition of uricase he observed was due to traces of Cu^{++}, inasmuch as diethyldithiocarbamate prevents the inhibition. It is also possible that some metal ion may be necessary to catalyze the oxidation of the enzyme and that the inhibition is not due to the Cu^{++} itself.

The inhibition of β-galactosidase by hydrogen peroxide is completely reversible by H_2S or cyanide, while that by iodine is not, indicating that here one may oxidize the SH groups more specifically with the peroxide (Knopfmacher and Salle, 1941). Reactivation of ATPase inhibited by hydrogen peroxide was observed by both Mehl (1944) and Ziff (1944), using cysteine or glutathione, so that specific oxidation of SH groups may occur with this enzyme. Simultaneously there is a suppression of the interaction of actin and myosin, which is believed to depend on SH groups (Bailey and Perry, 1947). Papain inhibited up to 90% by hydrogen peroxide can also be reactivated by cysteine, but beyond this there is apparently oxidation beyond the disulfide stage (Sanner and Pihl, 1963). Blocking the SH groups with

TABLE 5-5

INHIBITION OF ENZYMES BY HYDROGEN PEROXIDE

Enzyme	Source	Hydrogen peroxide (m*M*)	% Inhibition	Reference
Aconitase	*Aspergillus niger*	0.05	93	Bruchmann (1961 c)
		0.1	98	
		0.2	100	
	Yeast	0.05	100	Bruchmann (1961 a)
Adenosinase	*Vibrio cholerae*	0.3	100	Agarwala *et al.* (1954)
Adenosinetriphosphatase	Rat muscle (pH 6.3)	2.2	51	Mehl (1944)
	Rat muscle (pH 9.2)	2.2	95	
	Rabbit muscle	16	70	Ziff (1944)
	Rabbit muscle	0.2	60	Bailey and Perry (1947)
		0.5	72	
		1	82	
Adenylate deaminase	Rabbit muscle	9	0	Lee (1957)
Aldolase	Rabbit muscle	2	0	Herbert *et al.* (1940)
β-Amylase	Wheat	15	0	Ghosh (1958)
Ascorbate oxidase	*Curcurbita pepo*	0.04	100	Tokuyama and Dawson (1962)
Asparaginase	*Mycobacterium* sp.	20	0	Ott (1960)
Carbonic anhydrase	Spinach leaves	1	0	Chiba *et al.* (1954 a)
β-Fructofuranosidase	Yeast	0.1	100	Sizer (1942 a)
β-Galactosidase	*E. coli*	1	Stim 1	Knopfmacher and Salle (1941)
		5	14	
		50	16	

Glycerol-3-P dehydrogenase	*Trypanosoma rhodesiense*	0.4	57	Grant and Sargent (1961)
Glycerol-3-P oxidase	*Trypanosoma rhodesiense*	1	100	Grant and Sargent (1960)
Hydrogenlyase	*Aerobacillus polymyxa*	20	87	Crewther (1953)
Imidazolone-propionate hydrolase	Rat liver	1	0	Snyder *et al.* (1961)
Lactate dehydrogenase	Yeast	1	0	Gregolin and Singer (1963)
NADH:H_2O_2 oxidoreductase	*Streptococcus faecalis*	5.5	10	Dolin (1957)
		16.5	43	
	Clostridium perfringens	0.003	25	Dolin (1959)
		0.013	67	
		0.066	97	
NADH oxidase	*Streptococcus faecalis*	1.3	17	Dolin and Wood (1960)
	Trypanosoma rhodesiense	0.07	0	Fulton and Spooner (1959)
Papain	Papaya	2	96	Bersin and Logemann (1933)
Protease	*Aspergillus parasiticus*	25	0	Dhar and Bose (1962)
	Trifolium repens	2	0	Brady (1961)
		20	10	
Ribonuclease	Pancreas	10	0	Holden and Pirie (1955)
	Pea leaves	10	0	Holden and Pirie (1955)
	Ehrlich ascites tumor	0.005	5	Hilz and Klempien (1959)
		1	21	
Succinate dehydrogenase	—	2.9	28	Massart *et al.* (1940)
		14	100	
Uricase	Pig liver	—	Inh	Holmberg (1939)
Urocanase	Beef liver	4	0	Feinberg and Greenberg (1959)
Xanthine oxidase	Milk	0.00001	Stim 30	Bernheim and Dixon (1928)
		0.0001	Stim 48	
		0.001	Stim 76	
		0.1	0	
		1	80	

p-mercuribenzoate prevents their reaction with hydrogen peroxide. The single SH group of papain is about 5 times as susceptible to oxidation by hydrogen peroxide as the SH group of phosphoglyceraldehyde dehydrogenase. α-Chymotrypsin has two methionine residues, one being 3 residues away from the active site serine and the other 15 residues removed. The Met-3 is oxidized by hydrogen peroxide specifically, whereas both SH groups are oxidized in the urea-denatured enzyme (Schachter *et al.*, 1963). It is obvious that disulfide bonds cannot be formed intramolecularly in the native enzyme and it was shown that methionine sulfoxide is the product. Glutathione is also oxidized to the sulfoxide by hydrogen peroxide (Utzinger *et al.*, 1963).

Hydrogen peroxide generated during oxidation in enzyme preparations or cells is sometimes inhibitory and for many years it has been assumed that at least one function of catalase is to protect cells against it. Dixon (1925) was the first to demonstrate this with a purified enzyme system. When purines are oxidized by oxygen in the presence of xanthine oxidase there is a progressive inactivation of the enzyme, which is due to hydrogen peroxide formed, since it can be prevented by catalase. Hydrogen peroxide was claimed to stimulate xanthine oxidase at very low concentrations (between 0.00001 m*M* and 0.01 m*M*) (Table 5-5), and to inhibit above 0.1 m*M* (Bernheim and Dixon, 1928). The rate of activation is slow, maximal effects of 0.001 m*M* hydrogen peroxide occurring in around 100 min. The mechanism for this is unknown; metal impurities seem unlikely because they would be at extremely low concentrations. The inhibition of *Aspergillus* aconitase depends on the strain of the organism, the sensitivity varying over at least a 4-fold range of concentration (Bruchmann, 1961 a). Certain strains tend to accumulate citrate under specified conditions and this is believed to be due to the hydrogen peroxide formed and the particular sensitivity of aconitase in these strains (Bruchmann, 1961 b). Adding exogenous hydrogen peroxide augments the accumulation of citrate (Bruchmann, 1961 c).

Succinyl peroxide is a radiomimetic substance and has been found to inhibit several SH enzymes while having much less action on non-SH enzymes (Wills, 1959). The enzymes inhibited are amylase, β-fructofuranosidase, urease, succinate dehydrogenase, phosphoglyceraldehyde dehydrogenase, papain, tyrosinase, and cholinesterase. Urease, for example, is inhibited completely in 8 min by 0.033 m*M* succinyl peroxide. Reversal of inhibitions by cysteine is obtained only if the period of exposure to the peroxide is brief. It is questionable if the action is exerted by succinyl peroxide itself, since it is immediately hydrolyzed to persuccinate in aqueous solution, and peracids have long been known to oxidize SH groups (Freudenberg and Eyer, 1932; Swan, 1959). The inhibition of catalase by monoethyl peroxide is probably not due to SH group oxidation but to an analog type of inhibition (Blaschko, 1935).

Effects on Metabolism

Hydrogen peroxide inhibits brain respiration and especially the oxidation of succinate (Dickens, 1946 a). If one compares the effects on various tissues, the sensitivity to hydrogen peroxide depends on the relative concentrations of catalase; the more catalase, the less inhibition. In brain, 75 m*M* hydrogen peroxide inhibits respiration 36% and succinate oxidation 95% over a 60 min period. It was thought initially that the toxic effects of high oxygen tension on brain might be due to hydrogen peroxide released, but this was shown not to be true.

If *Lactobacillus* is grown anaerobically, the cells lose their iron enzymes and catalase; if they are then exposed to oxygen, hydrogen peroxide is formed and the cells are killed (Warburg *et al.*, 1957). Since cancer cells possess an anaerobic type of metabolism and contain much less catalase than normal cells, it was postulated that this may be the cause of the greater sensitivity of cancer cells to hydrogen peroxide. It was found that 1 m*M* hydrogen peroxide has no effect on the aerobic or anaerobic glycolysis of embryo tissue, but inhibits both almost completely in ascites cells. Since the catalactic activity of embryo tissue is around 10-fold that of the ascites cells, this could explain the differential susceptibility. Inasmuch as radiation of cells can induce hydrogen peroxide formation, this may be one reason for the more selective effects of radiation on cancer cells. Holzer and Frank (1958) extended these observations in ascites cells to show that hydrogen peroxide at 0.056 m*M* not only inhibits glycolysis 86%, but simultaneously reduces the NAD concentration very markedly (0.31 to 0.05 μmoles/ml). Triose-P and fructose-diP rise, indicating a block of the phosphoglyceraldehyde dehydrogenase. However, they found the extracted enzyme to be inhibited only 37% by 0.079 m*M* hydrogen peroxide, so that concentrations effectively blocking glycolysis would have little effect on this enzyme (assuming the same sensitivities of the intact and extracted enzymes). They thus postulated that the inhibition is due to a reduction of NAD and that this suppresses the oxidation of triose-P. Nicotinamide can protect both NAD and glycolysis from hydrogen peroxide, and Pantlitschko and Seelich (1960) showed that it could overcome the inhibition when added 1 hr after the hydrogen peroxide. Baker and Wilson (1963) confirmed the inhibition of anaerobic glycolysis in Ehrlich ascites carcinoma cells, although the effects were not as marked as observed previously — some inhibition at 0.3 m*M*, around 50% at 0.9 m*M*, and 80% at 2 m*M* — and further showed that during the oxidation of unsaturated fatty acids some hydrogen peroxide is formed and may depress glycolysis. Pütter (1961) studied the possible relationship between glycolysis and transplantability of ascites cells, but encountered the difficulty that the hydrogen peroxide used to inhibit glycolysis is fairly rapidly decomposed so that the inhibition disappears. Thus it requires above 1 m*M* to interfere with transplantability.

Hydrogen peroxide is by no means the ideal glycolytic inhibitor for this type of work.

Effects on Tissue Function and in Whole Animals

The spontaneous motility of the rat intestine is extremely sensitive to hydrogen peroxide inasmuch as 10% stimulation of the amplitude occurs with 0.00057 m*M* (Goodman and Hiatt, 1964). At 0.057 m*M* hydrogen peroxide the stimulation of contraction by acetylcholine is blocked and 67% of the total SH groups of the tissue are reacted. Although hydrogen peroxide at 0.001 m*M* has no definite effect on the spontaneous contractility, it reduces the effect of acetylcholine somewhat. Other SH reagents act similarly and it appears that the response to acetylcholine is dependent on SH groups. The contractility of nonconducting rabbit psoas muscle is blocked by 300 m*M* hydrogen peroxide after 7 min exposure, and this is not reversible with cysteine (Korey, 1950). However, little can be learned from concentrations of this magnitude.

An interesting relationship was discovered by Feinstein *et al.* (1954), in that a sublethal dose of iodoacetate (20 mg/kg) and a 20% fatal dose of hydrogen peroxide (15 meq/kg) given together kill all the animals. Inasmuch as iodoacetate also potentiates the lethality of X-irradiation in mice, this was considered as evidence that radiation may produce some of its effects by the release of hydrogen peroxide. It was noted that the toxicity of hydrogen peroxide is markedly increased by treating the animals with azide, a catalase inhibitor; however, hydroxylamine, which is a better catalase inhibitor, does not augment the effects of hydrogen peroxide.

TETRATHIONATE

Tetrathionate appears to be a fairly specific oxidant for SH groups under the proper conditions, but has been used very little in enzyme work. It was found to be capable of antagonizing cyanide poisoning in dogs at doses of 500 mg/kg (Chen *et al.*, 1934), and today we might interpret this as due to methemoglobin formation. Tetrathionate has been used in a method for the determination of protein methionine, which is demethylated to homocysteine and then oxidized (Baernstein, 1936). It has been used clinically in thromboangiitis obliterans, supposedly for an effect on the blood, an increase in the oxygen capacity being observed (Theis and Freeland, 1940). It is surprising that the blood glutathione increases after injection of tetrathionate. It has been applied occasionally to the reduction of the cytochrome components of the respiratory chain, since the initial work of Keilin and Hartree (1940), the tetrathionate apparently being oxidized to sulfite. It is thus, like hydrogen peroxide, both an oxidant and a reductant, which makes

its effects in complex systems more difficult to interpret. The first use of tetrathionate as a reagent for protein SH groups was by Anson (1941), who demonstrated that it would titrate denatured ovalbumin, although the reaction is slower than with ferricyanide, porphyrindin, or *p*-chloromercuribenzoate, not being complete in 3 min at neutrality. It has not been used extensively for this purpose, but it may well be a valuable reagent in certain types of work; a detailed description of the method is given by Chinard and Hellerman (1954).

Chemistry and Reaction with SH Groups

Sodium tetrathionate is prepared from the thiosulfate by oxidation with iodine in 90% ethanol. The precipitate is purified by redissolving it in an equal weight of water and filtering it into absolute ethanol, in which it reprecipitates. It is washed with ethanol and dried *in vacuo*. Sodium tetrathionate crystallizes with 2 waters of hydration. When it is kept at 0° in the dark, both the solid and 0.1 *M* solutions are stable for many weeks (Pollock and Knox, 1943), but it is unstable when kept under ordinary conditions. For all accurate work it is necessary to be certain that it is free of appreciable thiosulfate and other impurities, and it should be recrystallized as above.

Tetrathionate rapidly oxidizes simple thiols, such as cysteine, homocysteine, and glutathione, according to the reaction:

$$2\ R{-}SH + S_4O_6^{=} \leftrightarrows R{-}S{-}S{-}R + 2\ S_2O_3^{=} + 2\ H^+$$

In titrations of SH groups the thiosulfate is determined iodometrically. According to Baernstein (1936). it is specific for SH groups and does not react with other amino acid groups. It is not a strong oxidant, since the standard oxidation-reduction potential for the reaction

$$2\ S_2O_3^{=} \leftrightarrows S_4O_6^{=} + 2\ e^-$$

is + 0.08 v. Although it has always been assumed that tetrathionate oxidizes SH groups to disulfide, Pihl and Lange (1962) have obtained evidence that sulfenyl thiosulfate groups may be formed:

$$R{-}SH + S_4O_6^{=} \rightarrow R{-}S{-}S_2O_3^{=} + S_2O_3^{=} + H^+$$

Incubation of phosphoglyceraldehyde dehydrogenase with tetrathionate-S^{35} leads to the appearance of S^{35} bound to the protein, and the binding of each S^{35} is associated with the disappearance of one SH group.

One of the few thorough kinetic studies of SH group oxidants was made by Goffart and Fischer (1948). It was shown that tetrathionate oxidizes protein SH groups more slowly than cysteine or glutathione (Fig. 5-1).

Furthermore, the reaction is initially rapid but in most instances slows down suddenly, which is difficult to explain for the simple thiols. The oxidation proceeds much faster at pH 7 than at pH 5 (see accompanying tabulation).

Protein	Time for complete reaction (min)	
	pH 5	pH 7
Lens protein	140	30
Ovalbumin (denatured)	120	12
Myosin (denatured)	140	50

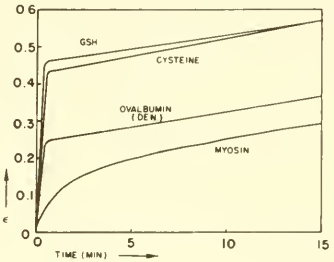


FIG. 5-1. Rates of reaction of 10 m*M* tetrathionate with the SH groups of thiols and proteins at pH 5. (From Goffart and Fischer, 1948.)

Inhibition of Enzymes and Metabolism

Succinate dehydrogenase is inhibited around 90% by 0.1 m*M* tetrathionate (Keilin and Hartree, 1940). This is an effect on the dehydrogenase SH groups according to these authors and Philips *et al.* (1947), who confirmed the inhibition on succinate dehydrogenases from several tissues. No inhibition of ascorbate oxidation, and hence of the cytochrome system, is observed even with 10 m*M*. Succinate protects the enzyme; when tetrathionate is 0.5 m*M*, succinate reduces the inhibition from 96% to 39%, and when it is 0.1 m*M* from 79% to 10% (pigeon breast enzyme). The inhibition is only partially reversible with glutathione or cysteine. In work with succinate dehydrogenase it may be well to consider the possibility that some of the inhibition results from a competitive action of the tetrathionate, since it has negative charges appropriately separated. Choline dehydrogenase from rat liver is also quite sensitive to tetrathionate, 38% inhibition re-

sulting from 0.2 m*M* and 93% from 0.6 m*M* (Gordon and Quastel, 1948). The only clear-cut demonstration of reaction with enzyme SH groups is that of phosphoglyceraldehyde dehydrogenase (Pihl and Lange, 1962). Here tetrathionate inhibits as well as *p*-chloromercuribenzoate, i.e., when 3 moles of inhibitor are reacted per mole of enzyme, the activity is reduced to zero in both cases. Also the inhibition is fully reversible with thiols. However, as mentioned above, the reaction does not appear to be a simple oxidation, but involves the formation of sulfenyl thiosulfate groups. The enzyme is very sensitive, since 0.005 m*M* inhibits completely (enzyme = 0.0005 m*M*) within 5 min.

In view of the potent inhibition of phosphoglyceraldehyde dehydrogenase one might anticipate tetrathionate to be a glycolytic inhibitor. Goffart and Fischer (1948) attempted to demonstrate a Lundsgaard effect in muscle, i.e., a typical contracture such as produced by iodoacetate and certain other SH reagents. Following injection into rabbits, the extremities become weak but the muscles remain elastic and the reflexes normal; if the gastrocnemius is stimulated, it does not go into contracture. Intraarterial injection produces a temporary contracture (or at least some inhibition of relaxation). Injection into frogs does not give an iodoacetate-like effect and the isolated frog rectus abdominis muscle gives only a temporary contracture-like reaction. It is doubtful if true contractures are observed, and in any case the tetrathionate concentration must be quite high. It is possible that the phosphoglyceraldehyde dehydrogenase in the muscle is protected by a permeability barrier to the doubly charged inhibitor, and by the presence of NAD and substrate on the enzyme. MacLeod (1951) found inhibition of glycolysis in human spermatozoa, but the tetrathionate concentration was 10 m*M* and the inhibition progressed very slowly, so that even after 3 hr the glycolysis is not completely depressed (around 50%). The motility decreases simultaneously with the reduction in glycolysis. One of the pitfalls of reversibility experiments is well illustrated here, for when cysteine is used there is a rapid toxic effect on the spermatozoa, this being due to the cystine arising as the result of the oxidation by tetrathionate.

Tetrathionate is reduced to thiosulfate by reaction with SH groups and it has been supposed that the rapid conversion into thiosulfate in rabbits and dogs is due to this (Gilman *et al.*, 1946). While this must be true in part, there is some evidence for enzyme systems catalyzing this reaction. Thus in various bacteria tetrathionate is readily reduced, while in others no reaction at all occurs (Pollock and Knox, 1943). Postgate (1956) has isolated cell-free systems reducing tetrathionate, thiosulfate, and sulfite from the anaerobe *Desulfovibrio desulfuricans*, the cytochrome system acting as an electron carrier for the tetrathionate reductase. Indeed, it is likely that tetrathionate can be oxidized through the cytochrome system in most cells. Thus tetrathionate must generally be rather labile in most biological situations.

Nephrotoxic Action

Intravenous injection of around 0.5 g/kg of sodium tetrathionate into dogs leads to the development of anuria within 30–60 min, a rapid reduction in creatinine clearance, and the appearance of proximal tubular necrosis (Gilman *et al.*, 1946). At the time of death there is no evidence of toxicity, symptomatic or histological, in any other tissue from such minimally lethal doses; higher doses, however, can cause a long-lasting ataxia, and in rabbits some difficulty in muscular relaxation. Inasmuch as simultaneous reduction of the tetrathionate to thiosulfate occurs, it was assumed that the renal damage is related to the oxidation of SH groups, the toxicity thus being related to that produced by the mercurials. Nevertheless, nephrotoxic doses in rabbits do not inhibit kidney succinate dehydrogenase at all *in vivo*, and yet this enzyme is very sensitive to tetrathionate (Philips *et al.*, 1947). Large doses of tetrathionate (1 g/kg of the sodium salt) in rabbits lead to a 77% loss of glutathione in the kidneys, 28% in the blood, 30% in the liver, and 20% in muscle after 90 min, most of the change occurring within 30 min (Goffart and Fischer, 1948). These results might indicate that tetrathionate has a greater effect on renal SH groups than those of other tissues, without implying that the toxicity is due to the loss of glutathione.

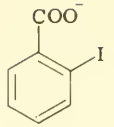
CHAPTER 6

o-IODOSOBENZOATE

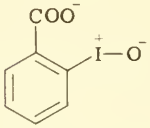
The most commonly used oxidant for enzyme SH groups at present is *o*-iodosobenzoate because it is probably the most selective for these groups. For this reason it deserves a somewhat more detailed treatment than the other oxidants and a separate chapter. *o*-Iodosobenzoate was first prepared by Meyer and Wachter (1892) and studied biologically by Heinz (1899) in Germany. The early interest stemmed from the use of iodine and organic iodine compounds in superficial infections. Indeed, Heinz was mainly concerned with the administration of sodium iodide and *o*-iodosobenzoate together so that by the oxidation of the iodide it would be possible to form "nascent" iodine in the tissues. Consequently there were early investigations on the antibacterial activity (Jahn, 1914) and the effects on phagocytosis (Arkin, 1912). The initial pharmacological study was by Loevenhart and Grove (1909, 1911) at the University of Wisconsin, but the results did not engender much clinical enthusiasm and, inasmuch as the actions at that time were not related to any metabolic site of attack, *o*-iodosobenzoate was little used by biochemists until it was introduced by Hellerman *et al.* (1941) for the estimation of protein SH groups. The application to enzyme characterization was slow but during the past several years it has come to be one of the most useful SH reagents. It differs from the arsenicals, the mercurials, and the alkylating agents in not introducing new groups or side chains onto the enzymes, since it is generally believed that the primary action is an oxidation of the SH groups to the disulfide state. However, the use of *o*-iodosobenzoate, like most SH reagents, in complex systems or cellular preparations is limited because of the number of components affected and the inherent difficulty in the interpretation of the results.

CHEMISTRY

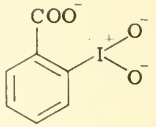
The structures of the different oxidation states of the iodinated benzoates may be written as:



o-Iodobenzoate



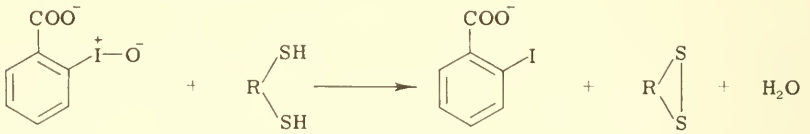
o-Iodosobenzoate



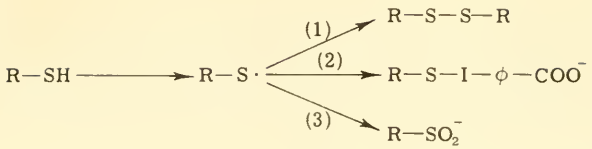
o-Iodoxybenzoate

It is possible that the aryl iodoso compounds, φ—I^+—0^-, can add a proton to form φ—I^+—OH since the corresponding protonated iodoxybenzene is known, but the ionization constant is unknown. Indeed, the carboxyl pK_a is not accurately known, but is probably around 6.0 to 6.5, in contrast to the pK_a for *o*-iodobenzoic acid (2.86), and *o*-iodosobenzoic acid may be precipitated from solution by passing CO_2 through solutions of the sodium salt. *o*-Iodosobenzoic acid may be easily prepared by oxidation of *o*-iodobenzoic acid with permanganate, crystallization by cooling, and recrystallization with CO_2, and can be determined iodimetrically (Loevenhart and Grove, 1911; Chinard and Hellerman, 1954). Commercial samples should probably be repurified for accurate work. The *m*- and *p*-iodosobenzoates are also strong oxidizing agents and might possibly have certain advantages over *o*-iodosobenzoate for particular purposes, but they have been almost completely ignored. It is interesting that *p*-iodosobenzoate reacts like *o*-iodosobenzoate with the SH groups of L-glutamate dehydrogenase, but no detailed comparison was made (Hellerman *et al.*, 1958). The *o*-iodoxybenzoate is also a potentially useful reagent but essentially nothing is known of its actions on proteins or enzymes.

The oxidation of SH groups by *o*-iodosobenzoate results in the formation of *o*-iodobenzoate. Whether the disulfide link is intra- or intermolecular depends on the thiol reacted; with cysteine or glutathione it is obviously intermolecular, but what evidence exists for proteins suggests that intramolecular oxidation is dominant. Oxidation apparently does not proceed beyond the disulfide stage:



at pH 7, and under proper conditions accurate titration of SH groups can be achieved. However, at lower pH's or in the presence of excess of *o*-iodosobenzoate, further oxidation to the sulfinate or sulfonate stages may occur, and groups other than SH may be attacked. Whether a free radical mechanism is involved in the oxidation of SH groups here is not known, but if so, one might postulate the following types of reaction:



Reaction (1) would involve combination with another R—S·, while reactions (2) and (3) would involve additional *o*-iodosobenzoate. Compounds of the type R—S—I—R′ are generally unstable, but on proteins such linkages may occasionally be more stable, as in the formatiom of sulfenyl thiosulfates (page 697).

At neutrality, 25°, and 1–5 m*M* *o*-iodosobenzoate, only SH groups are significantly oxidized; cystine, methionine, glucose, and ascorbate are not oxidized appreciably under these conditions. However, ascorbate is oxidized slowly by *o*-iodosobenzoate at pH 4.6 when both are present at 1 m*M* in acetate buffer, half-reaction time being around 80 min (Caraway and Hellerman, 1953). The nature of the buffer is important inasmuch as it is an acid-catalyzed reaction. It is interesting that *m*- and *p*-iodosobenzoates oxidize ascorbate almost instantaneously. NADH is not oxidized by *o*-iodosobenzoate at pH 4.6 (Schellenberg and Hellerman, 1958). The oxidizability of tyrosine phenolic groups by *o*-iodosobenzoate has not been thoroughly examined but there is no evidence from work with proteins that this reaction proceeds readily. The tyrosine groups of β-amylase seem to be resistant to *o*-iodosobenzoate since there is no change in absorption at 280 mμ (Englard *et al.*, 1951).

REACTION WITH PROTEIN SH GROUPS

In order to titrate selectively protein SH groups with *o*-iodosobenzoate, it is necessary to control the conditions carefully, as with all SH reagents. It is usual in titrations with *o*-iodosobenzoate to add a slight excess of the reagent and determine the amount not reduced by addition of KI and subsequent titration of the released I_2 with thiosulfate (Chinard and Hellerman, 1954). The test is best run at pH 7 and between 15° and 25°. The required reaction time varies with the protein tested but is usually less than 30 min. Ovalbumin denatured with guanidine is titrated quite satisfactorily and all of the SH groups are oxidized. Only a fraction of the SH groups of native ovalbumin or other proteins is oxidized.

The specific oxidation of protein SH groups to disulfide may be accompanied by changes in the protein structure, which could have important bearing on the mechanism of enzyme inhibition. Evidence for such structural alteration is given by the increased water-binding capacity of gels formed from serum proteins previously treated with *o*-iodosobenzoate (Jensen *et al.*, 1950). At about equimolar ratios, *o*-iodosobenzoate changes the

nature of the clots from soft and opaque to firm, elastic, and almost transparent, and simultaneously the water binding increases from 14.3 to 36.5 g/g. It is quite possible that more linear proteins, which may be reasonably flexible, can be altered quite markedly by the formation of disulfide bonds, and that particular regions on the surface may be made unavailable for other reactions.

Further evidence for structural changes induced by *o*-iodosobenzoate is the decrease in the viscosity of G-actin brought about by 2 m*M* of the reagent acting for 30 min at 25° and pH 7.8–8 (Bárány *et al.*, 1962). This is interpreted as an inhibition of polymerization. Simultaneously there is a decrease in the ability to bind Ca^{++}, as shown by the loss of G-actin-bound Ca^{45} upon tretment with *o*-iodosobenzoate. Although there appears to be some correlation between changes in viscosity and Ca^{++} binding as produced by various SH reagents, the mechanisms involved are not yet understood.

INHIBITION OF ENZYMES

Most SH enzymes are inhibited by *o*-iodosobenzoate (see Table 6.1) and in a few instances the inhibition is very marked. Enzymes without SH groups at or near their active centers, as shown by failure to be inhibited by SH reagents in general, are not affected by *o*-iodosobenzoate, except possibly in the single instance where it has been claimed to act as a competitive inhibitor on D-amino acid oxidase, although in such cases the concentration would usually have to be a good deal higher than for the oxidation of susceptible SH groups (Frisell *et al.*, 1949) (see page 342). Inasmuch as *o*-iodosobenzoate is used up in the reaction, inhibition is of a titration type and spontaneously irreversible; thus the degree of inhibition will often depend on the amount of enzyme present, or the amount of some SH containing impurity that also reacts with the reagent. In most complex systems, many enzymes will be inactivated to varying degrees, and probably little specificity is possible. However, the remarkable sensitivity of creatine kinase — definite inhibition at 0.00001 m*M* and complete inhibition at 0.00013 m*M* — might well make it possible to block this enzyme selectively (Ennor and Rosenberg, 1954). This inhibition may explain the observation of Bailey and Marsh (1952) that the fall in creatine phosphate in muscle homogenates is almost completely prevented by *o*-iodosobenzoate. It might be worthwhile to consider the use of *o*-iodosobenzoate in glycerinated and similar muscle preparations to determine the role of creatine kinase in the initiation of contraction or relaxation. Phosphoglyceraldehyde dehydrogenase seems to be less sensitive to *o*-iodosobenzoate than to iodoacetate or iodoacetamide, so that a specific block of glycolysis at this step would be impossible. It may be noted that several dehydrogenases are quite well inhibited by *o*-iodosobenzoate, notably the xanthine, malate, and aldehyde

TABLE 6-1

INHIBITION OF ENZYMES BY *o*-IODOSOBENZOATE

Enzyme	Source	Time[a] (min)	Concentration (m*M*)	% Inhibition[b]	Reference
Acetaldehyde dehydrogenase (NAD)	*Acetobacter suboxydans*		2	35	King and Cheldelin (1956)
			10	86	
Acetaldehyde dehydrogenase (NADP)	*Acetobacter suboxydans*		2	0	King and Cheldelin (1956)
			10	53	
			20	98	
Acetokinase	*E. coli*	30	0.3	50	Rose *et al.* (1954)
			2	100	
Acetylphosphatase	Horse muscle		—	(ni)	Harary (1957)
Adenosinetriphosphatase	Rabbit muscle		4	49	Bailey and Marsh (1952)
	Beef heart		5	0	Pullman *et al.* (1960)
	Eel electric organ	10	0.1	65	Glynn (1963)
			1	90	
Adenosinetriphosphate-P_i exchange enzyme	Rat liver	30	1	17	Plaut (1957)
			10	29	
	Rat liver	20	0.8	57	Cooper and Lehninger (1957)
			6	100	
Alanine dehydrogenase	*Bacillus cereus*		0.1	23	O'Connor and Halvorson (1960)
			1	48	
Alcohol dehydrogenase	Yeast	10	1	57	Barron and Levine (1952)
Aldehyde dehydrogenase	Yeast (K^+-activated)	0.5	0.02	83	Stoppani and Milstein (1957 b)
			0.06	75	

TABLE 6-1 (*continued*)

Enzyme	Source	Time[a] (min)	Concentration (m*M*)	% Inhibition[b]	Reference
		0.5	0.02	65	Stoppani and Milstein (1957 a)
			0.06	78	
	Yeast (NADP-linked)	0.5	0.02	94	Stoppani and Milstein (1957 a)
			0.06	97	
	Liver (NAD-linked)	1	0.2	72	Stoppani and Milstein (1957 b)
		1	0.2	67	Stoppani and Milstein (1957 a)
Alliinase	*Allium cepa* (onion)		0.1	0	Kupiecki and Virtanen (1960)
Allothreonine aldolase	Sheep liver		76	32	Karasak and Greenberg (1957)
D-Amino acid oxidase	Pig kidney		0.0133	78	Singer (1948)
	Pig kidney	15	0.156	40	Frisell and Hellerman (1957)
			0.625	97	
Aminoacylase	*Brassica campestris* (Polish rapeseed)	20	0.05	40	Ozaki and Wetter (1961)
6-Aminopyrimidine deoxyribonucleoside-5′-P deaminase	Sea urchin eggs		0.05	40	Scarano *et al.* (1960)
			0.1	60	
			0.2	75	
Amylo-1,6-glucosidase	Rabbit muscle	20	0.01	3	Larner and Schliselfeld (1956)
			0.1	62	
β-Amylase	Sweet potato	20	1	27	Englard *et al.* (1951)
			2.5	47	
			5	75	
			7	91	

Apyrase	Potato	20	5	16	Bárány and Bárány (1959 b)
Arginine kinase	Crayfish muscle		0.01	19	Morrison *et al.* (1957)
			0.1	67	
			1	100	
Arsenite oxidase	*Pseudomonas arsenoxydansquinque*	60	1	0	Legge and Turner (1954)
Arylsulfatase b	Beef cornea		0.2	10	Wortman (1962)
	Rabbit cornea		0.2	0	Wortman (1962)
Aspartase	*Propionibacterium peterssonii*	8–24 hr	10	15	Ellfolk (1953)
Aspartate carbamyl transferase	*E. coli*		4	0	Reichard and Hanshoff (1956)
Betaine aldehyde dehydrogenase	Rat liver		1	100	Rothschild and Barron (1954)
Cathepsin	Rat lymphosarcoma	120	0.5	13	Maver and Thompson (1946)
			2	16	
	Cod spleen	15	0.1	Stim 2	Siebert *et al.* (1963)
			1	33	
Choline acetylase	Squid ganglion	30	0.001	16	Reisberg (1954)
			0.01	49	
			0.1	66	
			1	70	
Choline oxidase	Rat liver		1	84	Rothschild *et al.* (1954)
Cholinesterase	Human erythrocytes		10	2	Markwardt (1953 b)
Creatine kinase	Sheep muscle		0.00001	13	Ennor and Rosenberg (1954)
			0.00013	100	
Crotonase	Beef liver		1	17	Wakil and Mahler (1954)

TABLE 6-1 (*continued*)

Enzyme	Source	Time[a] (min)	Concentration (m*M*)	% Inhibition[b]	Reference
	Beef liver	30	0.1	27	Stern and del Campillo (1956)
			1	56	
Cytochrome b_2	Yeast	30	1	88	Boeri *et al.* (1955)
Cytochrome oxidase	*Penicillium chrysogenum*		0.1	0	Sih *et al.* (1958)
Diaminopimelate decarboxylase	*E. coli*		0.14	50	Dewey *et al.* (1954)
Dihydroxyacid dehydratase	Spinach leaves		0.5	12	Kanamori and Wixom (1963)
Dipeptidase	Pig kidney		0.002	32	Traniello and Vescia (1964)
			0.01	100	
DT diaphorase	Rat liver		0.1	21	Ernster *et al.* (1962)
			1	68	
Equilin dehydrogenase	Rat liver		0.01	0	Mittermayer and Breuer (1963)
			1	30	
Formate hydrogenlyase	*Aerobacillus polymyxa*	30	2	46	Crewther (1953)
			40	100	
Fructokinase	Beef liver		0.068	50	Ponz and Llinás (1963)
Fumarase	Yeast	30	0.2	81	Favelukes and Stoppani (1958)
		11	0.5	48	
		30	0.5	61	
Glucose-6-P dehydrogenase	Rat liver		0.25	62	Glock and McLean (1953)
L-Glutamate dehydrogenase	Corn leaves	5	3	0	Bulen (1956)
	Beef liver		0.3	0	Olson and Anfinsen (1953)

D-Glutamate oxidase	Octopus hepatopancreas		0.001	8	Rocca and Ghiretti (1958)
			0.01	21	
			0.1	32	
			1	52	
Glutamate semialdehyde reductase	Rat liver		10	50	Smith and Greenberg (1957)
Glycerate kinase	*Brassica campestris*		0.037	61	Ozaki and Wetter (1960)
Glycine methyltransferase	Rabbit liver		1	70	Blumenstein and Williams (1963)
Glycolate oxidase	Pig kidney		0.1	91	Robinson *et al.* (1962)
Homogentisate oxidase	Rat liver		0.1	96	Crandall (1955)
			1	99	
Homoserine deaminase	Rat liver		0.01	16	Matsuo and Greenberg (1959)
			0.1	76	
Hydrogenase	*E. coli*		1	30	Joklik (1950 b)
L-α-Hydroxy acid oxidase (long-chain)	Pig kidney		0.1	100	Robinson *et al.* (1962)
3-Hydroxyanthranilate oxidase	Beef liver		0.1	(i)	Stevens and Henderson (1959)
5-Hydroxytryptophan decarboxylase	Rat kidney		1	47	Buzard and Nytch (1957)
Inositol dehydrogenase	*Aerobacter aerogenes*	15	0.55	89	Larner *et al.* (1956)
			1	100	
Isocitrate dehydrogenase	Pig heart	15	0.4	16	Lotspeich and Peters (1951)
			0.81	50	
			1.21	68	
Isocitrate [illegible]ase	Yeast	5	0.001	0	Olson (1959)
			0.01	8	
			0.063	50	
			0.1	70	

TABLE 6-1 (*continued*)

Enzyme	Source	Time[a] (min)	Concentration (m*M*)	% Inhibition[b]	Reference
Lactate dehydrogenase	*Propionibacterium pentosaceum*	5	1	5	Molinari and Lara (1960)
			2	2	
			5	36	
	Beef heart	30	1	0	Neilands (1954)
L-Lactate oxidase	*Tetrahymena pyriformis*	10	0.1	27	Eichel and Rem (1962)
			1	29	
			2	29	
Lipase	Wheat germ		1	50–89	Singer (1948)
	Pancreas		1	0	Wills (1960)
Lombricine kinase	Earthworms	5	0.3	100	Gaffney *et al.* (1964)
Malate dehydrogenase (decarboxylating)	Pigeon liver		0.002	29	Rutter and Lardy (1958)
			0.01	64	
			0.03	82	
			0.06	91	
Maltase	Pig intestine	60	1.4	0	Larner and Gillespie (1956)
NADH: cytochrome-c oxidoreductase	Pig heart		10	100	Vernon *et al.* (1952)
	Pig heart		0.1	0	Mahler (1955)
			10	100	
	Pig liver		1	42	Mahler *et al.* (1958)
	Bacillus cereus		0.01	22	Doi and Halvorson (1961)
NADH oxidase	*Tetrahymena pyriformis*	7	0.1	55	Eichel (1956 a)
			1	82	
			10	96	

	Lactobacillus casei		1	73	C. F. Strittmatter (1959)
	Pig heart		0.1	22	Huennekens *et al.* (1955)
Nicotinamide deamidase	*Neurospora crassa*	120	0.01	0	Sundaram *et al.* (1958)
	Corcyra cephalonica	120	0.1	0	Rajagopalan *et al.* (1960)
Nitrate reductase	*E. coli*		2.5	20	Joklik (1950 a)
Ornithine carbamyltransferase	Rat liver		1	46	Reichard (1957)
Penicillinase	*Aerobacter cloacae*		2	29	Smith (1963 b)
Pentose-P isomerase	Rabbit muscle	10	1	(i)	Dickens and Williamson (1956)
Phosphatase (acid)	Mouse liver		0.1	17	Macdonald (1961)
	Human prostate	14 hr	10	0	Tsuboi and Hudson (1955 a)
	Human erythrocytes	60	1	100	Tsuboi and Hudson (1955 b)
Phosphatase (alkaline)	Intestine		13.3	50	Lazdunski and Ouellet (1962)
Phosphoenolpyruvate carboxylase	Yeast	15	0.7	0	Cannata and Stoppani (1963 a)
	Yeast		0.7	2	Cannata and Stoppani (1963 b)
	Yeast		0.66	16	Cannata and Stoppani (1963 c)
Phosphoglucomutase	Rabbit muscle	10	0.02	1	Milstein (1961)
			0.4	12	
			2	22	
6-Phosphogluconate dehydrogenase	Rat liver		0.25	76	Glock and McLean (1953)
Phosphoglucose isomerase	Human erythrocytes		2	0	Tsuboi *et al.* (1958)
Phosphoglycerate kinase	Rabbit muscle		4.4	0	Rao and Oesper (1961)
Phosphoglycerol dehydrogenase	*Lactobacillus casei*		1	27	C. F. Strittmatter (1959)
Phosphohalidase (DFPase)	Rabbit kidney		—	(ni)	Mazur (1955)
Phosphomevalonate kinase	Pig liver		5	88	Hellig and Popják (1961)

TABLE 6-1 (*continued*)

Enzyme	Source	Time[a] (min)	Concentration (m*M*)	% Inhibition[b]	Reference
Protease	*Etroplus suratensis*	15	1	0	Sundaram and Sarma (1960 b)
	Rastrelliger kanagurta	15	1	12	Sundaram and Sarma (1960 a)
Proteinase	Ox lens		1	91	Waley and van Heyningen (1962)
Pyrophosphatase	Wool follicles	30	10	100	Gillespie (1956)
Pyruvate decarboxylase	Yeast	10	0.019	5	Kuhn and Beinert (1947)
			0.15	13	
	Yeast		0.1	45	Stoppani *et al.* (1953)
			1	66	
	Acetobacter suboxydans		2	50	King and Cheldelin (1954)
Ribonuclease	*Euglena gracilis*		1	0	Felling and Wiley (1960)
	Pea leaves		2.5	50	Holden and Pirie (1955)
	Pancreas	30	0.1	10	Ledoux (1954)
			1	20	
			10	50	
	Pancreas		10	0	Davis and Allen (1955)
	Pancreas	10	3.4	0	Rabinovitch and Barron (1955)
Serine deaminase	*E. coli*		0.038	42	Lenti and Grillo (1955)
			0.38	100	
	Rat liver	30	1	~90	Selim and Greenberg (1960)
Sorbitol dehydrogenase	*Acetobacter suboxydans*		0.1	0	Cummins *et al.* (1957)
			5	49	
Δ^1-Steroid dehydrogenase	*Pseudomonas testosteroni*		1	90	Levy and Talalay (1959)

Δ^4-5α-Steroid dehydrogenase	*Pseudomonas testosteroni*		1	100	Levy and Talalay (1959)
Steroid hydroxylase	Beef adrenals	60	1	5	Ryan and Engel (1957)
Succinate dehydrogenase	Pigeon breast muscle	20	0.5	100	Barron and Singer (1945)
	Beef heart	30	0.4	50	Slater (1949)
	Beef heart mitochondria		0.2	100	Singer *et al.* (1956 b)
	Pig heart		0.21	75	Stoppani and Brigone (1956)
			0.42	93	
Thiosulfate transulfurase (rhodanese)	Beef liver	30	0.1	31	Sörbo (1951)
			1	63	
Threonine aldolase	Sheep liver		76	86	Karasek and Greenberg (1957)
Threonine dehydrogenase	Bullfrog liver	30	0.1	1	Hartshorne and Greenberg (1964)
Transhydrogenase	Rat heart		0.17	21	Humphrey (1957)
Tripeptidase	Human erythrocytes		3.7	50	Tsuboi et *al.* (1957)
Tyrosine: α-ketoglutarate transaminase	Rat liver		0.04	50	Kenney (1959)
UDP-glucose dehydrogenase	Pea seedlings	10	1	34	Strominger and Mapson (1957)
UDP glucuronyltransferase	Mouse liver		0.3	55	Storey (1964)
Uricase	Pork liver		1	0	Mahler *et al.* (1955 b)
Urocanase	Beef liver		1	0	Feinberg and Greenberg (1959)
Vitamin K_1 reductase	Dog liver		1	0	Wosilait (1960)
Xanthine oxidase	Milk		0.0025	100	Harris and Hellerman (1956)
D-Xylulokinase	*Aerobacter aerogenes*	10	1	14	Bhuyan and Simpson (1962)
			10	100	

[a] Generally the time for preincubation of the enzyme with *o*-iodosobenzoate before the reaction is started; is most cases this important parameter is not stated.

[b] The designations (*i*) and (*ni*) indicate that the enzyme is inhibited or not inhibited respectively, actual figures not being given; Stim indicates stimulation.

dehydrogenases. At the present time, *o*-iodosobenzoate is more useful in the study of pure enzymes as an indicator of SH groups than in cellular systems, but has been little investigated in the latter and may possess potentialities as a metabolic blocker if applied properly.

Titration of Enzyme SH Groups

Muscle phosphoglyceraldehyde dehydrogenase contains 11 cysteine residues and reacts rapidly with 11 moles of *p*-chloromercuribenzoate per mole of enzyme. Segal and Boyer (1953) reported that 7.3–7.45 moles of *o*-iodosobenzoate react with each mole of this enzyme, indicating 14.6–14.9 reducing groups. Theoretically one would expect 5.5 moles of *o*-iodosobenzoate to be reduced by each mole of enzyme, assuming that all the SH groups are oxidized to the disulfide level. Segal and Boyer thus suggested that some of the SH groups may be oxidized beyond the disulfide stage. Actually only 10 of the 11 SH groups could form intramolecular disulfide bonds, so the extra SH group must either remain unoxidized, form a disulfide link with another molecule of the enzyme (which is unlikely), or be oxidized to some state other than the disulfide. Since oxidation to the S—O^- or SO_2^- state would require 2 molecules of *o*-iodosobenzoate for each SH group, 7 moles of *o*-iodosobenzoate would react with each mole of enzyme if oxidation of the extra SH group occurred in this way. Rafter (1957) investigated this problem further and, under his conditions, found 10–11 moles of *o*-iodosobenzoate to react with each mole of the enzyme, indicating 20–22 reducing equivalents. Furthermore, the *o*-iodosobenzoate-treated enzyme still possesses 30% of its initial SH groups, as determined by reaction with *p*-chloromercuribenzoate. These results point to reaction of *o*-iodosobenzoate with groups other than SH groups, or to oxidation of a fraction of the SH groups beyond the disulfide stage. There is no evidence for reaction with other groups and the enzyme is completely reactivated by cysteine. Thus one might assume that 4 SH groups are oxidized to the disulfide level, 4 are oxidized beyond this, and 3 remain unreacted; this would require 10 moles of *o*-iodosobenzoate per mole of enzyme. Another possibility is that some of the SH groups form E—S—I—φ—COO^- residues. It is interesting that although *o*-iodosobenzoate abolishes the usual phosphoglyceraldehyde dehydrogenase activity and the arsenolysis reaction, it simultaneously increases phosphatase activity 6-fold, this phosphatase activity being dependent on NAD. The esterolytic activity with *p*-nitrophenylacetate as substrate is inhibited completely in 10 min when 4–5 moles of *o*-iodosobenzoate have reacted per mole of enzyme (Olson and Park, 1964). No substrate protection was observed. Yeast phosphoglyceraldehyde dehydrogenase contains fewer SH groups than the muscle enzyme — 2–4 per molecule — and reacts with 6 molecules of *o*-iodosobenzoate, so that here too an anomalous effect is seen. Barron and Levine (1952) report 11.9 SH groups in yeast alcohol dehydro-

genase by *o*-iodosobenzoate titration and 9.3 by an amperometric method, and thus the value with *o*-iodosobenzoate is a little high in this case also.

Kinetics of Inhibition

The rate of oxidation of simple thiols by *o*-iodosobenzoate is usually quite rapid, but protein or enzyme SH groups vary greatly in the rapidity with which they react with this reagent. The inhibition of succinate oxidase by *o*-iodosobenzoate at 0.2 m*M* and 37° requires about 30 min to become maximal (Slater, 1949). This is shown in Fig. I-12-12, where the maximal inhibition of around 35% indicates that insufficient *o*-iodosobenzoate was present for reaction with all of the enzyme and nonenzyme material. At 16° it is evident that there are two phases, one complete within 10 min and the other incomplete after 2 hr (Fig. I-12-13). Although the first phase undoubtedly represents oxidation of SH groups, it is not clear if the slow reaction is further oxidation of other SH groups or secondary inactivation of the enzyme.

The inhibition of ribonuclease by *o*-iodosobenzoate also shows two phases (Figs. 6-1 and 6-2), one a fairly rapid reaction inhibiting around 20% and

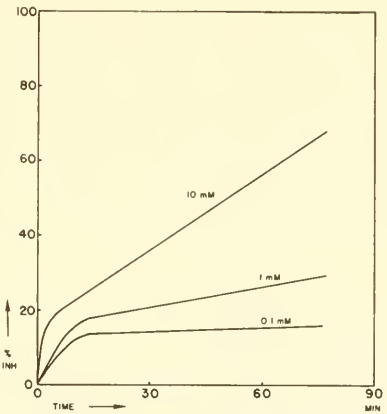


Fig. 6-1. Rates of inhibition of ribonuclease by *o*-iodosobenzoate at pH 7. The times indicate the duration of the incubation of the enzyme and inhibitor. (From Ledoux, 1954.)

the other a much slower one that is linear at least over 1–2 hr (Ledoux, 1954). The rate of inhibition during the second phase is dependent on the concentration of *o*-iodosobenzoate and, since this phase starts from essentially the same degree of inhibition for each concentration, it seems that

the slow phase is a further reaction with enzyme groups rather than a secondary inactivation. It has been emphasized several times with other enzymes, e.g. β-amylase (Englard *et al.*, 1951) and phosphoglucomutase (Milstein, 1961), that the reaction with *o*-iodosobenzoate is slow. For this reason, many of the results given in Table 6-1 are not comparable, since different times of incubation with the inhibitor were used and usually the times were not given. Unless preincubation of the enzyme with *o*-iodosobenzoate for a reasonably long period (at least 30 min) is done, it is likely that the inhibitions determined are partial and do not accurately represent the true effect of the oxidant on all of the enzyme present.

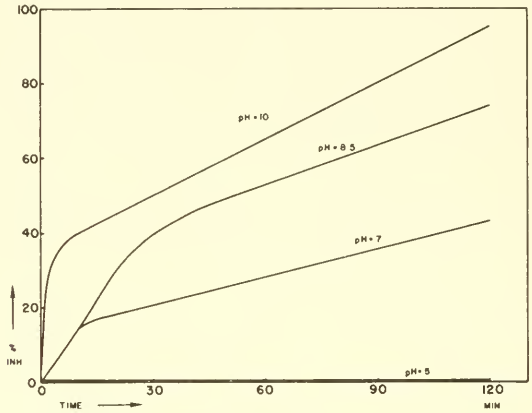


FIG. 6-2. Rates of inhibition of ribonuclease by 1 m*M* *o*-iodosobenzoate at various pH's. The times indicate the duration of the incubation of the enzyme and inhibitor. (From Ledoux, 1954.)

Effects of pH

It has been emphasized that in titrations of protein SH groups a pH near neutrality must be maintained if specificity is desired; below a pH of 7 the oxidizing power of *o*-iodosobenzoate increases, and oxidation of other protein groups may occur. Also the solubility of *o*-iodosobenzoate decreases rapidly below pH 7. One might expect, therefore, that the inhibition of enzymes might decrease as the pH is raised above 7, but just the opposite has been observed. β-Amylase is inhibited more strongly and more reproducibly at pH 7.8 than at pH 7 (Englard *et al.*, 1951), succinate dehydrogenase is inhibited more at pH 7 than at pH 6 (Stoppani *et al.*, 1953), and ribonuclease is inhibited more rapidly and completely as the pH is increased from 7 to 10, no inhibition occurring at pH 5 (Fig. 6-2) (Ledoux, 1954). The very rapid initial oxidation of ribonuclease at pH's above 8.5 may be due

to the ionization of the SH groups. Penicillinase is inhibited 29% by 2 m*M* *o*-iodosobenzoate at pH 7.4 but not at all at pH 6 (Smith, 1963 b). On the basis of these results, one might conclude that one should avoid pH's of 7 or below in enzyme work. Unfortunately, there are no data on the specificity of *o*-iodosobenzoate at higher pH's.

Protection of Enzymes against Inhibition by *o*-Iodosobenzoate

Protection of an enzyme by addition of some thiol with the *o*-iodosobenzoate does not tell one anything about the mechanism of inhibition, since the inhibitor is simply depleted by oxidation of the thiol. However, protection by substances interacting with the enzyme active center provides some evidence for the site of the inhibition by *o*-iodosobenzoate. The normal substrate of an enzyme has been shown frequently to protect against *o*-iodosobenzoate, if it is present during the incubation of the enzyme with the inhibitor. Thus, alcohol dehydrogenase is protected by ethanol (Barron and Levine, 1952), fumarase by either fumarate or malate (Favelukes and Stoppani, 1958), D-amino acid oxidase by alanine (Frisell and Hellerman, 1957), choline oxidase by choline (Rothschild *et al.*, 1954), glutamate semialdehyde reductase by glutamate semialdehyde (Smith and Greenberg, 1957), succinate oxidase by succinate (Thorn, 1959), and homogentisicase by homogentisate (Tokuyama, 1959). In some instances the protection may be very marked; e.g., fumarase is completely protected against 0.5 m*M* *o*-iodosobenzoate by 25 m*M* fumarate, and a 58% inhibition of alcohol dehydrogenase is reduced to 6.5% by ethanol. Coenzymes can likewise protect in certain instances: aldehyde dehydrogenase is protected by NAD and NADP (Stoppani and Milstein, 1957 a,b), alcohol dehydrogenase is protected by NAD (Barron and Levine, 1952), and D-amino acid oxidase is protected by FAD (Frisell and Hellerman, 1957). It is interesting that the different aldehyde dehydrogenases are protected to different degrees by their coenzymes. The K^+-activated yeast enzyme is protected by both NAD and NADP, as well as by acetaldehyde, whereas the NAD-linked liver enzyme is protected only by NAD and not by NADP. These observations may be taken to mean that the inhibition is the result of a reaction of *o*-iodosobenzoate with SH groups at or near the active center, and that when the active center is covered by substrate or coenzyme the oxidant is unable to attack these SH groups. Such effects must be taken into account when *o*-iodosobenzoate is used in cellular preparations. In one instance the effect of substrate is abnormal. The lactate dehydrogenase of *Propionibacterium pentosaceum* is inhibited more readily in the presence of lactate than in its absence (see accompanying tabulation) (Molinari and Lara, 1960). This was explained by assuming that lactate increases the fraction of free SH groups, suggesting that the SH groups may be involved in the electron transport.

o-Iodosobenzoate (m*M*)	% Inhibition	
	No lactate	Lactate 50 m*M*
1	5	30
2	2	36
5	36	54

Reactivation of o-Iodosobenzoate Inhibition

If the only action of *o*-iodosobenzoate is the oxidation of SH groups to disulfide bonds, one might expect some reversal of the inhibition by thiols, and this has been observed with certain enzymes. The inhibition of D-amino acid oxidase is reversed completely by cysteine (Rocca and Ghiretti, 1958) but in most cases only partial reactivation is possible, for example, succinate oxidase by dimercaprol (Thorn, 1959) and by glutathione (Slater, 1949), amylo-1,6-glucosidase by glutathione (Larner and Schliselfeld, 1956), threonine aldolase by dimercaprol (Karasek and Greenberg, 1957), and alcohol dehydrogenase by glutathione (Barron and Levine, 1952). It is difficult to interpret partial reactivation since failure to reverse the inhibition completely may be due to a variety of factors. No reactivation by thiols has been reported for a few enzymes: Acid phosphatase cannot be reactivated by cysteine or thioglycolate (Tsuboi and Hudson, 1955 b), nor xanthine oxidase by cysteine (Harris and Hellerman, 1956), nor β-amylase by glutathione or dimercaprol (Englard *et al.*, 1951), nor 5-hydroxytryptophan decarboxylase by thiols (Buzard and Nytch, 1957). However, these failures cannot be immediately attributed to other actions of *o*-iodosobenzoate and perhaps the most likely explanation is progressive secondary inactivation consequent to the protein distortion induced by disulfide bond formation. Some failures might also be due to attempting reactivation in the presence of oxygen, which can often oxidize the thiols to disulfides, which in turn can inhibit the enzyme, as pointed out by Slater (1949).

Variation of the Inhibition with the Substrate Used

The degree of inhibition of lipase by 1 m*M* *o*-iodosobenzoate varies with the substrate (Singer, 1948; Singer and Hofstee, 1948 a). The inhibition by *p*-chloromercuribenzoate is also dependent on the substrate used and Singer postulated that the mercurial is bound near the substrate site so that it sterically interferes with the binding of the substrates, the interference being greater the larger the substrate molecule. However, how this explanation could apply to *o*-iodosobenzoate is not clear, since the simple formation of

a disulfide link near the substrate site would not obviously produce a steric effect. Inasmuch as the general problem of dependence of inhibition on the substrate will come up several times with other inhibitors, it will be well to suggest some of the possible mechanisms by which such effects can arise.

Substrate	% Inhibition by *o*-iodosobenzoate (1 m*M*)
Triacetin	50
Tripropionin	67
Monobutyrin	74
Tributyrin	89

(1) It is conceivable that the formation of a disulfide structure can distort the enzyme structure at or near the substrate site so that inhibition will result, and this inhibition might not be the same for each substrate, because of either steric factors or changes in the spatial position of the enzyme groups involved in the hydrolysis. (2) The SH reagent might not primarily react with the enzyme but with the substrate, as suggested by Wills (1960) for the inhibition of pacreatic lipase by *p*-chloromercuribenzoate. Since this enzyme is not inhibited by *o*-iodosobenzoate, a relation with substrate cannot be established. Wills believes that the mercurial is adsorbed onto the glyceride-water interface and, in order to examine this possibility, shook tributyrin with 10 m*M* *p*-chloromercuribenzoate, washed it, and then used this as a substrate; marked inhibition was noted, indicating a rather strong affinity of the glyceride for the mercurial. However, again this explanation would not seem to hold for *o*-iodosobenzoate, since it should inhibit the pancreatic lipase as well as the wheat germ lipase (with which Singer worked) if the substrates are altered. Also it is not too surprising that a molecule like *p*-chloromercuribenzoate would adsorb at an oil-water interface, since it possesses polar and nonpolar regions, but *o*-iodosobenzoate has polar groups at either end. (3) A group, such as —S—I—φ—COO^-, is actually added to the enzyme near the substrate site and interferes sterically as *p*-chloromercuribenzoate may do.

Another explanation involves the basic kinetics of such inhibitions. It seems to have been generally assumed that when an irreversible inhibitor reduces the affinities of each substrate of an enzyme equally, the inhibitions will all be the same, which can readily be shown not to be true. Let us assume that K_m is equal to K_s, the true dissociation constant of the ES complex, so that the uninhibited rate is given by:

$$v = \frac{V_m(\mathrm{S})}{(\mathrm{S}) + K_s}$$

If only the affinities of the substrates are altered by the inhibitor, we may write for the inhibited rate:

$$v_i = \frac{V_m(S)}{(S) + \alpha K_s}$$

where α is a factor indicating the magnitude of the effect of the inhibitor on the substrate binding ($\alpha > 1$). The inhibition is then given by:

$$i = \frac{\alpha - 1}{(S') + \alpha} \tag{6-1}$$

where (S′) is the specific concentration of the substrate, $(S)/K_s$. Thus, even though α is the same for each substrate, the inhibition will vary with (S′). Superficially it might appear that an irreversible inhibitor reducing substrate binding would produce inhibitions independent of the substrate concentration, but such is not the case. Some of the confusion arises from associating this type of inhibition with noncompetitive inhibition, which it is not in any sense. The inhibitions by *o*-iodosobenzoate, and probably most SH reagents, are usually competitive, as shown by the protection afforded by the substrate when it is present during the incubation with the inhibitor, and K_s is altered rather than k_2, the rate constant for the breakdown of the ES complex into products. It may be noted that even though K_m is not K_s, but the more complex $(k_{-1} + k_2)/k_1$, a similar expression for the inhibition will be found, and the substrate concentration will play a role in the degree of inhibition produced. Furthermore, if the inhibition actually is noncompetitive and k_2 is altered rather than K_s, it can easily be shown that the inhibition is given by:

$$i = \frac{1 - \beta}{1 + \dfrac{\beta k_2}{k_{-1}[(S') + 1]}} \tag{6-2}$$

where β is the factor by which k_2 is changed by the inhibitor ($\beta < 1$). Here the variation of the inhibition with the specific concentration of the substrate is different than in the previous case, in that the inhibition rises as (S′) increases, as long as (S′) does not greatly differ from unity. Indeed, at high substrate concentrations, $i = 1 - \beta$, the usually expected purely noncompetitive inhibition and, likewise, if $K_m = K_s$, $i = 1 - \beta$.

It is consequently not necessary to assume some complex mechanism involving steric factors when the inhibition is found to vary with the substrate used, unless the specific concentrations of all the substrates are kept equal. It was stated by Singer (1948) in his study of lipase that the substrate concentration was chosen so as to "just saturate the enzyme and thereby give optimal activity." However, calculation of the values of (S′)

from the K_m's given by Singer and Hofstee (1948 b) shows that, for the substrates used with *o*-iodosobenzoate, (S′) varies from 4 to 23 at least and, furthermore, the variation of the inhibition with (S′) is as one would expect from Eq. 6-1, i.e., it decreases with increasing (S′). Thus the results with *o*-iodosobenzoate can be explained quite simply. However, it is not implied that this will explain all of the results obtained by Singer, and it is quite possible that with *p*-chloromercuribenzoate, where a bulky group is added to the enzyme, steric factors also play a role. The purpose of the foregoing treatment is to indicate the importance of keeping (S′) constant when comparing inhibitions with different substrates.

INHIBITION OF METABOLISM

Very little quantitative work has been done on the effects of *o*-iodosobenzoate on glycolysis, respiration, the tricarboxylate cycle, or other metabolic pathways, so that the following is not so informative as indicative of possibly interesting experiments to be done. From Table 6-1 it is evident that few glycolytic enzymes have been tested and these few are not particularly sensitive to *o*-iodosobenzoate. Only one investigation of glycolysis *in vivo* has apparently been reported, that of Harting (1947), who found *o*-iodosobenzoate at 1 m*M* to stimulate scallop muscle anaerobic glycolysis, as does *p*-chloromercuribenzoate. This may not be due to direct action on the glycolytic system, but to some effect on the muscle membranes facilitating glucose entry. Glycolysis in muscle homogenates is definitely inhibited by 4 m*M* *o*-iodosobenzoate (Bailey and Marsh, 1952). The changes in the pH and phosphate fractions with time are modified, as shown in Table 6-2. The fall in pH is quite strongly inhibited and the normal decrease in ATP is accelerated, presumably by inhibiting ATP formation. The effects on fructose-1,6-diP are interesting; in the control there is an initial accumulation followed by a fall to low levels — *o*-iodosobenzoate blocks the accumulation partially, but what does accumulate remains, indicating some inhibition of aldolase or phosphoglyceraldehyde dehydrogenase. Part of the depression of the early accumulation may be due to the low levels of ATP, but it is likely that some inhibition is exerted on the enzymes forming fructose-1,6-diP, perhaps hexokinase. The prevention of the fall in creatine-P is undoubtedly due to the potent inhibition of the transfer of the phosphate to form ATP. The minor accumulation of phosphoglyceraldehyde produced by *o*-iodosobenzoate may also point to some block of the dehydrogenase, not unlikey at this rather high concentration.

One might expect *o*-iodosobenzoate to inhibit respiration fairly strongly since several dehydrogenases and cycle enzymes are quite sensitive. The respiration of sea urchin spermatozoa is depressed almost completely by 0.3–1 m*M* *o*-iodosobenzoate, although lower concentrations around 0.1 m*M*

Table 6-2

Effects of o-Iodosobenzoate on Glycolysis in Muscle Homogenates[a]

Conditions	Time (min)	Δ pH	Δ ATP	Δ CrP	Δ FrPP	Δ TrioseP
Controls	3	−0.28	−14	−27	+27	+ 3
	10	−0.76	−19	−29	+10	+ 4
	30	−1.03	−23	−29	− 2	+ 3
	60	−1.04	−23	−29	− 3	+ 3
o-Iodosobenzoate (4 m*M*)	3	−0.14	−19	− 2	+ 9	+ 5
	10	−0.26	−20	− 5	+13	+ 7
	30	−0.35	−20	−10	+13	+ 7
	60	−0.38	−20	−11	+13	+ 7

[a] The values for the phosphate fractions are changes in the per cents of the total acid-soluble phosphorus. (From Bailey and Marsh, 1952.)

may stimulate, a phenomenon seen with other SH reagents ($HgCl_2$, *p*-chloromercuribenzoate, arsenite, and iodoacetamide) (Barron *et al.*, 1948). The respiration of Ehrlich ascites tumor cells is inhibited 50% by 0.35 m*M* *o*-iodosobenzoate, 0.1 m*M* inhibiting 11% and 1 m*M* 93% (Shacter, 1957). Thus the susceptibility of respiration is confirmed but there are no data for locating the principal sites of action.

The binding of K^+ in liver mitochondria is believed by Gamble (1957) to be related to the sites for oxidative phosphorylation, although it is not directly dependent on ATP. The evidence comes from the ability of 2,4-dinitrophenol to lower mitochondrial K^+ markedly. *o*-Iodosobenzoate at 0.03 m*M* produces effects similar to 2,4-dinitrophenol, which does not necessarily imply an uncoupling action of the *o*-iodosobenzoate, but indicates some effect on the electron transport chain. Scott and Gamble (1961) have found mercurials to stimulate the K^+ exchange rate of mitochondria and simultaneously to reduce the bound K^+. These effects are also produced by *o*-iodosobenzoate: the exchange rate is doubled by 0.08 m*M*, the mitochondrial K^+ is half reduced by 0.15 m*M*, and oxidative phosphorylation is 50% inhibited by 0.08 m*M*. These potent actions of *o*-iodosobenzoate point to important effects on mitochondrial oxidative systems that apparently play a role in the depression of respiration.

EFFECTS ON ANIMAL TISSUE FUNCTIONS

The injection of *o*-iodosobenzoate into animals or its application to *skeletal muscle* preparations does not produce rigor so readily as does iodoacetate. In the whole animal, indeed, the actions on muscle seem to be of little importance, and the paralysis sometimes seen is more likely explained by a central effect. Applied directly to isolated frog muscle in reasonably high concentration, *o*-iodosobenzoate can lead to a loss of excitability and the development of contracture, whereas *o*-iodobenzoate, although it depresses excitability somewhat, does not induce contracture (Jahn, 1914). The turtle biceps muscle is also slowly and irreversibly shortened by *o*-iodosobenzoate (and iodoacetamide) at concentrations much higher than would be reached *in vivo* (Pisanty, 1948). If the mechanism of iodoacetate in contracture is a block of glycolysis, *o*-iodosobenzoate does not seem to share this selectivity, which confirms what little is known from the results on enzymes and metabolism (see page 721). The ability of myosin to associate with actin and to split ATP depends on SH groups and is inhibited by *o*-iodosobenzoate as well as by other SH reagents (Bailey and Perry, 1947), and the binding of Ca^{++} by G-actin is depressed parallel to the reduction in polymerization by *o*-iodosobenzoate (Bárány *et al.*, 1962). In these respects, *o*-iodosobenzoate is more potent and rapidly acting than iodoacetamide, and such effects may play a role in the contractures observed at high concentration, although a metabolic site of action is not excluded. The contractile response to ATP by nonconducting psoas muscle fibers is abolished by 0.5-1 m*M* *o*-iodosobenzoate, and this is reversible if the fibers are incubated for 90–120 min in 10 m*M* cysteine (Korey, 1950).

The *heart* appears to be more sensitive to *o*-iodosobenzoate than is skeletal muscle. In the initial pharmacological study by Loevenhart and Grove (1911), intravenous injection into rabbits, cats, and dogs was found to produce a rapid fall in the blood pressure, little change in the cardiac rate, and a decrease in cardiac output with dilation of the heart. *o*-Iodoxybenzoate acts very similarly but *o*-iodobenzoate is inactive, indicating that the oxidizing activity is essential. Minimal effects are given in the cat by 13.2 mg (50 μmoles), so that the total concentration is probably around 0.5 m*M*. However, inasmuch as Jahn (1914) showed that blood appreciably reduces the action of *o*-iodosobenzoate — due to reaction with hemoglobin, other proteins, and glutathione — the concentration of free *o*-iodosobenzoate is undoubtedly much less. Jahn also showed that the perfused frog heart is depressed by as little as 0.038 m*M* *o*-iodosobenzoate and that 0.38 m*M* causes a prolonged depression of the amplitude, although not standstill or contracture. The results of Méndez (1946) and Méndez and Peralta (1947) on the frog heart differ from those of Jahn, in that concentrations of 0.2–0.4 m*M* were found to cause an increase in the contractile amplitude, and 0.83 m*M* to produce systolic standstill within 20 min. Furthermore, the

rate always increases up to the final failure, whereas Jahn observed only slowing. No conduction disturbances were noted. Since partial reversal can be achieved by lengthy perfusion with *o*-iodosobenzoate-free medium, in tissues some reduction of disulfide groups may occur. The dog heart-lung preparation is quite resistant to *o*-iodosobenzoate, 100 mg producing no effect, although high doses increase the venous pressure, presumably by initiating cardiac failure (Méndez and Pisanty, 1949).

The effects of *o*-iodosobenzoate on *smooth muscles* are at least superficially similar to those on skeletal and heart muscle (Alanis, 1948). Isolated rabbit intestine and the uterus in several species are put into a form of contracture, although this is eventually followed by relaxation and loss of all rhythmic activity. These actions are similar to those of iodoacetamide and arsenicals.

Although the effects of *o*-iodosobenzoate in the whole animal indicate marked effects on the *central nervous system*, no analysis of this has been made so that sites and mechanisms are completely unknown. Frog nerve axons are unaffected by 1 m*M* *o*-iodosobenzoate as measured by excitability and conduction (Jahn, 1914), but the central actions are undoubtedly on synaptic mechanisms. Neuroblastic damage has been found in developing mice and rats after injections of *o*-iodosobenzoate, as with other SH reagents, but this probably relates more to growth and differentiation than function (Hicks, 1953).

EFFECTS IN WHOLE ANIMALS

The earliest study of *o*-iodosobenzoate by Heinz (1899) is not very illuminating since he administered potassium iodide simultaneously to generate "nascent" iodine. However, he showed it to be irritant to the eye, the gastric mucosa, and the peritoneum, and that this action seems to be due to something other than its acidic properties. Loevenhart and Grove (1911) confirmed the inflammatory action in the eye and subcutaneously, and showed that intraperitoneal injections can be fatal as a result of the congestion produced. Whether this is related in any way to the vesicant activity of many SH reagents is not known. On the other hand, Bernheim *et al.* (1932) found that injection into rabbits of *o*-iodosobenzoate inhibits conjunctival edema induced by mustard oil. However, this could well be a nonspecific action, since *o*-iodobenzoate and benzoate are somewhat active (as the ammonium salts), and might well be mediated through the adrenal cortex.

Loevenhart and Grove (1909, 1911) investigated the pharmacological properties of *o*-iodosobenzoate and related compounds because they believed that the oxygen of this substance is physiologically active and can be used by the tissues; e.g., *o*-iodosobenzoate alone does not oxidize phenolphthalin to phenolphthalein, but does if some serum is present, this being interpreted

as an action mediated by peroxidase, the *o*-iodosobenzoate acting like hydrogen peroxide — furthermore, the taste of *o*-iodosobenzoate is almost exactly like hydrogen peroxide. Injection of 10–20 μmoles of *o*-iodosobenzoate into animals causes an immediate and marked depression of the respiration usually lasting 2–3 min, from which recovery occurs spontaneously. *o*-Iodoxybenzoate is somewhat more potent but *o*-iodobenzoate is inactive. Higher doses are required to elicit the circulatory depression described above and the apnea is not secondary to the fall in blood pressure. Antagonism between *o*-iodosobenzoate and cyanide on the respiration (the latter stimulates respiration) is also observed and felt to support the concept that *o*-iodosobenzoate acts by giving up its active oxygen. Jahn (1914) observed rather nonspecific toxic effects in frogs, followed by a slowly developing paralysis and loss of reflexes, death occurring when reflex activity has dropped to zero and cardiac failure is evident. *o*-Iodobenzoate is less than one tenth as toxic. The relative inactivity of *o*-iodobenzoate in all of these studies makes it very unlikely that any of the actions of *o*-iodosobenzoate are due to the former compound, which undoubtedly is formed in the tissues. Jahn postulated an enzyme that splits the iodine from *o*-iodobenzoate since he found both organic and inorganic iodine in the urine after *o*-iodosobenzoate, the product presumably being salicylate.

Very interesting effects on the blood are observed following intravenous infusion of 0.5 millimole of *o*-iodosobenzoate into rabbits (Loevenhart and Grove, 1911). Over a period of 3 days there is a slight depression of the erythrocytes (around 15%) and negligible effects on coagulation mechanisms but there appears early a very marked leucocytosis, this being confined almost entirely to the polymorphonuclears, which increase from 2,160 to 11,362 in 24 hr. It is not known if this stems from a reaction with SH groups or an action on some metabolic system.

One factor which must be taken into account in considering the effects of any SH reagent on the whole animal is the possible release of active substances. Thus *o*-iodosobenzoate at fairly low concentrations (0.1 m*M*) releases catecholamines from the isolated chromaffine granules of the adrenal medulla (D'Iorio, 1957). This was thought to be an effect on the SH groups located in the granule membranes, but there is no evidence for any mechanism. On the other hand, the release of histamine from rat peritoneal mast cells by Compound 48/80 is inhibited by *o*-iodosobenzoate, and presumably histamine is not released by *o*-iodosobenzoate alone (VanArsdel and Bray, 1961).

The intravenous lethal dose in rabbits is 150–200 mg/kg (0.57–0.76 millimole/kg) and such values have generally been found in most animals. Dr. Loevenhart courageously ingested a total of 1.3 g within 5.5 hr without the slightest effect. The lethal dose of iodosobenzene is the same as that of *o*-iodosobenzoate, indicating that the carboxylate group is not essential for

the toxicity (Luzzato and Satta, 1910). Probably the only useful role for the carboxylate group is to increase the solubility.

EFFECTS ON SEA URCHIN EGG DEVELOPMENT

The effects of 0.66 m*M* *o*-iodosobenzoate in sea water on the development of *Arbacia* eggs was studied by Runnström and Kriszat (1952). It was found that fertilization and cleavage proceed quite normally up to the blastula stage (perhaps with a slight delay), but after 6 hr the controls are hatched whereas the treated ones are not. After 20 hr the controls are bilateral early plutei, but 80–90% of the treated larvae are still within their membranes, the formation of the entoderm being suppressed in these. The animal region is characterized by a high cylindrical region of epithelium carrying a ciliary tuft, whereas the cells at the vegetal pole are flattened. The treated larvae contain no pigment and the pigment initially present has disappeared. This effect of animalization of the larvae can be brought about by other enzyme inhibitors (iodoacetate, parapyruvate, etc.) and has been confirmed for *o*-iodosobenzoate by Ranzi (1955). If the larvae after 6 hr exposure to *o*-iodosobenzoate are removed to normal sea water, some recovery occurs and fairly normal plutei may be formed, although the arms are lacking and the archenteron shows no differentiation. The general conclusion was that oxidation of certain SH groups suppresses primarily the differentiation of the entomesoderm.

A more detailed study of the earliest stages of *Arbacia* egg development was made by Monroy and Runnström (1952). The high concentration of 2.64 m*M* *o*-iodosobenzoate does not prevent the fertilization reaction or the formation of the fertilization membrane, but the membrane is somewhat thicker and more refractile than normally. At 80 min the controls are in 2- and 4-cell stages with the membrane unchanged, whereas the treated eggs are all in the 2-cell stage with conspicuous membranes. One hour later three fourths of the treated eggs are cytolyzed with escape of pigment. The membrane thickening and the escape of pigment seem to be correlated. If the eggs are first centrifuged, thickening of the membrane occurs only at the pole where the pigment is located. Thus the membrane changes do not appear to be due to a direct action of the *o*-iodosobenzoate. Possibly the nature of the membrane and its later changes during development depend on substances formed in the egg and metabolic inhibitors interfere in the production or action of these substances.

The exposure of *Paracentrotus lividus* eggs to 0.35–0.7 m*M* *o*-iodosobenzoate does not affect subsequent fertilization or suppress cleavage, although hatching is prevented (Hagström, 1963), confirming the earlier results of Runnström and Kriszat (1952) on *Arbacia* eggs. There are, nevertheless, differences in the response. First, cleavage is somewhat accelerated: The

controls at 135 min after fertilization are 2% in the 2-cell stage, 50% in the 4-cell stage, and 48% in the 8-cell stage, whereas those treated with *o*-iodosobenzoate are 8% in the 4-cell stage and 92% in the 8-cell stage. Second, there is no obvious disturbance in development, e.g., no evidence of animalization, and the ciliated embryos inside their membranes appear to be normally active. Differentiation in *Paracentrotus* is thus less susceptible than in *Arbacia* to *o*-iodosobenzoate. Higher concentrations of *o*-iodosobenzoate may produce other effects on eggs but whether these actions are mediated through SH group oxidation is not known. The eggs of *Hemicentrotus pulcherrimus* and *Urechis unicinctus* elevated the fertilization membrane when incubated for 10 min in 10 m*M* *o*-iodosobenzoate at pH 4 and then returned to normal sea water (Isaka and Aikawa, 1963). It was suggested that the vitelline and plasma membranes are connected by hydrogen bonds and that *o*-iodosobenzoate and other SH reagents react with SH groups in the plasma membrane, weakening these bonds and allowing separation of the membranes. Movements during cleavage have been supposed to involve contractile proteins as in muscle, and threads formed from fibrous proteins obtained from *Hemicentrotus* eggs contract when metal ions (e.g., Mg^{++}, Cu^{++}, Cd^{++}, etc.) are added (Sakai, 1962). This contraction is blocked by 5 m*M* *o*-iodosobenzoate and high concentrations of other SH reagents, indicating that SH groups are necessary.

EFFECTS ON BACTERIA AND VIRUSES

The early interest in the antibacterial actions of iodine led Arkin (1911), in connection with the pharmacological studies of Loevenhart and Grove at Wisconsin, to investigate the effects of *o*-iodosobenzoate and related compounds on various bacteria. *Eberthella typhosa*, *E. coli*, *S. aureus*, and *B. pyocyaneus* are all killed by exposures of 24 hr to 1 m*M* at 37°, not surprisingly. *o*-Iodoxybenzoate is even more potent, but *o*-iodobenzoate does not kill even at 10 m*M*. Jahn (1914) found the growth of *E. coli* to be inhibited by 0.38 m*M* *o*-iodosobenzoate, but not by 38 m*M* *o*-iodobenzoate, indicating the importance of the oxidative action and confirming the results in animals. Chinard (1942) considered the possibility of using *o*-iodosobenzoate locally in infected wounds. He observed marked inhibition of the growth of *E. coli* at 0.02 m*M* with eventual death of the bacteria in 72 hr, and death of hemolytic streptococci at 0.38 m*M*. If the *o*-iodosobenzoate is injected with these streptococci subcutaneously into mice, no infections are seen, but all the control mice die. The flagellar activity of *B. brevis* is well inhibited by 2 m*M* *o*-iodosobenzoate at 30 sec and maximally at 5 min (De Robertis and Peluffo, 1951). Yeast is more resistant, since it requires 3.8 m*M* to inhibit the growth 50% (Loveless *et al.*, 1954). No analyses at all have been made of the sites or mechanisms of action. It is likely that

the activity against bacteria will be strongly influenced by the media used and the other conditions; most of the media for pathogens contain substances readily reacting with *o*-iodosobenzoate. Phagocytosis of staphylococci and streptococci by human leucocytes is stimulated by *o*-iodosobenzoate, but this is indirect since it occurs only in the presence of serum, and is perhaps an activation of serum opsonin (Arkin, 1912).

The psittacosis virus is 30–75% inactivated by exposure to 0.1 m*M* *o*-iodosobenzoate for 1 hr at 37°, only *p*-chloromercuribenzoate of all the agents tested being more potent (Burney and Golub, 1948). In addition, it is the most effective substance in reducing viral growth in chick embryo cultures without inhibiting culture growth. The selectivity is probably not great enough to warrant clinical interest.

CHAPTER 7

MERCURIALS

The mercurials occupy a rather special niche in the subject of enzyme inhibition; they are very useful for demonstrating the presence and importance of SH groups in enzyme reactions, but apparently lack specificity toward particular enzymes or classes of enzymes. Since so many enzymes contain reactive SH groups at or near the active center, the mercurials would seem to inhibit more enzymes than they leave unaffected. When a mercurial acts on living cells, one cannot state which enzymes are affected most readily. In other words, they are reasonably specific with regard to the molecular group attacked, but quite nonspecific at the enzyme or cellular levels. The mercurials will, in addition, react with nonenzymic proteins and may modify complex systems by mechanisms unrelated to metabolism. The mercurials are thus at present generally useless as tools to study the relationship of a particular enzyme to the over-all metabolism, growth, or function of a cell or organism. Nevertheless, with judicious use, they may give some insight into the broader metabolic basis of function, as in certain studies of gastric acid secretion, renal transport, and mitosis. Their primary use, however, is the detection and titration of SH groups on enzymes. They are often stated to be the most specific SH reagents; this may be questioned, but without doubt they are among the most reactive reagents and seldom does one find SH groups resistant to the mercurials and capable of reacting with other SH reagents. Like all inhibitors, they are valuable only when used in the proper system. It is always tempting to use inhibitors such as the mercurials which will almost always produce definite effects, but unfortunately the results usually cannot be interpreted satisfactorily. We shall emphasize the quantitative side of mercurial action and the inhibitions of pure enzymes, discussing only briefly effects observed on complex systems, inasmuch as little useful information can be derived from this latter work.

The medical use of the mercurials can be traced back for over 3000 years, although their modern therapeutic applications began with the rediscovery of the diuretic action of mercurous chloride in 1849 (since then this action has been rediscovered several times), the demonstration of the antiseptic

action of mercuric chloride by Koch in 1881, and the introduction of organic mercurials for diuresis, antisepsis, and other chemotherapeutic purposes from 1900 to 1920. The marked toxicity of inorganic mercury was recognized in antiquity and became a more critical problem over 400 years ago, especially in processes such as fur felting for hats and more recently in the widespread use of mercurials as plant fungicides for various rots and rusts. There was a good deal of experimentation and speculation on the nature of mercurial antisepsis between 1900 and 1940, but little of this is pertinent to our present purposes. The early work was much concerned with the examination of the validity of certain vague concepts, such as the Arndt-Schulz law (which states that drugs stimulate in low concentration and inhibit in high concentration), oligodynamic action, and the Ostwald adsorption theory. Despite the fact that the combination of mercurials with thiols, e.g. cysteine, has been known since 1875 at least, investigations on the metabolic effects and enzyme inhibition are very sparse before 1930. Actually the mercurials have been intensively used by biochemists for the characterization of enzymes for only the past several years. Of the some 1350 publications on the effects of mercurials on isolated enzymes, only 4% were issued prior to 1950, 16% from 1950 to 1956, and 80% from 1956 through 1964. By the time this volume goes to press, approximately half of the publications on this aspect of the mercurials will have appeared after 1960. These figures indicate essentially that each newly isolated enzyme is subjected to one or more mercurials for the purpose of detecting SH groups. One of the major aims of this chapter is to attempt to determine the validity and usefulness of such determinations.

CHEMICAL PROPERTIES

The most commonly used inorganic mercury compound in inhibition work is mercuric chloride ($HgCl_2$). Some fundamental properties of the Hg^{++} ion and its halides are summarized in Table 7-1. It may be noted that although the linearity of HgX_2 molecules is established and the configuration of certain HgX_4 complexes appears to be tetrahedral, the nature of the $HgCl_3^-$ and $HgCl_4^=$ ions is not clear and a planar arrangement is possible. The aqueous solubility of $HgCl_2$ increases with the concentration of NaCl, KCl, or other halide present; thus the solubility of $HgCl_2$ in Krebs-Ringer medium is around 12.5 and in sea water around 27 g/100 ml (Barnes and Stanbury, 1948). The deficiency in the ionic character of $HgCl_2$ is indicated by the high solubility in ethanol (26.3 g/100 ml) and even in ether (4.55 g/100 ml). Indeed, $HgCl_2$ has been said to be reasonably lipid-soluble, a fact of some importance in considering the distribution in the tissues. $HgBr_2$ and HgI_2 are much less soluble than $HgCl_2$ in water and seem to have no advantages over $HgCl_2$ in enzyme studies.

TABLE 7-1

SOME PROPERTIES OF MERCURY, THE MERCURIC ION, AND THE MERCURIC HALIDES

Radii	
Hg atom	1.59 Å
Hg^{++} ion	0.66 Å
Bond lengths	
Hg-Cl	2.20 Å
Hg-Br	2.40 Å
Hg-I	2.55 Å
Bond ionic character	
Hg-Cl in $HgCl_2$	28%
Bond types	
$HgCl_2$	Linear (sp)
$HgCl_4^=$	Tetrahedral (sp^3)
Electronegativity	
Hg(II)	1.9
Solubility in water (g/100 ml solution)	
$HgCl_2$	6.8 (25°)
	8.9 (37.5°)
Solubility product	
$HgCl_2$	1.06×10^{-18} (25°)
$K_{sp} = (Hg^{++})(Cl^-)^2$	2.95×10^{-18} (37.5°)
pH of saturated solution	
$HgCl_2$	4.7 (25°)
Redox equilibrium	
$Hg^{++} + Hg(I) \leftrightarrows Hg_2^{++}$	
$K = (Hg_2^{++})/(Hg^{++})$	129.2
Redox potentials ($E^\circ_{25^\circ}$)	
$Hg_2^{++} \leftrightarrows 2\,Hg^{++} + 2e^-$	− 0.92 v
$2\,Hg \leftrightarrows Hg_2^{++} + 2e^-$	− 0.79 v
$Hg \leftrightarrows Hg^{++} + 2e^-$	− 0.85 v

Equilibria between Hg^{++} and Halide Ions

In aqueous solution $HgCl_2$ does not dissociate simply into Hg^{++} and Cl^- ions, but forms a series of complexes, the relative concentrations of which depend on the Cl^- concentration and the pH. The following species are the most important: Hg^{++}, $HgCl^+$, $HgCl_2$, $HgCl_3^-$, and $HgCl_4^=$. This applies to acid solutions where hydrolysis and hydroxyl complexes can be ignored (see next section). Higher Cl complexes with Hg^{++} can be neglected in biological work, as can univalent Hg_2^{++} and its complexes (since no equilibrium with metallic mercury occurs). Sillén and his collaborators in Stockholm have summarized their extensive investigation of the halide complexes of mercury (Sillén, 1949) and we shall follow their values for the equilibrium constants (it should be noted that their work was done at 25° so that small corrections should be applied for solutions at other temperatures). However, we shall differ in two ways from Sillén in the expression of the constants. In the first place, we shall use dissociation rather than association constants, in conformity to the usage throughout this book. In the second place, we shall indicate the individual dissociations by K's and the cumulative dissociations by β's, in conformity with the usual terminology in metal-ligand complexes and chelates (Bjerrum *et al.*, 1957). The fundamental dissociations and their constants can be formulated as in Table 7-2. The tight binding of the first two Cl^- ions is evident, but the next two are bound only weakly, due perhaps to the change in bond configuration and the increasing negativity; that the latter is not a major factor is indicated by the similar behavior of the ammonia complexes. The constants for the Br^- and I^- complexes are much less than for Cl^-, i.e., the former ions are more tightly bound to Hg^{++}, but such equilibria are seldom of importance in biological systems.

The relative concentrations of the various complexes depend in simple solutions mainly on the Cl^- concentration. The fractions of the total mercury in particular complexes may be calculated from the following equations:

$$\begin{aligned}
(Hg^{++})/(Hg_t) &= 1/A \\
(HgCl^+)/(Hg_t) &= (Cl^-)/\beta_1 A \\
(HgCl_2)/(Hg_t) &= (Cl^-)^2/\beta_2 A \\
(HgCl_3^-)/(Hg_t) &= (Cl^-)^3/\beta_3 A \\
(HgCl_4^=)/(Hg_t) &= (Cl^-)^4/\beta_4 A
\end{aligned} \tag{7-1}$$

where

$$A = 1 + \frac{(Cl^-)}{\beta_1} + \frac{(Cl^-)^2}{\beta_2} + \frac{(Cl^-)^3}{\beta_3} + \frac{(Cl^-)^4}{\beta_4}$$

The Cl^- concentration varies over a wide range in the media used. In isolated enzyme work it may be very low (unless KCl or NaCl is added); it is 102 mM

TABLE 7-2

DISSOCIATION CONSTANTS FOR MERCURIC CHLORIDE COMPLEXES[a]

Equilibrium	Definition of K	K	pK	Definition of β	β	pβ
$Hg^{++} + Cl^- \rightleftarrows HgCl^+$	$K_1 = \frac{(Hg^{++})(Cl^-)}{(HgCl^+)}$	1.82×10^{-7}	6.74	$\beta_1 = \frac{(Hg^{++})(Cl^-)}{(HgCl^+)}$	1.82×10^{-7}	6.74
$HgCl^+ + Cl \rightleftarrows HgCl_2$	$K_2 = \frac{(HgCl^+)(Cl^-)}{(HgCl_2)}$	3.31×10^{-7}	6.48	$\beta_2 = \frac{(Hg^{++})(Cl^-)^2}{(HgCl_2)}$	6.03×10^{-14}	13.22
$HgCl_2 + Cl \rightleftarrows HgCl_3^-$	$K_3 = \frac{(HgCl_2)(Cl^-)}{(HgCl_3^-)}$	1.41×10^{-1}	0.85	$\beta_3 = \frac{(Hg^{++})(Cl^-)^3}{(HgCl_3^-)}$	8.51×10^{-15}	14.07
$HgCl_3^- + Cl^- \rightleftarrows HgCl_4^=$	$K_4 = \frac{(HgCl_3^-)(Cl^-)}{(HgCl_4^=)}$	1.00×10^{-1}	1.00	$\beta_4 = \frac{(Hg^{++})(Cl^-)^4}{(HgCl_4^=)}$	8.51×10^{-16}	15.07

[a] The cumulative constants are, of course, given by: $\beta_1 = K_1$, $\beta_2 = K_1K_2$, $\beta_3 = K_1K_2K_3$, and $\beta_4 = K_1K_2K_3K_4$. Values of β's from Sillén (1949).

in serum, 126 mM in Krebs-Ringer bicarbonate medium, 143 mM in Tyrode solution, 154 mM in physiological saline, and 515 mM in sea water. The distribution between species of complexes for three situations (low, moderate, and high Cl^-) is shown in Table 7-3, and the distribution over a complete spectrum of Cl^- concentrations is illustrated in Fig. 7-1. It so happens

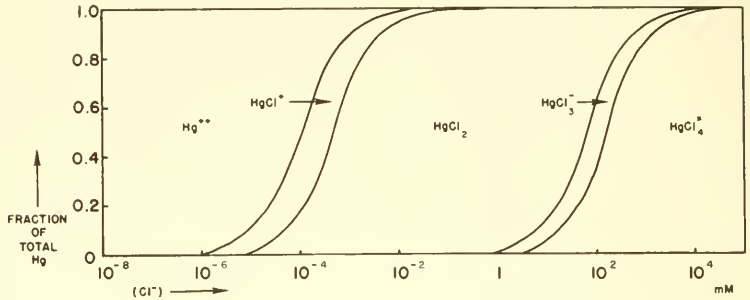


Fig. 7-1. Curves showing the distribution of the different chloride complexes of Hg^{++} with Cl^- concentration. (From Sillén, 1949.)

that, in most media used in cell and tissue preparations, the concentrations of $HgCl_2$, $HgCl_3^-$, and $HgCl_4^=$ are roughly equal, whereas in sea water the predominant form is $HgCl_4^=$. In the media for isolated enzyme study, in which Cl^- is often low, the predominant form may be $HgCl_2$ or even $HgCl^+$.

Table 7-3

Distribution of Mercuric Chloride Complexes as Fractions of the Total Mercury in Media of Different Cl^- Concentration

Fraction	$(Cl^-) = 1$ mM	Krebs-Ringer medium $(Cl^-) = 126$ mM	Sea water $(Cl^-) = 515$ mM
$(Hg^{++})/(Hg_t)$	6.03×10^{-8}	1.26×10^{-12}	9.8×10^{-15}
$(HgCl^+)/(Hg_t)$	3.31×10^{-4}	8.68×10^{-7}	2.8×10^{-8}
$(HgCl_2)/(Hg_t)$	0.9925	0.331	0.0428
$(HgCl_3^-)/(Hg_t)$	7.09×10^{-3}	0.296	0.156
$(HgCl_4^=)/(Hg_t)$	7.09×10^{-5}	0.373	0.801

It has often been assumed in the past that the mercuric ion Hg^{++} is the predominant form or the active inhibitor, but it is now realized that this is not the case. The importance of such complexes in inhibition studies is 2-fold. In the first place, the equilibria for the binding of mercury to SH

groups are modified, since one may say that the Cl^- ions are competing with the SH groups for Hg^{++}; this affects the dissociation constants for R—S—Hg^+ and R—S—Hg—S—R complexes (see page 740). In the second place, the penetration of the inhibitor into cells will depend on the relative concentrations of these complexes. A few investigators have realized the implications of such complexes and have attempted to take into account the equilibria under their experimental conditions. Jowett and Brooks (1928) calculated the relative concentrations of the complexes in a 0.2 m*M* solution of $HgCl_2$ in Locke's medium in a study of the effects of $HgCl_2$ on tissue glycolysis and respiration, and concluded that the dominant penetrating species is $HgCl_2$, although they were uncertain as to the form effective on the enzymes. Barnes and Stanbury (1948) realized that Hg^{++} is extremely low in sea water in their investigation of the toxic actions of $HgCl_2$ on a copepod, and assumed that the prevalent species were $HgCl_3^-$

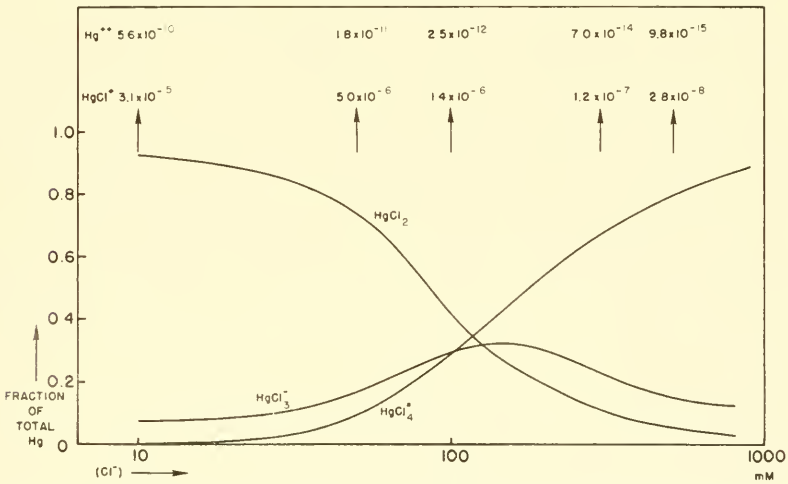


Fig. 7-2. Fraction of Hg in various forms in acid medium with varying Cl^- concentration. The figures at the top give the concentrations of Hg^{++} and $HgCl^+$ at selected Cl^- concentrations.

and $HgCl_4^=$. Green and Neurath (1953) likewise discounted the importance of Hg^{++} in the inhibition of trypsin in a medium containing 10 m*M* Cl^-. In order to facilitate estimation of the relative concentrations of the complexes in a narrower range of Cl^- concentration as commonly used in inhibition studies, Fig. 7-2 is presented. However, before considering these complexes further, it will be necessary to discuss their so-called hydrolysis in a pH range around neutrality.

Equilibria between Hg^{++} and Hydroxyl Ions

It has generally been assumed that the Hg^{++} ion is hydrated and that this ionizes according to the following equations:

$$Hg(H_2O)_2^{++} \leftrightarrows HgOH(H_2O)^+ + H^+ \qquad pK_{a_1} = 3.70$$

$$HgOH(H_2O)^+ \leftrightarrows Hg(OH)_2 + H^+ \qquad pK_{a_2} = 2.60$$

This is essentially saying that the hydrated ion is a dibasic acid (Hietanen and Sillén, 1952). It is evident that at pH's near neutrality, $Hg(OH)_2$ will be the predominant form. For our purpose and comparison of these equilibria with those for Cl^-, it might be better to express the reactions as simple complexing with OH^- ions. Thus $pK_{OH_1} = 10.3$, and $pK_{OH_2} = 11.4$:

$$Hg^{++} + OH^- \leftrightarrows HgOH^+ \qquad K_{OH_1} = \frac{(Hg^{++})\ (OH^-)}{(HgOH^+)} = K_w/K_{a_1} = 5 \times 10^{-11}$$

$$HgOH^+ + OH^- \leftrightarrows Hg(OH)_2 \qquad K_{OH_2} = \frac{(HgOH^+)(OH^-)}{[Hg(OH)_2]} = K_w/K_{a_2} = 4 \times 10^{-12}$$

which may be compared to $pK_{Cl_1} = 6.74$, and $pK_{Cl_2} = 6.48$ (Table 7-2). Now in a Cl^--free medium, even at pH 5, the ratio $[Hg(OH)_2]/(Hg^{++})$ will be 5000, and at pH 7 will be 50,000,000, so that Hg^{++} will be negligible. Although the affinity of Hg^{++} for OH^- is greater than for Cl^-, when Cl^- is present in appreciable concentration (e.g. 10–150 mM) it will compete effectively for the Hg^{++} ion since at neutrality its concentration will be 10^5 to 10^6 times that of OH^-. Therefore one would predict that, in the usual media for inhibition studies, the Cl^- complexes will predominate over the OH^- complexes although, particularly as the pH is increased above 7, it is clear that complexes of the type $HgCl(OH)$, $HgCl(OH)_2^-$, $HgCl_2(OH)^-$, and $HgCl_3(OH)^=$ may contribute significantly to the total population. The data given by Sneed and Brasted (1955) allow one to calculate the constants for the following equilibria:

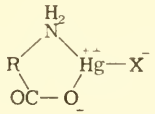
$$HgCl^+ + OH^- \leftrightarrows HgCl(OH) \qquad \frac{(HgCl^+)\ (OH^-)}{[HgCl(OH)]} = 3.2 \times 10^{-8}$$

$$HgCl_3^- + OH^- \leftrightarrows HgCl_3(OH)^= \qquad \frac{(HgCl_3^-)\ (OH^-)}{[HgCl_3(OH)^=]} = 5.0 \times 10^{-8}$$

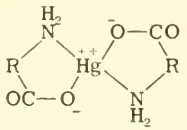
The affinities of the Cl^- complexes for OH^- are thus of the same order of magnitude, and less than for the Hg^{++} ion. At pH 7, $(HgCl^+)/HgCl(OH) = 0.32$ and $(HgCl_3^-)/HgCl_3(OH)^= = 0.5$, so it is seen that these OH^- complexes are indeed significant. The importance of these OH^- and mixed complexes for inhibition studies is, of course, the same as that of the Cl^- complexes, but the concentration of Hg^{++} will be even less than calculated in the previous section.

Complexes of Hg^{++} with Various Ligands

Most metal ions, including Hg^{++}, form strongly ionic covalent bonds with ligand atoms capable of donating electron pairs, both Cl^- and OH^- being simple examples of this. We would expect that Hg^{++} might complex readily with a variety of substances, many of which occasionally occur in media used for inhibition studies. It is usually stated that Hg^{++} reacts with SH groups selectively and that other groups on proteins seldom contribute to the binding; it is necessary to look into this matter quantitatively, and obtain some idea of the relative affinities of the various groups for Hg^{++}. Complexes of Hg^{++} with ammonia are well known so that binding to amino groups might be predicted and, since some interaction with carboxylate groups is likely, it may be anticipated that amino acids would provide effective ligands. Indirect evidence for such complexes was obtained by Salle and Ginoza (1943) by showing that several amino acids reduce the bactericidal activity of $HgCl_2$. The minimal lethal concentration of $HgCl_2$ is increased 6 times by glycine, aspartate, glutamate, arginine, and lysine at 67 mM, and 120 times by cysteine. This indicates appreciable complexing with amino acids under physiological conditions, although the reaction with the SH group of cysteine is evidently stronger than with other groups. Haarmann (1943 a,b) claimed that whereas 1 equivalent of Hg is bound to certain amino acids at pH 7, as much as 4 to 8 equivalents may be bound at pH 11, some loosely and some tightly. A definitive investigation was made by Perkins (1952, 1953) and a number of stability constants were determined. Two major complexes were assumed, probably with the following structures:



Complex I



Complex II

The composite constant, $\beta_2 = K_1K_2$, where K_1 and K_2 are defined by the following equilibria:

$$Hg^{++} + AA^- \leftrightarrows HgAA^+ \qquad K_1 = \frac{(Hg^{++})\ (AA^-)}{(HgAA^+)}$$

$$HgAA^+ + AA^- \leftrightarrows Hg(AA)_2 \qquad K_2 = \frac{(HgAA^+)\,(AA^-)}{Hg(AA)_2}$$

was determined in each case, and these values are given in Table 7-4 along with the dissociation constants for a number of ligand complexes. The form of the amino acid necessary for chelation with Hg^{++} is $^-OOC—R—NH_2$

Table 7-4

Dissociation Constants for Various Mercuric Complexes[a]

Ligand	pK_1	pK_2	$p\beta_2$	Reference[b]
Inorganic ions				
Cl^-	6.74	6.48	13.22	(1)
Br^-	9.05	8.28	17.33	(1)
I^-	12.87	10.95	23.82	(1)
OH^-	10.3	11.4	21.7	(2)
CN^-	—	—	34.7	(3)
SCN^-	—	—	17.4	(3)
Pyrophosphate	—	—	17.45	(4)
Nitrogenous compounds				
Methylamine	8.6	9.3	17.9	(5)
Triethylamine	7.8	7.8	15.6	(5)
1,2-Diaminopropane	—	—	23.5	(5)
1,2,3-Triaminopropane	19.6	—	—	(5)
Ethylenediamine	—	—	23.4	(5)
Ethanolamine	8.5	8.8	17.3	(5)
Diethanolamine	7.8	7.8	15.6	(5)
Triethanolamine	6.9	6.2	13.1	(5)
2,2′-Diaminodiethylamine	21.8	—	—	(5)
Triethylenetetramine	25.3	—	—	(6)
Ammonia	8.8	8.7	17.5	(7)
Pyridine	5.1	4.9	10.0	(3)
Piperidine	8.7	8.7	17.4	(5)
Imidazole	—	—	16.7	(8)
Ethylenediaminediacetate	9.75	6.05	15.8	(5)
Ethylenediaminetetraacetate	22.1	—	—	(5)
Hexamethylenediaminetetraacetate	21.4	—	—	(5)
Amino acids				
Glycine	10.3	8.9	19.2	(5)
			18.2	(9)
Glycylglycine	—	—	12.4	(9)
Alanine	—	—	18.4	(9)
Leucine	—	—	17.5	(9)
Proline	—	—	20.5	(9)

TABLE 7-4 (*continued*)

Ligand	pK_1	pK_2	$p\beta_2$	Reference [b]
Serine	—	—	17.5	(10)
Tyrosine	—	—	17.1	(10)
Arginine	—	—	17.4	(10)
Histidine	—	—	21.2	(8)
Methionine	6.52	4.93	11.45	(11)
Ethionine	7.25	5.92	13.17	(11)
Cysteine	14.21	—	—	(11)
S-Methylcysteine	7.20	5.81	13.01	(11)
Purines and pyridimidines				
Adenine	—	—	11.5	(12)
Adenosine	—	—	8.5	(12)
Thymine	—	—	21.2	(12)
Thymidine	—	—	21.2	(12)
Cytosine	—	—	10.9	(12)
Miscellaneous				
Acetate	4.0	—	—	(13)
Cyclohexene	4.3	—	—	(5)
Penicillamine	16.15	—	—	(11)

[a] β_2 is the cumulative dissociation constant for HgL_2, and is K_1K_2.

[b] References:

(1) Sillén (1949).
(2) Hietanen and Sillén (1952).
(3) Simpson (1961).
(4) Yamane and Davidson (1960).
(5) Bjerrum *et al.* (1957).
(6) Chaberek and Martell (1959).
(7) Bjerrum (1941).
(8) Brooks and Davidson (1960).
(9) Perkins (1952).
(10) Perkins (1953).
(11) Lenz and Martell (1964).
(12) Ferreira *et al.* (1961).
(13) Gurd and Wilcox (1956).

and, in the calculations of the constants, only that fraction of the amino acid at the pH used was considered.

The fact that simple amines complex with Hg^{++} to approximately the same degree as the amino acids indicates that the amino group is the important ligand, the carboxylate group perhaps contributing slightly to the stability. Ring nitrogen atoms are probably not as effective as amino groups. Hg^{++} reacts with both the amino and imidazole groups of histidine, but more tightly with the former, the pK's being 10.6 and 7.5, respectively (Simpson, 1961). The effect of the pH on the stability of these complexes is well illustrated by the constants for the following equilibria with histidine (Brooks and Davidson, 1960):

$Hg^{++} + 2$ hist$^-$	$\leftrightharpoons$ Hg $(hist)_2$	$p\beta_2 = 21.2$
Hg^{++} + hist$^-$ + H-hist	$\leftrightharpoons$ Hg (hist) $(H\text{-hist})^+$	$p\beta_2 = 18.4$
$Hg^{++} + 2$ H-hist	$\leftrightharpoons$ Hg $(H\text{-hist})_2^{++}$	$p\beta_2 = 15.0$

where hist designates the $^-OOC—R—NH_2$ form and H-hist the $OCC—R—NH_3^+$ form. These complexes with histidine were assumed to be linear and it was claimed that chelation must play only a small role in Hg^{++} complexes due to the tendency of Hg^{++} to form linear complexes. This brings up an interesting point of importance in understanding the reactions of $HgCl_2$ with proteins and enzymes. Certainly some of the most stable complexes of Hg^{++} — as with 1,2,3-triaminopropane, triethylenetetramine, and EDTA — must be chelates and nonlinear, and it is also well known that Hg^{++} reacts with dimercaprol (BAL) to form a ring with the two SH groups. Whether chelation is or is not important in any case probably depends on several factors, such as the spatial arrangement of the ligand groups, the intrinsic affinity of the Hg^{++} for these groups, and the entropy changes accompanying the formation of the complex. Certainly the third and fourth ligands generally add to the HgL_2 complex much less readily than the first two, as we have seen for Cl^-. This is also true for ammonia, the successive constants being given by $pK_1 = 8.8$, $pK_2 = 8.7$, $pK_3 = 1.0$, and $pK_4 = 0.78$. Thus the formation of HgL_3 and HgL_4 type of complexes must involve some additional factors increasing the stability.

In any event it is clear that the complexes of Hg^{++} with amino acids and many other compounds are stable enough so that, when these substances are present in the media used for the study of inhibition, a significant fraction of the Hg may be in the form of such complexes. For example, it may be calculated for a solution containing 1 mM glycine and a total concentration of Hg of 0.1 mM that $(Hg^{++}) = 6.3 \times 10^{-18}$ M. If the Cl^- concentration is appreciable, this will reduce the binding to these other ligands. One may visualize the situation somewhat as follows. In most media there will be several substances — Cl^-, OH^-, buffers, amino acids, substrates, etc. — capable of complexing with Hg^{++}. The end result will

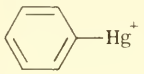
be that the Hg^{++} concentration will be extremely low, the Hg being partitioned between numerous complexes of different types, each one reducing to some extent the reaction of Hg with enzymes. It must be clearly understood that when inhibition is stated below to be by $HgCl_2$ or $Hg(NO_3)_2$ or Hg acetate, it refers only to what was added and not to the dominant form present or the active inhibitor.

Complexes of Hg^{++} with Nucleotides and Nucleic Acids

Complexes between Hg^{++} and certain purines and pyrimidines, especially thymine, are quite stable (Table 7-4) (Katz, 1962), and complexes with phosphates are probably formed readily; thus, one would expect nucleotides and nucleic acids also to bind Hg^{++} rather well. Inagaki (1940) found various nucleotides, such as AMP, GMP, and IMP, to be precipitated by mercuric compounds, but unfortunately no further work has been done on these complexes. One would like to know the nature and extent of the interactions of mercurials with ATP, NAD, FAD, and related substances. However, the studies of the reactions between Hg^{++} and nucleic acids have recently been accelerated, and it is obvious that the results could be very important in understanding the effects of mercurials on cellular growth and proliferation. Hg^{++} has been found to complex with nucleic acids from thymus (Katz, 1952), plants (Trim, 1959), pneumococci (Dove and Yamane, 1960), and tobacco mosaic virus (Katz and Santilli, 1962 b). The general effects on the nucleic acids may be summarized briefly as follows: a decrease in viscosity; an increase in turbidity, sedimentation constant, aggregation, and the dimer:monomer ratio; an increase in the flexibility of the chains with the assumption of a more compact configuration; and a change in the ultraviolet absorption spectra (Katz, 1952; Thomas, 1954; Yamane and Davidson, 1961). It was originally believed that the complexing is with the phosphate groups, but the nature of the absorption spectrum changes, the stoichiometry of the reactions, and the release of H^+ indicate that the bases are the sites of binding, the Hg:base combining ratio being 1 : 2 in most cases, Hg apparently bridging the double strands of the DNA helix (Katz, 1962). The single-stranded tobacco mosaic virus RNA, however, gives a combining ratio of 1 : 1, as expected (Katz and Santilli, 1962 a). It may be noted that the combining ratio is 1 : 2 for guanine oligoribonucleotides, such as GpGpG (Lipsett, 1964). These complexes are usually completely reversible upon adding various Hg^{++} complexers (Cl^-, cyanide, EDTA, or thiols), and indeed the pneumococcal transforming DNA after demercuration retains all of its activity (Dove and Yamane, 1960), and the tobacco mosaic virus after removal of the Hg^{++} regains its infectivity (Singer and Fraenkel-Conrat, 1962), these observations indicating that the original configurations of the nucleic acids can be restored despite the apparently marked structural modifications occurring during reaction with Hg^{++}.

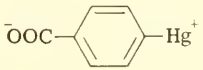
Organic Mercurials

It will be convenient to discuss some of the general properties of the organic mercurials before coming to the important problem of the reaction of mercurials with SH groups. Many organic mercurials were developed for chemotherapy, disinfection, and diuretic activity and, although some of these have been occasionally used in inhibition work, mercurial inhibitors are generally simpler structurally. The chemical properties and structure-action relationships will be taken up later for the antiseptics (page 970) and diuretics (page 917). The aryl mercurials (such as *p*-chloromercuribenzoate) were introduced as enzyme inhibitors by Hellerman (1937) and the alkyl mercurials (such as methylmercuric chloride) as protein reactants by W. L. Hughes (1950). The accompanying formulas are for the ions which



Phenylmercuric ion

Methylmercuric ion



p-Mercuribenzoate ion

p-Mercuriphenylsulfonate ion

eventually complex with SH groups and other ligands. They are used as chlorides, hydroxides, nitrates, or acetates but, once they have been added to the medium, the ions as formulated complex with various ligands which may be present, essentially as Hg^{++} does. Thus it is correct to speak of phenylmercuric acetate or *p*-chloromercuribenzoate as the mercurial used, but this terminology does not give an accurate representation of the forms present in solution. For example, it makes no difference in the final inhibition whether one uses *p*-chloromercuribenzoic acid or sodium *p*-hydroxymercuribenzoate, the two forms of the *p*-mercuribenzoate ion commonly available. For the sake of compression, we shall use the abbreviations shown in the following tabulation in the remainder of this chapter.

Ion	Abbreviation
Phenylmercuric	PM
Methylmercuric	MM
p-Mercuribenzoate	*p*-MB
p-Mercuriphenylsulfonate	*p*-MPS

It will be useful to summarize briefly some of the important differences between the organic mercurials and $HgCl_2$.

(a) *Functionality.* $HgCl_2$ is bifunctional in the sense that it can react with two ligands to form L-Hg-L complexes, whereas the organic mercurials are monofunctional in that they can react with only one ligand to give R-Hg-L. Hg^{++} can also form cyclic mercaptides with two adjacent SH groups but the organic mercurials cannot. These differences are often very important in the reactions with thiols and enzymes, and in fact one of the major reasons for the preference of many investigators for the organic mercurials, especially in the quantitative titration of SH groups, is their monofunctional nature.

(b) *Aqueous solubility.* The organic mercurials are less soluble than $HgCl_2$ and occasionally this has created a problem if higher concentrations are required. However, in most titration or inhibition studies, the concentration required is seldom over 1 m*M*, and this can be easily attained in most cases. Phenylmercuric chloride is soluble to the extent of only 0.16 m*M* in distilled water, but a good deal more soluble in salt solutions, and the introduction of anionic groups increases the solubility. It is well known that *p*-chloromercuribenzoic acid, which for many years was the only commonly used mercurial, does not readily go into solution at neutral pH. It is therefore usual, to dissolve it in dilute KOH or NaOH solutions (0.01–0.05 *M*) and adjust to the desired pH with HCl.* However, the sodium salt is now commercially available and dissolves readily. The phenylsulfonic acid is also more soluble than the benzoic acid derivative. The solubility will be determined, as with $HgCl_2$, by the concentrations of various complexing substances in the medium; thus the solubility is reasonably high in most physiological media containing over 100 m*M* Cl^-, or various other ions such as pyrophosphate or sulfate, concentrations around 10 m*M* of mercurial being readily obtained.

(c) *Lipid solubility.* The unsubstituted alkyl and aryl mercurials are more soluble than the Hg^{++} ion and its complexes in lipids. Hughes (1957) has estimated that the simple alkyl mercurials are around 100 times more soluble in lipids than in water. This property will presumably allow the organic mercurials to penetrate more readily than inorganic mercury into cells and tissues, and evidence for this is provided by the greater central nervous system toxicity of the organic mercurials (page 951). In this connection,

* Although I have no definite evidence that strongly alkaline media are detrimental to *p*-MB, I would prefer not to use 1 *M* NaOH solution to dissolve the material, as has been done by some (e.g., Snodgrass *et al.*, 1960), since, as we shall see, the C—Hg bond is weak and dissociation is a possibiltiy, and, furthermore, such strongly alkaline solutions are not necessary. For most work it is satisfactory to dissolve *p*-chloromercuribenzoic acid in 0.02 *M* KOH or NaOH at approximately 2 mg/ml or 7.4 m*M*.

if maximal penetration is desired, it is probably best to use the alkyl or the unsubstituted-phenylmercurials, since the COO^- and SO_3^- groups will reduce the permeability.

(d) *Configuration.* Although $HgCl_2$ is linear, as shown by Raman spectra and electron diffraction, the organic mercurials for some reason are apparently not. Dipole moment studies (e.g., for phenylmercuric chloride, $\mu = 2.99$) suggest that the angle of the C—Hg—X bonds is around 130° or higher (Sipos *et al.*, 1955). The moment is directed as follows:

$$\overset{+}{R} - Hg - \overset{-}{X}$$

(e) *Molecular size.* The organic mercurials are, of course, larger than the simple complexes of Hg^{++}. This may be of importance in the reaction with the SH groups of proteins and enzymes, since steric factors may impede the approach of the mercurial to SH groups not exposed on the surface. A reduction in volume was one of the reasons for the introduction of the alkyl mercurials by W. L. Hughes (1950). In addition, penetration into cells will depend to some extent on the molecular size. Other factors will be discussed relative to enzyme inhibition.

(f) *Affinities for ligands.* The organic mercurial ions in solution tend to complex with various ligands in the same way as the Hg^{++} ion, forming complexes of the type R-Hg-L. The affinities seem to be somewhat less for the organic mercurial ions than for Hg^{++}, although very few have been studied. Simpson (1961) gave the dissociation constants for MM complexes (Table 7-5), and generally the pK's are around 1.3 units less than for the Hg^{++} complexes. Nevertheless, the affinities are of sufficient magnitude so that at pH 7 there is perhaps 500 times as much MM-OH as MM^+, and if much Cl^- is present there may be 100 times as much MM-Cl as MM-OH (Hughes, 1957). Rowland (1952) determined the equilibrium constant, $K = (RHg\text{-}OH)\ (H^+)\ (Cl^-)/(RHg\text{-}Cl)$, for a variety of diuretic mercurials, and found a mean value for pK of 9.9, so that at pH 7 and $(Cl^-) = 100$ mM the ratio (RHg-Cl)/(RHg-OH) is around 100 as for MM. Ledoux (1953) reported the interaction of *p*-MB with nucleic acid and from the spectral changes assumed a complex to be formed with the carbonyl group of pyrimidines. Various complexes of PM and amino acids were obtained by Smalt *et al.* (1957) but were claimed to dissociate rather readily. The remarkably tight complex of PM and thyroxine has been investigated by Frieden and Naile (1954). One must thus assume that in solution in most physiological media the organic mercurials will exist in a variety of complexes, and that this will be an important factor in determining the degree of reaction with proteins and enzyme SH groups.

One characteristic of the organic mercurials is the weakness of the C—Hg bond, the energy of which is only 15–19 kcal/mole (Cottrell, 1954), so that

Table 7-5

Dissociation Constants for Complexes with Methylmercuric Ion [a]

Ligand	pK
Cl^-	5.45
Br^-	6.7
I^-	8.7
OH^-	9.5
CN^-	14.2
SCN^-	6.1
Acetate	3.6
Phenolate	6.5
$HEDTA^{3-}$	6.2
Ammonia	8.4
Pyridine	4.8
Imidazole	7.3
Histidine (NH_2 group)	8.8
Histidine (imidazole group)	6.4

[a] From Simpson (1961).

inorganic Hg may be split off more readily than is usually supposed. The exchange reaction:

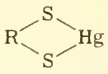
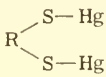
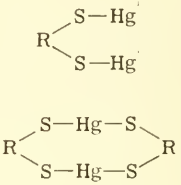
$$Hg^{203}Cl_2 + {}^-OOC—\varphi—Hg^+ \leftrightharpoons HgCl_2 + {}^-OOC—\varphi—Hg^{203+}$$

is fairly fast, the rate constant being 5.4 liters $mole^{-1}sec^{-1}$ at 25° with an activation energy of 12 kcal/mole (Cerfontain and van Aken, 1956), and this indicates the instability of the C—Hg bond. It is possible to produce Hg^{203}-labeled *p*-MB by this reaction. This problem has assumed a good deal of importance in diuretic action and will be discussed more fully in this connection.

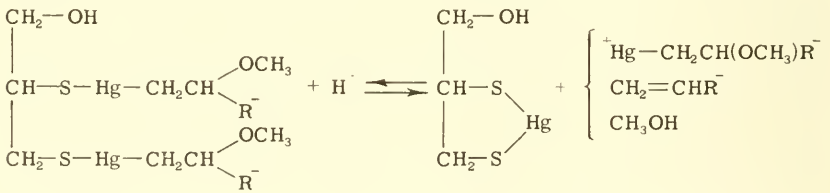
The synthesis of *p*-MB has been described by Whitmore and Woodward (1941). It may be purified by repeated solutions in dilute NaOH and precipitations with excess HCl (Boyer, 1954). For the accurate titration of SH groups it is suggested that the purity of the *p*-MB be checked by iodometric titration or spectrophotometrically by absorption measurement at 232 mμ at pH 7 ($\varepsilon_M = 1.69 \times 10^4$) or 234 m$\mu$ at pH 4.6 ($\varepsilon_M = 1.74 \times 10^4$). The stability of *p*-MB solutions has not been determined quantitatively, but Cunningham *et al.* (1957) found that heating to 80°–82° for 90 min with various buffers and at different pH's does not destroy more than 2–4%, and MacDonnell *et al.* (1951) stated that solutions are stable for a month at room temperature. Nevertheless, I would advise making solutions daily for accurate work.

Reactions of the Mercurials with SH Groups

The various types of mercaptide which can be formed are indicated in the accompanying tabulation. The complexes formed under particular cir-

	Monofunctional organic mercurials $R'—Hg^+$	Bifunctional inorganic mercurials Hg^{++}
Monothiols R—SH	R—S—Hg—R′	R—S—Hg^+ R—S—Hg—S—R 
Dithiols 		 (—R—S—Hg—S—)$_n$

cumstances will depend on the relative concentrations of thiol and mercurial, the presence of ligands capable of complexing with the mercurials, the spatial arrangement of the SH groups, the pH, and the nature of the R and R′ groups. Monofunctional mercurials, such as *p*-MB and PM, react with cysteine, glutathione, and 2-mercaptoethanol in 1 : 1 molar ratio to form R—S—Hg—R′ type of complexes (Benesch and Benesch, 1952; Hoch and Vallee, 1960). These reactions can be conveniently followed polarographically. Reactions with dithiols are more complex. The dimercaptide formed from PM and dimercaprol (BAL) is insoluble, but if hydrophilic groups occur on the mercurial the product is usually soluble. However, the instability of the C—Hg bond may allow further reaction to form the cyclic mercaptide, as occurs with mersalyl (Benesch and Benesch, 1952). This reaction, where R = —CH_2CONH—*o*—OCH_2COO^-, leads to the splitting



off of one mersalyl and the formation of inorganic mercury from the other, essentially the reverse of the reaction whereby the mercurial diuretics are synthesized (the oxymercuration of alkenes). Such reactions presumably do not occur with the simpler organic mercurials. It has also been shown that diphenethynyl mercury and glutathione react to form GS—Hg—SG and phenylacetylene (Tanaka, 1961). Mercurials of the type R_2Hg might be expected to be unreactive with SH groups; inasmuch as they are quite toxic, Webb *et al.* (1950) studied their reactions and found that, although most thiols are not attacked, dithiozne is reacted as follows:

$$R—Hg—R + R'—SH \rightarrow R—Hg—S—R' + RH$$

This type of cleavage of the C—Hg bond occurs at physiological temperature and pH.

The primary products of the reactions between $HgCl_2$ and cysteine, glutathione, thioglycolate, and other monothiols are the dimercaptides of the type R—S—Hg—S—R, and it is difficult to study the initial formation of a monomercaptide R—S—Hg^+ (Shinohara, 1935; Stricks and Kolthoff, 1953; Stricks *et al.*, 1954). The reactions between $HgCl_2$ and dithiols are complex and several types of mercaptide may be formed, as indicated in the tabulation above. The occurrence of cyclic mercaptides and polymercaptide linear complexes will depend mainly on the spatial configuration of the SH groups. The pH apparently plays some role in determining the nature of the complexes, since as the pH increases above 2.5, more of the forms $Hg_2(SG)_2$ and $Hg_3(SG)_2$ appear (Kolthoff *et al.*, 1954).

We next turn to the problem of the stability of mercaptides and it is important to establish the dissociation constants for the fundamental complexes formed in simple reactions, such as the following:

$$R—S^- + Hg^{++} \leftrightharpoons R—S—Hg^+$$

$$R—S^- + R'—Hg^+ \leftrightharpoons R—S—Hg—R'$$

Table 7-6 shows a few of the recently determined constants for Hg^{++} and MM. If one assumes that pK_1 and pK_2 are similar in magnitude, which is reasonable, it is seen that pK_1 (which applies to the reactions above) lies between 20 and 22, with a mean value of 21.3. This range may thus be taken provisionally as indicating the usual affinity between mercurials and thiols in the absence of competing protons and ligands. Comparison of these values with those in Tables 7-4 and 7-5 shows that the affinity of mercurials for SH groups is far greater than for any other single ligands, and that, in a mixture of thiols and various other complexing ligands, a mercurial will be predominantly associated with the thiols. The variations of pK with the temperature and the ionization of auxiliary groups on the thiol are shown, as summarized from the studies of Stricks and Kolthoff (Table 7-7). Proto-

Table 7-6

Some Dissociation Constants for Simple Mercaptides[a]

Mercurial	Thiol	pK_1	$p\beta_2$	Reference
Hg^{++}	Cysteine$^-$	20.1	—	Simpson (1961)
		20.5	—	Perkins (1953)
		—	43.57	Stricks and Kolthoff (1953)
	Glutathione$^-$	—	41.58	Stricks and Kolthoff (1953)
	Thioglycolate$^-$	—	43.82	Stricks *et al.* (1954)
Methyl-Hg^+	Cysteine$^-$	15.7	—	Simpson (1961)
	Human seralbumin	22.0	—	Hughes (1957)
	Bovine seralbumin	22.6	—	Hughes (1957)
	Bovine HbO_2	22.1	—	Hughes (1957)
	Bovine HbCO	22.6	—	Hughes (1957)

[a] The pK_1's for the mercaptides with seralbumin and hemoglobin have been recalculated (page 755), assuming pK_a for the SH groups to be 8.7 and the pK for complexing with I^- to be 8.7. These values should not be considered as accurate because of the assumptions involved.

Table 7-7

Dissociation Constants for Mercaptides with Hg^{++} Illustrating the Effects of pH and Temperature

Constant[a]	Cysteine		Glutathione		Thioglycolate	
	12°	25°	12°	25°	12°	25°
pK_1	41.82	40.25	42.29	40.96	45.66	44.31
pK_2	45.27	43.60	43.54	41.92	45.85	44.33
pK_3	45.40	43.57	43.47	41.58	45.52	43.82
pK_e	7.39	7.10	7.97	7.85	—	—
pK_f	10.72	10.48	9.28	9.15	—	—

[a] The constants are defined as follows:

$$K_1 = \frac{(Hg^{++})\,(R)^2}{(HgR_2^{++})} \qquad K_2 = \frac{(Hg^{++})\,(R)\,(R^-)}{(HgR_2^{+})} \qquad K_3 = \frac{(Hg^{++})\,(R^-)^2}{(HgR_2)}$$

$$K_e = \frac{(H^+)\,(HgR_2^{+})}{(HgR_2H^{++})} \qquad K_f = \frac{(H^+)\,(HgR_2)}{(HgR_2H^{+})}$$

where $R = {}^+H_3N—X—S^-$ and $R^- = N_2H—X—S^-$ for cysteine and glutathione. and $R = HOOC—X—S^-$ and $R^- = {}^-OOC—X—S^-$ for thioglycolate. (From Stricks and Kolthoff, 1953; Stricks *et al.*, 1954.)

nation of an amino group on cysteine or glutathione reduces somewhat the affinity of the thiol for Hg^{++}, as might be anticipated, while ionization of the thioglycolate carboxyl groups has little effect. With the constants in Table 7-7 it is possible to predict the relative concentrations of the various species present; when complexing ligands are in significant concentration, appropriate corrections must be made (page 737). The variations with temperature allow the calculation of certain basic thermodynamic parameters. For the formation of the mercaptides, ΔF is -55 to -59 kcal/mole and the entropy changes are positive and usually rather large; for the equilibria expressed by pK_1 (see legend in Table 7-7), ΔS is $+27$ cal/deg for cysteine, $+54$ cal/deg for glutathione, and $+68$ cal/deg for thioglycolate.

We must now inquire into the effect of pH on mercaptide formation and particularly consider the reactions with the SH group and the ionized S^- group. Taking the two following equilibria:

$$R—Hg^+ + R'—S^- \leftrightarrows R—Hg—S—R' \qquad K_S = \frac{(R—Hg^+)\,(R'—S^-)}{(R—Hg—S—R')}$$

$$R—Hg^+ + R'—SH \leftrightarrows R—Hg—S—R' + H^+ \qquad K_{SH} = \frac{(R—Hg^+)\,(R'—SH)}{(R—Hg—S—R')(H^+)}$$

and the ionization of SH — i.e., $K_a = (H^+)\,(R'—S^-)/(R'—SH)$ — it is easy to show that:

$$pK_S = pK_{SH} + pK_a \tag{7-2}$$

The pK_a for SH groups varies from 7 to 10, and in Volume I a mean value of 8.7 was assumed for protein SH groups. In any event, pK_S and pK_{SH} will differ quite markedly. This is, of course, essentially a competition between H^+ ions and the mercurial for the S^- group. At physiological pH, SH will predominate over S^-, and the apparent pK for mercaptide dissociation will be smaller than those given in Tables 7-6 and 7-7.

Just as H^+ competes with the mercurial for the S^- groups, so various complexing ligands may compete with the S^- group for the mercurial. Despite the fact that the pK's for thiols are much greater than for most other ligands, a very significant effect on the equilibrium may be exerted. Let us write for the usual reaction of mercaptide formation in physiological media:

$$R—Hg—X + R'—SH \leftrightarrows R—Hg—S—R' + X^- + H^+$$

where X represents some ligand such as Cl^- or OH^-. The equilibrium is given by:

$$K = \frac{(R—Hg—X)\,(R'—SH)}{(R—Hg—S—R')\,(X^-)\,(H^+)}$$

If we designate the equilibrium with X by K_x=(R—Hg^+)(X^-)/(R—Hg—X), this taken in conjunction with the expressions for K_S, K_a, and K leads to:

$$pK = pK_S - pK_a - pK_x \tag{7-3}$$

Thus in any experimental situation the observable pK will be less than the true pK_S for the reaction of R—Hg^+ and S^- by the sum of pK_a and pK_x, each of these expressing the competitions involved. Thus in the work of Hughes (1957) on the reaction of human seralbumin with MM, a pK of 4.6 was found; if pK_a = 8.7 and pK_x = 8.7 (Table 7-5), one may calculate pK_S to be 22.0, X being I^- in this case.

The rates of mercaptide formation increase with pH as would be expected if the reactive form of the thiol is the ionized R′—S^-. It is not so easy to decide on the reactive forms of the mercurial. It seems unlikely that the Hg^{++} or R—Hg^+ ions are the only reactive species because of their extremely low concentrations in most physiological media, and it is possible that the S^- group makes a sideways attack on the Hg atom utilizing a pair of the six *s* electron pairs to displace the X^- ligand.

It is generally considered that mercurials do not react with disulfide (S—S) bonds, and there is sufficient evidence that this is true for many proteins and enzymes at physiological conditions. However, Cunningham *et al.* (1957) have shown that *p*-MB catalyzes the splitting of S—S bonds in cystine, insulin, and ribonuclease at pH 7 if incubation is carried out at 80°. At this temperature, this may well be a matter of equilibria between S—S and SH groups, i.e., between native and denatured forms of the proteins, with *p*-MB shifting the equilibria by reacting with the SH groups. It is quite possible that in certain enzymes the S—S groups exist in a state where reaction with mercurials is significant, and such a reaction should not be completely ignored.

Certain metabolically important cofactors, such as coenzyme A and lipoate, are thiols, and it is of some interest to inquire into whether the mercurials react readily with them. Surprisingly little quantitative work has been done and most of the evidence is indirect. For example, Galston *et al.* (1955) found that *p*-MB increases the yield of peroxidase, catalase, and tyrosinase in plant breis when added to the preparation medium; since coenzyme A inactivates these enzymes, it was assumed that *p*-MB protects the enzymes by forming a mercaptide with the coenzyme A. In coenzyme A-deficient rats, the toxicity of mercurials is increased, and the mercurials inhibit the coenzyme A-dependent acetylation of sulfanilamide (Leuschner *et al.*, 1957). Mersalyl and $HgCl_2$ reduce the coenzyme A level of yeast 25% at 22.5 mM and 0.3 mM, respectively (Estler *et al.*, 1960). Sanner and Pihl (1962) followed the reaction between *p*-MB and coenzyme A by changes in the absorption at 255 mμ and showed that the thiol could be titrated by the mercurial. Turning to DL-α-lipoate, one finds that its

administration prevents mercurial poisoning in mice, and that it reduces the inhibition of pyruvate oxidase by $HgCl_2$ at high concentrations (Grunert, 1960; Grunert and Rohdenburg, 1960). It is interesting that with lower concentrations of $HgCl_2$ (1.3 m*M*), lipoate increases the inhibition of pyruvate oxidation in intact cells of *Streptococcus faecalis*, the mercaptide possibly entering the cells more readily than the complexes of Hg^{++}. It would thus appear that mercurials react with coenzyme A and lipoate, but how rapidly and how tightly are not known.

Finally, we consider the problem of the splitting of thioesters by the mercurials. Sachs (1921) showed that acetylthioethyl esters are rapidly split by $HgCl_2$ to acetate and mercurothioethanol, and in general all acyl mercaptans seemed to behave in this way. Thus Lynen *et al.* (1951) in their early studies on active acetate and coenzyme A investigated acetyl-CoA and found it to be split by approximately 100 m*M* Hg acetate, a result of questionable significance in physiological work. Stern (1956) studied acetoacetyl-CoA, a thioester of importance in lipid metabolism and possessing a strong absorption maximum around 303 mμ, and found that $HgCl_2$ at concentrations higher than 0.001 m*M* produces a rapid decrease in this absorption, 0.1–0.2 m*M* completely abolishing it, this corresponding to about a 1 : 1 molar ratio between Hg and thioester. The following reaction sequence was suggested:

$$Hg^{++} + AcAc{-}S{-}CoA^{-} \rightarrow Hg{-}AcAc{-}S{-}CoA^{+}$$
$$Hg{-}AcAc{-}S{-}CoA^{+} + H_2O \rightarrow Hg^{++} + AcAc^{-} + HS{-}CoA$$
$$Hg^{++} + HS{-}CoA \rightarrow Hg{-}S{-}CoA^{+} + H^{+}$$

The initial reaction is apparently with the enolate group of the acetoacetyl radical. Stern believes that this reaction, occurring at such low concentrations of Hg^{++} and so rapidly, may well be of great importance in the effects of the mercurials on metabolism. However, Gibson *et al.* (1958) reported that *p*-MB does not react with or split succinyl-CoA at a significant rate, and Sanner and Pihl (1962) found a variety of thioesters to be resistant to *p*-MB (e.g., acetyl-CoA, succinyl-CoA, and benzoyl-CoA). From this limited work one might conclude that $HgCl_2$ can split some thioesters but that *p*-MB cannot. But Vagelos and Earl (1959) found that, in contrast to most thioesters, malonyl semialdehyde pantetheine reacts readily with *p*-MB, and proposed that β-carbonyl thioesters may be susceptible. The possible role of these reactions in metabolic inhibitions is at present unknown.

REACTIONS WITH PROTEINS

It was believed in years past that mercury, in common with other heavy metals, is adsorbed onto proteins, denaturing and precipitating them, but recent work has shown that under appropriate conditions stoichiometric

combinations of mercury with proteins occur, and that denaturation and precipitation are by no means a general phenomenon. These definite complexes in most cases are formed through the SH groups of the proteins, and methods whereby these groups may be titrated quantitatively with the mercurials have been devised. Some may feel that a discussion of the complexes of mercury with the proteins is out of place in a book on metabolic inhibition, but actually much can be learned from the thorough and illuminating investigations on mercaptalbumin, hemoglobin, and other proteins reported in the past few years. One must also realize that in any system containing nonenzymic proteins, particularly cellular preparations, reaction of mercury with these proteins not only may have definite effects on the enzyme inhibition, but may be responsible, at least in part, for metabolic or functional changes.*

Protein Groups Reacting with Mercurials

The mercurials react rapidly with certain free and exposed protein SH groups, more slowly with others, and not at all with some which are presumably buried within the protein structure or otherwise sterically unavailable (page 643). Many SH groups react only after denaturation of the protein, a process which apparently exposes them for attack by the mercurials. Since mercurials often initiate denaturation, they may produce a progressive unloosening of the protein structure and themselves make available SH groups originally unreactive, the process continuing until it is irreversible, the reformation of the normal configuration not being possible when the mercurial is removed. Much work with proteins and enzymes, to be discussed later, provides evidence that SH groups are the primary site of mercurial binding, and often the only site under certain conditions. The problem to be considered here is whether protein groups other than SH can under any circumstances contribute to the binding.

Examination of the constants for the complexing of mercurials with SH groups and with groups normally present in proteins leads to the immediate

* There is some inherent and unavoidable difficulty and ambiguity in the terminology of the mercurials. If one uses the inorganic $HgCl_2$, how should the inhibitor be designated — as $HgCl_2$, Hg, Hg^{++}, Hg^{II}, or otherwise? We have seen that in most, if not all, media the mercuric ion will exist in a variety of complexes, probably of different reactivities, so that it is impossible to designate the situation accurately. Similar problems arise with other heavy metal ions, e.g., copper and zinc. We shall, therefore, arbitrarily designate inorganic divalent mercury as Hg^{++}, without implying that this is either a predominant form or an active form. It is fundamentally and finally the Hg^{++} ion which complexes and reacts with the various substances present, so that when this is so written it must be understood that all of the complexes are implied. The designation of the organic mercurials as *p*-MB, PM, etc., similarly is noncommittal with respect to the forms present or active.

conclusion that no single group can compete very effectively with the SH groups for the mercurials. Indeed, these other groups probably have their orbitals occupied by competing with the various ligands present in the media. However, there are at least three factors which must be taken into consideration. (1) Certain fortuitous arrangements of two or more non-SH groups, perhaps allowing chelation of the mercurial, can increase the affinity markedly, as seen in Table 7-4. It is quite possible that occasionally such situations occur on protein surfaces, although generally the opportunities for successful chelation must be rare; e.g., the binding to glycylglycine is less than to glycine, and increase in the length of the polypeptide chain will probably reduce affinity except in very special cases. (2) The most reactive SH group or groups on a particular protein may not happen to have a strong affinity for a mercurial, due to steric factors or an unfavorable electric field, so that non-SH groups can compete more effectively. Although there is little quantitative evidence, one gets the impression that usually the SH groups of proteins do not bind most mercurials as tightly as do the SH groups of simple thiols, such as cysteine or glutathione, especially since one can often remove a mercurial from a protein quite readily by adding one of these thiols. (3) As pointed out by W. L. Hughes (1950), mercurials will complex with non-SH groups when the SH groups become saturated, or actually before in many cases. Since excess mercurial is often present, especially in enzyme studies, such secondary complexes must be considered, even though the SH groups are reacted first.

On the experimental side, it has been observed that some proteins bind more Hg^{++} than corresponds to the SH content and that some of this is relatively weakly bound. Haarmann (1943 a) found that with increase in the pH progressively more Hg^{++} is bound to various proteins, although only a fraction is really tightly attached to the protein, and postulated that CONH groups might bind Hg^{++}. More recently, Perkins (1958, 1961) reported the binding of 104–130 g-atoms of Hg/10^5 g seralbumin and, following treatment with bromoacetate (blocking SH and amino groups), the binding increased to 190 g-atoms/10^5 g protein at pH 5.5. The SH groups could have accounted for only 1 g-atom/10^5 g protein and Perkins felt that numerically the only possible binding sites are the COO^- groups. This is a situation in which there is excess Hg^{++} present for reaction with non-SH groups and, inasmuch as the dissociation constants are not known, it is impossible to compare the affinities for the various groups. Nevertheless, these results conclusively establish the non-SH binding of mercurials under certain conditions, and it would be well to bear this in mind in enzyme inhibition work.

Examples of Reactions with Specific Proteins

(A) *Ovalbumin.* Although not much work has been done with this protein, the results illustrate some of the problems one encounters. Anson

(1941) found it difficult to determine if *p*-MB reacts with ovalbumin or not, since ferricyanide and nitroprusside cannot be used to detect these SH groups (they do not react with ovalbumin) and iodine oxidizes the group whether free or combined with *p*-MB. Using an indirect method involving the determination of the binding of *p*-MB to cysteine in the presence of ovalbumin, Anson claimed that either *p*-MB does not react at all with the ovalbumin SH groups, or, if so, the binding is much less strong than with cysteine. If the ovalbumin is denatured, demonstrable binding of the mercurial occurs. W. L. Hughes (1950) reported that MM reacts with ovalbumin very slowly, the reaction requiring a day to come to equilibrium. However, MacDonnell *et al.* (1951) obtained a crystalline derivative of ovalbumin treated with *p*-MB by adjusting the pH to 4.7 and adding ammonium sulfate to opalescence. Three of the four SH groups of ovalbumin react and the fourth reacts only after denaturation. The complex is very stable and is not dissociated by cysteine or prolonged dialysis, although 6-day dialysis against cysteine at pH 7.9 dissociates about 75% of

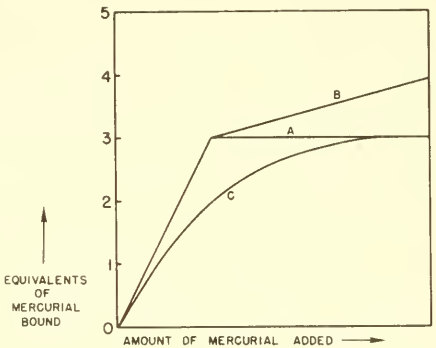


FIG. 7-3. Theoretical curves for the reaction of a mercurial with a protein, such as ovalbumin with three reactive SH groups. See text for explanation.

the mercurial. In such cases as this, if one plots the amount of mercurial combined, or the disappearance of SH groups from the protein, against the amount of mercurial added, one may obtain different curves (Fig. 7-3). If the reactions with the free SH groups are equivalent, a linear relationship to saturation will be obtained (curves A and B), but if interaction between the SH groups occurs (i.e., if the binding of a mercurial reduces the binding of the next) or the SH groups combine with the mercurial with different affinities, a curve concave downward will be obtained (curve C). If no further reaction with protein groups occurs after saturation of the SH groups, the curve will be horizontal (curve A), but if other groups with

less affinity for the mercurial continue to react, a sloping or curved line will be obtained (curve B). It is important to construct such curves whenever possible in order to understand the binding characteristics.

(B) *Hemoglobin.* Crystalline human oxyhemoglobin reacts with 2 moles of MM per mole of protein but the rate is rather slow at pH 7.5 (W. L. Hughes, 1950). The hemoglobins of other species may contain either two or four SH groups that react readily. Green *et al.* (1954) crystallized a derivative of horse hemoglobin in which two SH groups had been reacted with *p*-MB and showed that, although the crystals are isomorphous with normal hemoglobin, the X-ray diffraction pattern is somewhat different. Ingram (1955) established that Hg^{++}, MM, and *p*-MB all combine readily with four SH groups of horse hemoglobin in the native state, and with six in the denatured form; ox and human hemoglobins are similar but the latter presents eight SH groups when denatured. In native hemoglobin, Hg^{++} probably reacts with two SH groups simultaneously; at least 2 equivalents of Hg^{++} reduce the free SH groups to zero. However, *p*-MB likewise blocks two SH groups and since reaction of a molecule of *p*-MB with two SH groups is impossible, it is likely that a pair of SH groups is so close

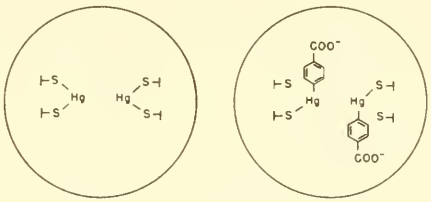


FIG. 7-4. The reactions of the SH group pairs on hemoglobin with Hg^{++} and *p*-MB, according to the concept of Ingram (1955).

that each group cannot react with the large mercurial. It is difficult to say in the case of Hg^{++} if the effect is steric or due to the formation of a bridge between the two SH groups in a pair, but the latter mechanism is favored. The situation as represented by Ingram is shown in Fig. 7-4. It is interesting that Hg^{++} and *p*-MB compete for the SH groups, and that the former is bound more tightly, probably due to the reaction with two of the SH groups.

Since the earliest studies of hemoglobin SH groups, there has been difficulty in establishing the exact number of reactive and unreactive groups, due to the fact that the nature of the reactions and the stoichiometry were uncertain. The details are not pertinent to our purpose and have been well reviewed by Huisman (1959). Further complications have arisen in the recognition that different types of hemoglobin react differently with the mercurials and that the pH is an important factor. Murayama (1958) claimed

that adult hemoglobin contains two SH pairs, sickle cell hemoglobin three SH pairs, and hemoglobin C no pairs, while Huisman obtained quite different results indicating more reactive SH groups than were found by most previous workers. At pH 7, adult hemoglobin reacts with 3 p-MB molecules and fetal hemoglobin with 2, whereas at pH 4.6 the values are 6 and 4, respectively, the lowering of the pH presumably altering the configuration of the protein. Studies of mercaptalbumin show that Hg^{++} can induce the formation of dimers of the protein, but Allison and Cecil (1958) believe that only monomers occur in the case of hemoglobin, and found that Hg^{++} and PM give the same titer, while the results of Cecil and Snow (1962) are more in accord with those of Ingram, 2.2 reactive SH groups of a total of 6 in adult hemoglobin being detected, the 3.8 sluggish SH groups reacting differently with Hg^{++}, PM, and *p*-MPS. These studies of hemoglobin not only show the influence of many factors on the interaction of proteins with mercurials, but point out the difficulties of SH titration of even relatively simple proteins.

Reaction of hemoglobin with mercurials brings about striking changes in the characteristics of oxygenation: The affinity of hemoglobin for O_2 may be unaffected or increased, but the interactions between the heme groups are reduced or abolished by all the mercurials (A. F. Riggs, 1952; Wolbach and Riggs, 1955; Riggs and Wolbach, 1956; A. Riggs, 1959). Mersalyl and Hg^{++} at pH 6.8 increase the affinity for O_2 quite markedly, P_{50} decreasing to about one third of normal, but *p*-MB and MM do not alter the affinity. All these mercurials lower the interaction constant n from around 2.9 to 1 (or near 1), the latter corresponding to complete loss of heme-heme interaction. Glutathione completely reverses these effects. It is very interesting that the maximal effects are produced at ratios close to 2 moles of mercurial to 1 mole of hemoglobin; however, as the amount of mersalyl is increased, the changes in heme interaction and O_2 affinity progressively disappear, so that at a ratio of 15–16 moles of mercurial per mole of hemoglobin there is no longer an effect. This curious reversal is unexplained. The observation may have some bearing on the use of mercurials for enzyme inhibition, and Riggs and Wolbach (1956) state, "Our observations suggest that the attempt to inhibit an enzyme with only a single high concentration of mercurial may lead to spurious conclusions." I know of no example in which enzyme inhibition is lost at higher mercurial concentrations, but in any case it would presumably be a rare phenomenon. Occasionally one finds stimulation of enzyme activity at low mercurial concentrations and this reverses to inhibition as the concentration is increased, but it is not known if this has any relation to the above reversal. Mercuration of oxyhemoglobin increases the rate constants for the dissociation of O_2 from three of the hemes, but decreases the rate constant for the dissociation of the last O_2, this being in fair accord with the effects on the O_2 dissociation curve ob-

served by Riggs (Gibson and Roughton, 1955). Oxygenation of hemoglobin facilitates reaction of the SH groups with *N*-ethylmaleimide and iodoacetamide but not with *p*-MB (Benesch and Benesch, 1962). The mechanisms by which these effects are produced are not clear, but hypotheses have been offered based on the spatial arrangement of the SH groups and the hemes. Riggs (1959) considers the hemoglobin molecule to consist of two halves, each with a pair of reactive SH groups and a pair of hemes, the SH groups perhaps lying between the hemes. Any mercurial which can form a bridge between the 2 SH groups of a pair — such as Hg^{++}, or mersalyl if the C—Hg bond is ruptured and inorganic Hg^{++} is released — increases the affinity of the hemes for O_2, whereas mercurials reacting only with a single SH group — such as *p*-MB and MM — do not have this effect. Any interaction with the SH groups reduces the interaction between the hemes, probably by bringing about reversible structural changes in the protein configuration. On the other hand, Klotz and Klotz (1959) favor a mechanism involving disturbances in the water structure around and between the hemes. Whatever the explanation, the bearing on the effects of mercurials on enzyme active centers by reaction with adjacent SH groups is obvious.

(C) *Mercaptalbumin.* Mercaptalbumin is one fraction of the serum albumins containing a single reactive SH group whereas the other albumins contain none. It was isolated as the crystalline mercury salt by Hughes (1947) and its reactions have been studied in detail, so that it has become the classic example of protein-mercurial interaction (W. L. Hughes, 1950). The three major reactions may be represented as follows:

Reaction 1: $\text{alb—SH} + HgX_2 \leftrightharpoons \text{alb—S—HgX} + H^+ + X^-$

Reaction 2: $\text{alb—S—HgX} + \text{alb—SH} \leftrightharpoons \text{alb—S—Hg—S—alb} + H^+ + X^-$

Reaction 3: $\text{alb—S—Hg—S—alb} + HgX_2 \leftrightharpoons 2\ \text{alb—S—HgX}$

where alb indicates mercaptalbumin and X some ligand (e.g. Cl^-). The first reaction is mercaptide formation, the second dimerization, and the third dissociation of the dimer by excess HgX_2. Formation of the dimer increases the turbidity and, if some ethanol is added, crystals form. These crystals are colorless diamond-shaped orthorhombic plates, containing channels or enclosures of fair size, with liquid within them, and permeable to various salts, sugars, and dyes (Low and Weichel, 1951). Hughes and Dintzis (1964) have described procedures for crystallizing the dimers from ethanol-water mixtures at low temperatures. Viscosity and sedimentation studies (Low 1952) led to the representation of the dimer as in A of Fig. 7-5, while the results of X-ray diffraction study are compatible also with structure B. The lengths of the dimer would be around 140–150 Å, monomer mercaptalbumin being of molecular weight 66,000. The structure is independent of the smaller ions making up the crystal; e.g., the dimer will

form crystals with HgI^{-}_{3}, the interactions being purely electrostatic and not involving SH groups (Lewin, 1951).

Reaction 1 is quite rapid, but reaction 2 is slow because it involves two large molecules of similar charge. The dimerization requires about 25 min for half-reaction and 2 hr for equilibrium when Hg^{++} is mixed with mer-

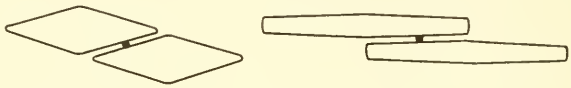


Fig. 7-5. Possible forms of the Hg-mercaptalbumin dimer. The small solid circle represents Hg^{++}. (From Low, 1952.)

captalbumin in a 0.5 : 1 ratio (Edelhoch *et al.*, 1953). Dimerization is an endothermic reaction, rise in the temperature favoring formation of the dimer, ΔH° being about 7 kcal/mole. The temperature effect indicates an activation energy for dimerization of 17–21 kcal/mole. The constant for the equilibrium (alb—S—HgCl) (alb—SH)/(alb—S—Hg—S—alb) was found to be 3.2×10^{-14} at pH 4.5 and 25°. Reaction 1 is reversible by substances forming stable compounds with Hg^{++}, and reaction 2 is reversible by ligands forming HgX_n complexes. Dimerization is also reversed by reac-

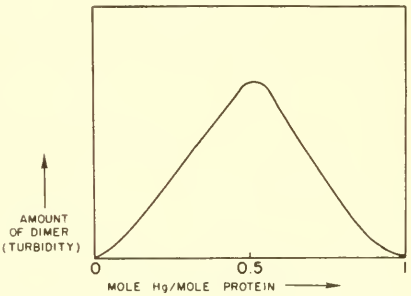


Fig. 7-6. The formation and dissociation of the Hg-mercaptalbumin dimer as the molar ratio of Hg^{++} to protein is increased. (From Edelhoch *et al.*, 1953.)

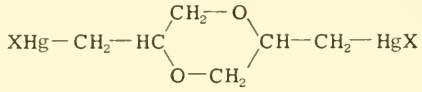
tion 3 (Fig. 7-6), Hg^{++} competing with mercaptalbumin for the alb—S—Hg monomer. The dissociation of the dimer by cysteine is very rapid (apparently within a few seconds) and yet the dimer is not split nor the Hg^{++} dissociated readily by dialysis, so that this would seem to be one of those interesting situations in which a complexer appears to take the metal from the protein rather than merely combining with free metal ions (Straessle,

1954). The disulfide dimer, alb—S—S—alb, however, is dissociated by cysteine very slowly. The dimerization is accompanied by an increase in levorotation, and this implies that the mercaptalbumin molecule undergoes some unfolding in the region of the reactive SH groups as a necessary prelude to dimerization; this may be thought of as a partial denaturation, adding one more item of evidence for configurational changes induced by mercurials (Kay and Marsh, 1959).

Organic mercurials such as *p*-MB, PM, and MM react in a 1 : 1 ratio with mercaptalbumin and, of course, no dimer is formed. The equilibrium constant for the reaction:

$$\text{alb—SH} + \text{CH}_3\text{—HgI} \leftrightharpoons \text{alb—S—Hg—CH}_3 + \text{H}^+ + \text{Cl}^-$$

has been found by W. L. Hughes (1950) to be 3.5×10^{-5} ($pK = 4.46$), from which the value for the dissociation constant of the mercaptide in Table 7-6 was calculated. On the other hand, bifunctional organic mercurials, such as:



can link two mercaptalbumin molecules together (Straessle, 1951; Edsall *et al.*, 1954). The pK for the equilibrium (dimer)/(monomer$^+$) (alb$^-$) is 18.2 at pH 4.75 and 25°, the corresponding pK for the Hg^{++} dimer equilibrium being 13.5.* This difference of some 4.7 pK units between the two dimers is undoubtedly due to the fact that the mercaptalbumin molecules must approach about 10 Å closer in the Hg^{++} dimer than in the bifunctional mercurial dimer, and the steric and electrostatic factors could easily account for the some 6.7 kcal/mole difference.

The importance of these results with mercaptalbumin for inhibition studies with the mercurials is clear. First, the possibility of the dimerization of certain enzymes by Hg^{++} leads to the concept that inhibition may occasionally result through steric sequestration of the active sites and not necessarily through the reaction of SH groups at the active sites. It is also possible that occlusion of active sites could occur by linking a nonenzyme protein to the protein through a Hg bridge. It is known that Hg^{++} often produces an increase in turbidity of enzyme solutions and even precipitation, although this can be due to other factors as well. Second, this reasonably well understood and quantitatively investigated system provides a model on which the effects of ligands, pH, temperature, and other factors

* Gurd and Wilcox (1956) give values of 17.2 and 12.6 for the pK's, respectively, due to different assumptions regarding the ligand constants.

may be better appreciated. Third, it presents one clear instance in which mercurials react specifically with protein SH groups, since there is no evidence that other groups are even involved in the mercuration of mercaptalbumin. Last, it is significant in the use of mercurials in whole animals that the serum contains sufficient mercaptalbumin to bind most, if not all, the mercurial present, a factor that must be considered in the penetration, distribution, and actions of the mercurials in animals.

Effect of pH

The competition between H^+ and the mercurial for the S^- group and the effect this has on the over-all equilibrium have been discussed. On the basis of only the ionization of the SH group, one would predict that mercurials would react more rapidly and more completely at higher pH's (particularly above pK_a's of the SH groups). However, there are many other factors which may be important. Actually, it has generally been observed that the rate of reaction of *p*-MB with proteins is decreased with a rise in the pH. Both the extent and rate of reaction of *p*-MB with ovalbumin are affected by pH: At pH 4.6, 4 moles of *p*-MB react rapidly with 1 mole of protein, whereas at pH 7, only 3.2 moles of *p*-MB react in 24 hr (Boyer, 1954). Reduction of the rate with increasing pH has also been reported for β-lactoglobulin and 3-PGDH (Boyer and Segal, 1954), it being much faster at 4.6 than at 7. The work of Huisman (1959) with hemoglobin illustrates an important point; the rate and extent of reaction may be influenced differently by pH, inasmuch as the rate of mercaptide formation is faster between 7 and 11.2 than at 4.6, but more SH groups are reactive at 4.6. One also recalls that *p*-MB is dissociated more rapidly from ovalbumin at pH 7.9 than in the acid pH range (MacDonnell *et al.*, 1951). Another factor often overlooked is the effect of pH on the secondary denaturation of the protein following mercuration. The rate of thermal denaturation of seralbumin is increased 13.2-fold at pH 3.6, while at pH 7 there is no denaturation by Hg^{++} at 1.85 mM, while denaturation of β-lactoglobulin is increased 89-fold at pH 3.6 and not at all at pH 7, this indicating that an acid pH favors secondary configurational changes resulting from binding of the Hg^{++} (Stauff and Ühlein, 1958). The reaction of mercurials with non-SH groups is also pH-dependent, since the complexing of the azomercurial studied by Horowitz and Klotz (1956) with glycine is maximal between pH 6.5 and 9.5; at low pH's the $^+H_3N{-}CH_2{-}COO^-$ form of glycine dominates and is less reactive, while at high pH's there is competition by OH^-.

$$R{-}Hg{-}OOC{-}CH_3 + H_2N{-}CH_2{-}COO^- \leftrightharpoons R{-}Hg{-}OOC{-}CH_2{-}NH_2 + CH_3{-}COO^-$$

The following factors, in addition to the ionization of SH groups, must thus be considered. (1) The pH may vary the number of reactive SH groups

or their individual reactivity, by effects on the structure of the protein, or on the association of subunits. (2) The pH will determine the over-all charge on the protein; e.g., with increased pH the protein will become more negatively charged and possibly repel negatively charged mercurials, such as *p*-MB, *p*-PMS, or the higher Cl^- complexes with Hg^{++}. (3) Rise in pH will increase the OH^- concentration and this ion will compete with the SH groups for the mercurial. (4) The pH may determine the degree of hydrogen bonding of SH groups and thus their reactivity with mercurials. It is likely that the dependence on the pH will depend on the mercurial used, but insufficient data are available for comparisons.

A final effect of pH involves dimerization where it occurs. The rate and degree of dimerization in the presence of Hg^{++} will depend on the total protein charge, being maximal at the isoelectric point, all else being equal. Straessle (1951) reported that the dimerization of mercaptalbumin with a bifunctional mercurial is slower at pH 6 than at 4.75, and Edelhoch *et al.* (1953) found the rate of dimerization with Hg^{++} to be increased 60 times when the pH is decreased from 6 to 4.75, and doubled with further decrease to 4.25. It was calculated that a charge of 9 charge units would account for this, and titration data indicated a change of 10 units over this pH range, so the electrostatic mechanism seems to be correct.

Effects of Mercurials on Protein Structure and Properties

The importance of secondary changes in protein structure upon reaction with a mercurial cannot be overemphasized in studies of enzyme inhibition and its reversibility, but unfortunately little exact information is available. Configurational changes have been postulated to explain certain results, such as have already been mentioned in regard to mercaptalbumin (page 757) and hemoglobin (page 755), and additional examples will be presented in connection with enzyme inhibition, but in most instances the evidence is indirect and tenuous. Nevertheless most investigators agree, I believe, in accepting that such changes occur in certain cases; the problems are the nature of the changes and the mechanisms by which they are induced.

Higher concentrations of Hg^{++} and most organic mercurials decrease the solubility of proteins, and may precipitate or coagulate them. This gave rise to the early concept of the mercurials as denaturing agents. However, it would appear that the primary effect is seldom denaturation (in the sense of disruption of the polypeptide chain structure), and that the altered properties of the protein are more directly related to modification of side-chain groups and the introduction of new groups. Prolonged contact of proteins with mercurials occasionally leads to true denaturation as a secondary reaction, but complete reversibility can usually be achieved by removing the mercurial; this indicates that if structural changes occur they are probably localized, and that the normal configuration can be restored. Such direct

structural effects should be distinguished from preferential reaction of mercurials with denatured protein in cases in which native and denatured protein exist in equilibrium under nonphysiological conditions (e.g., at low pH's or high temperatures) (Habeeb, 1960). They should also be distinguished from changes brought about in proteins evident after precipitation. The thermal coagulation of serum proteins is enhanced by *p*-MB and the coagula are firmer, more elastic, and more transparent, the water bound to the clot being around 4 times greater (Jensen, *et al.*, 1950), but this does not provide evidence that protein configuration before coagulation is altered by the mercurial. High Hg^{++} concentrations weaken keratin fibers so that they break under less tension (Hoare and Speakman, 1963). This would be expected if interchain disulfide bonds are disrupted. Changes in gross protein properties seldom provide information on the more important and subtler localized modifications which are believed to occur.

In view of the significance of configurational changes in enzyme inhibition, and in the belief that more examples will be postulated and established in the coming years, we may summarize some of the possible mechanisms by which such effects can be brought about. (1) In those cases in which there are equilibria between SH and S—S groups, or where there is a cyclic oxidation and reduction, and in which the S—S bonds contribute to the stability of a local configuration, mercaptide formation may loosen the structure. (2) The SH groups themselves may contribute to the stability, perhaps by hydrogen bonding or the binding of cofactors, so that mercuration may again enhance dissolution of the native structure. (3) The introduction of a charged group, such as occurs with *p*-MB or *p*-MPS, will alter the local electric field, and this may favor instability. (4) Hg^{++} and organic mercurials which dissociate to form Hg^{++} can bind to two groups simultaneously and thereby distort protein configuration. (5) Reaction of mercurials with non-SH groups, especially N- and O-containing groups, may reduce hydrogen bonding between polypeptide chains. It is not necessary that the affinity for these groups be especially high, and there is some indirect evidence that it is often the excess mercurial, above that required to saturate the SH groups, which is responsible for denaturation. There is increasing reason for believing that proteins are not rigid structures but often exhibit a fair degree of flexibility (page I-199), so that it is reasonable that reversible modifications of the structure may be fairly easily induced.

Estimation and Titration of Protein SH Groups with Mercurials

The older methods for the determination of SH groups, using nitroprusside, ferricyanide, iodine, or other reagents, are now considered to be generally unreliable when applied to proteins, due mainly to lack of specificity, and, in addition, these methods are often rather laborious. Ampero-

metric titration with Ag^+ is still commonly used and is often useful when combined with mercurial titration; one should consult Leach (1960) for a discussion of some of the difficulties of this method. Amperometric titrations at a rotating platinum electrode using Hg^{++} or MM have been shown to be accurate and reliable in some cases (Saroff and Mark, 1953; Kolthoff *et al.*, 1954; Leach, 1960), but these technique have not yet been extensively applied to enzymes. Leach has listed the requirements for an ideal SH reagent for titrations: It should (1) be specific for SH groups, (2) be highly reactive, (3) have a small molecular size, (4) be preferably monofunctional, (5) be devoid of charge or other reactive groups so that all protein SH groups are equally favored despite their different environments, (6) be soluble, stable, and reactive over a range of pH, and (7) show well-defined reduction steps for amperometric use. MM fulfills most of these criteria and perhaps it has been neglected in enzyme work. Past work on many proteins has indicated that it is often well to use more than one method in order to increase the reliability.

The most commonly used method at present is the spectrophotometric titration with *p*-MB developed by Boyer (1954), since it is convenient and appears to be generally accurate. Furthermore, the sensitivity is as high as with the amperometric methods, namely, around 0.01–0.1 m*M* SH. Reaction of *p*-MB with SH groups leads to an increase in the absorbancy at 250 mμ at pH 7 (Fig. 7-7); at pH 4.6 the maximal increment occurs at 255 mμ, but it is usually preferable to titrate enzymes at the more physiological pH of 7 to determine the number of reactive SH groups, unless the mercaptide formation occurs too slowly. The increase in absorbancy is a linear function of the SH groups reacted, for both simple thiols and proteins (Fig. 7-8), but the absorbancy change is somewhat different for different SH groups, a fact of no importance in titrations. The *p*-MB may be titrated with protein (as in Fig. 7-8), the end-point being the sharp break between the two linear segments, or the protein may be titrated with *p*-MB; in both cases one determines when all the reactive SH groups are transformed into mercaptides. Although the details of the method may be found in the original paper of Boyer (1954) and the excellent review of R. Benesch and R. E. Benesch (1962), it may be useful in interpreting such titrations applied to enzymes to note briefly certain precautions and difficulties.

(1) The only mercurial which can be used is *p*-MB, and since it is a rather large molecule with a negative charge, steric or electrostatic factors may reduce its reaction with certain SH groups. PM and *p*-MPS exhibit absorbancy shifts but at lower wavelengths where protein absorbs much more strongly than at 250–255 mμ.

(2) The addition of excess *p*-MB occasionally results in a further small increment in the absorbance, so that the flat portion of the curve may not be exactly horizontal, and this may indicate reaction with non-SH groups

or less readily reacting SH groups. Such behavior seems to be rare and does not seriously interfere with the determination of the end-point.

(3) The time relations must be considered. It is common practice to incubate the protein and *p*-MB for 10–15 min to allow reaction, but a decision as to this depends on one's definition of a reactive SH groups. In some proteins, additional SH groups are reacted when the incubation is prolonged; is such cases one is not certain if these groups were initially exposed, or if they arise during a progressive denaturation of the protein.

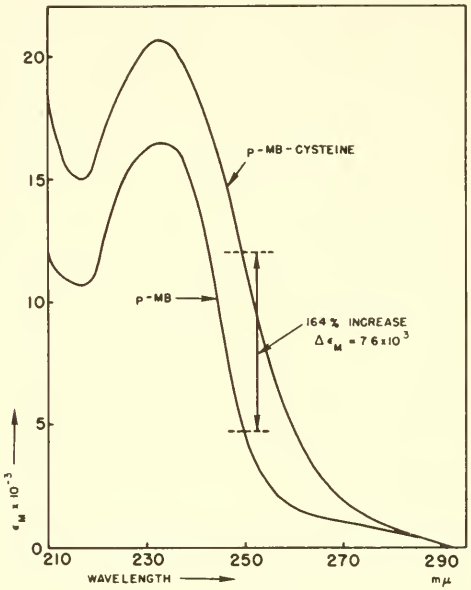


FIG. 7-7. The spectral absorption curves for *p*-MB and its mercaptide with cysteine, at pH 7 in 60 m*M* phosphate buffer. (From Boyer, 1954.)

(4) Both proteins and *p*-MB absorb significantly at 250–255 mμ and the appropriate controls must be run. For example, when protein is titrated with *p*-MB, equivalent increments of the mercurial are added to the blank cell.

(5) Protein or enzyme solutions should be as pure as possible, since even small amounts of certain impurities may cause large errors, and the solutions should be clear so that light scattering is reduced.

(6) Special consideration should be given to the pH since it has been shown that both the rate and extent of reaction are markedly affected, as in Boyer's experiments with ovalbumin. Although reaction may be more

rapid at pH 4.6 than at 7, it is preferable, as mentioned above, to use as physiological a pH as possible if the normal state of the protein or enzyme is to be established.

(7) Salt effects on the reaction of *p*-MB with proteins are also often of some magnitude, so that attention must be given to the ionic composition of the medium and the buffers used. It is preferable in most cases to use as low concentrations of salt and buffer as possible, if maximal reactivity of the SH groups is desired, but occasionally it is useful to add some salt, such as KCl, to reduce the *p*-MB reactivity in order to eliminate non-SH group effects.

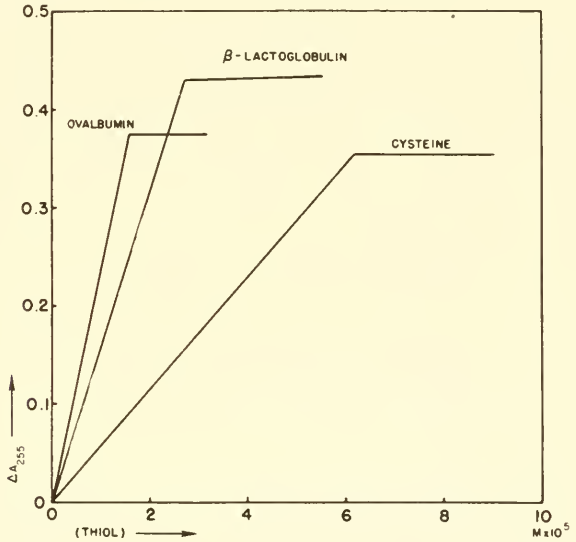


FIG. 7-8. Titration of cysteine and proteins with *p*-MB at pH 4.6 in 330 m*M* acetate medium. The reaction times are: cysteine and ovalbumin 15 min, and lactoglobulin 20 hr. (From Boyer, 1954.)

(8) It is worth noting that Boyer found EDTA to interfere, presumably due to a complex with *p*-MB, so it is advisable to omit this substance.

(9) Masking of the reactive SH groups, e.g. with alkylating agents, abolishes the absorbancy changes on adding *p*-MB, this providing evidence that it is indeed the SH groups which are responsible for the changes. Titration of proteins or enzymes treated with various agents can thus provide information on the disappearance of SH groups.

(10) The *p*-MB must be pure, should be analyzed iodometrically or spectrophotometrically, should be standardized against glutathione (details are

given by Benesch and Benesch), and should be used in freshly made solutions.

(11) The presence of two or more SH groups close together on the protein may prevent the reaction of each with *p*-MB, as is the case with hemoglobin. This will lead to low values for the total number of SH groups in proteins or enzymes.

A typical titration of an enzyme is shown in Fig. 7-9. The titration of 3-phosphoglyceraldehyde dehydrogenase at pH 4.6 presents a clear end-point indicating a rapid reaction of the SH groups. This yields 10.3 SH groups per molecule of enzyme (assumed molecular weight of 118,000).

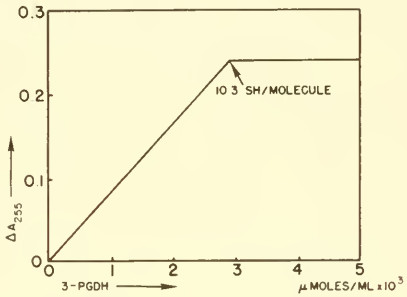


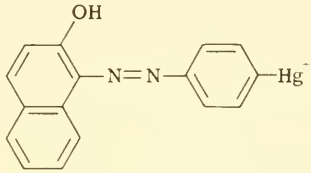
FIG. 7-9. Titration of 5 times recrystallized 3-phosphoglyceraldehyde dehydrogenase with *p*-MB at pH 4.6 and 0.03 μmole/ml. (From Boyer and Segal, 1954.)

The reaction of the SH groups occurs more slowly at pH 7 and a sharp end-point was not obtained by incubations up to 15 min; however, longer incubations would probably have given a sharp break in the curve. Here the end-point yields 8.3 SH groups per enzyme molecule, suggesting that 2 SH groups become much more reactive when the pH is lowered. A value of 10.7 half-cystines per molecule for this enzyme has been reported (Velick and Ronzoni, 1948), so it is evident that most of these SH groups are free and reactive.

Colored Mercurials and Histochemical Determination of Protein SH Groups

Various colored mercurials, usually azobenzene derivatives, have been known for many years but were not applied to biological material until Bennett (1948 a) studied the reaction of *p*-mercuriphenylazo-β-naphthol with tissue thiols. Direct visualization of thiol distribution in the tissues is possible, but the dye has a very low solubility in water and at the usual pH's so low a molecular extinction coefficient that its use is limited. How-

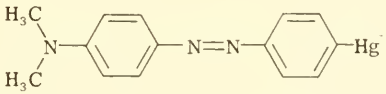
ever, Flesch and Kun (1950) found that the addition of strong acid intensifies the color markedly. It has been claimed that this mercurial is as specific as PM for SH groups, but one wonders if this complex molecule does not



p-Mercuriphenylazo-β-naphthol

through other groups occasionally react with various tissue components (β-naphthol derivatives being fairly potent enzyme inhibitors), although previous treatment of the tissue with Hg^{++} or iodoacetamide is said to prevent staining. Aqueous solutions of thiols, proteins, or tissue homogenates are shaken with an amyl acetate solution of the mercurial dye, and a red precipitate slowly forms in the aqueous phase as the reaction proceeds; the amount of precipitate is proportional to the number of SH groups and can be determined colorimetrically after centrifuging and redissolving in acid solution. Fragments of dehydrated tissues may also be placed in butanol or propanol solutions of the mercurial for several hours, and the staining demonstrated histologically (Bennett, 1951). Bennett ran controls with phenylazo-β-naphthol to determine if this portion of the molecule contributed to the binding, and generally found little or no staining. This mercurial has been used to investigate thiol distribution in muscle (Bennett, 1948 b), skin (Mescon and Flesch, 1952), and a variety of other tissues (Bennett, 1951).

Another colored mercurial, 4-mercuri-4′-dimethylaminoazobenzene, has been used by Horowitz and Klotz (1956) to determine protein SH groups. The solubility in water is so low that colorimetric determinations cannot



4-Mercuri-4′-dimethylaminoazobenzene

be made, but it dissoves sufficiently in 100 m*M* glycine (due to the formation of a glycinate complex) that reactions with SH groups in aqueous medium can be carried out. However, it is also possible to determine the amount of the mercurial removed from heptanol when shaken with an aqueous solution containing the protein, although equilibrium usually requires several hours. The specificity of reaction appears to be satisfactory,

since the amount bound to bovine seralbumin increases with the dye concentration until the molar ratio of dye to protein is 0.66, following which no more is bound although the dye concentration is increased 50-fold. This ratio corresponds quite closely to the known SH content of the protein. Iodination of the seralbumin prevents the reaction with the mercurial. Ovalbumin reacts readily with two of its SH groups, slowly with a third, and more slowly with the fourth, the dye perhaps differentiating the relative reactivities more closely than does *p*-MB. This method has a high sensitivity and can be used for very low concentrations of protein.

A more recently examined mercurial dye, 4-(*p*-mercuriphenylazo)-1-naphthylamine-7-sulfonate, must also be dissolved in glyine buffer (Nosoh, 1961). Absorption at 470 mμ is determined and the titration of glutathione and proteins appears to be quite satisfactory.

INHIBITION OF ENZYMES

The early concept of the mercurials as nonspecific denaturing and coagulating agents for enzymes has gradually been abandoned in favor of a picture in which definite and often isolatable mercurial complexes are formed under the proper experimental conditions. A selective reaction with SH groups on enzymes is now generally assumed and the mercurials are extensively used for the detection of these groups. The possibility of reaction with other than SH groups has been discussed (pages 737 and 753) and should never be ignored. We shall note instances in which a selective action on SH groups is well established, and a few examples of inhibition not involving SH group. We shall also see that mercurial inhibition does not necessarily imply an SH group within the active center or the participation of an SH group in the catalysis. In this connection, it is well to bear in mind the different groups which are introduced on the surfaces of enzymes when the different mercurials are used (Fig. 7-10), inasmuch as the steric and electrostatic effects of these side chains may be critical in producing inhibition.

Crystalline Mercuri-enzymes

The crystallization of the mercuric derivative of mercaptalbumin was not the first instance of such a procedure. Warburg and Christian (1941, 1942) introduced this technique for the isolation of fermentation enzymes and obtained the crystalline Hg-enolase complex from yeast, whereas the normally active Mg-enolase could not be crystallized. Kubowitz and Ott (1941) in Warburg's laboratory also crystallized the Hg^{++} complexes of lactate dehydrogenases from Jensen sarcomata and rat muscle. The Hg^{++} complexes in all cases are enzymically inactive, but dialysis against cyanide solution removes the Hg^{++} and restores the activity. There is no better evidence for the homogeneous, stoichiometric, and reversible Hg^{++} deriva-

tives of enzymes than such complexes, which is the reason they are discussed briefly at this point. Warburg and Christian suggested that the isolation of mercuri-enzymes might be generally useful, but this technique either was not used or was unsuccessful until Kimmel and Smith (1954) reported the crystallization of mercuri-papain. Krebs (1930) had shown that papain is very sensitive to Hg^{++}, 50% inhibition requiring only 0.005 mM, and this

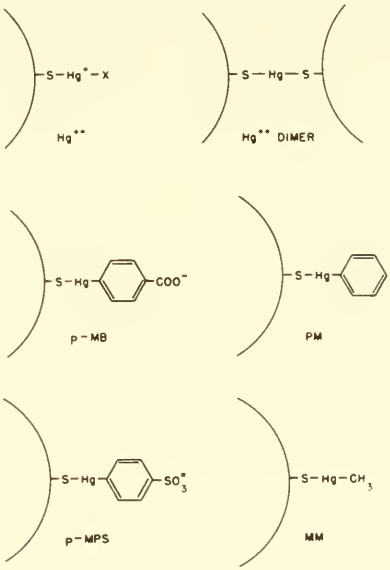
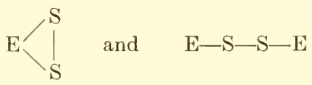


FIG. 7-10. The side chains introduced onto proteins by various mercurials. The S—Hg—R bonds are not actually linear but are shown in this way for convenience.

indicated that a tight complex is formed and might be susceptible to crystallization. Twice recrystallized papain (1.5–2%) was reacted with 1 mM Hg^{++} in 70% ethanol in the cold; within 24 hr a precipitate formed and in 3–4 days 90% of the activity was in crystalline form. These crystals are long rectangular plates, often large enough to be visible to the eye, and are soluble in water. The properties of mercuri-papain have been reviewed by Kimmel and Smith (1957) and we shall discuss only those aspects relevant to enzyme inhibition.

Mercuri-papain contains 0.49% Hg and has a minimal molecular weight of 41,400; this corresponds to 1 Hg atom per molecule of mercuri-papain. Since the molecular weight of reduced papain is around 20,500, mercuri-papain must be a 1 : 2 complex or dimer to be represented by E—S—Hg—

S—E. However, the situation is more complex, the pH being an important factor in determining the type of complex occurring, and it is likely that the crystalline mercuri-papain is the least soluble form of several possible derivatives (Smith *et al.*, 1954 b). Sedimentation studies at pH 4 indicate a monomer or 1 : 1 complex, probably to be designated by HS—E—S—Hg^+, while at pH 8 there is a heavy component corresponding to a hexamer, possibly cyclic with alternating —S—S— and —S—Hg—S— bonds. It is interesting that there are two electrophoretic peaks at pH 4, one of unit + charge greater than the other; since dissociation of the dimer must result in equal proportions of HS—E—SH and HS—E—S—Hg^+, this would tend to confirm the dimeric structure. Oxidized papain is a mixture of



and does not react with Hg^{++}; thus it is very important in studying the combining ratios to be certain that the papain is fully reduced. Mercuri-papain is actually purer than papain, as indicated by electrophoretic studies, has fewer N-terminal residues detected by the fluorodinitrobenzene technique (Thompson, 1954), and has some 10% greater activity following removal of the Hg^{++} with cysteine and EDTA, and it is also more stable. The proteolytic enzyme, pinguinain, also forms stable complexes with Hg^{++} which are stable for much longer times than the pure enzyme (Messing, 1961). Other enzymes to be crystallized as the mercury complexes are a lysozyme from papaya latex (Smith *et al.*, 1955) and 3-phosphoglyceraldehyde dehydrogenase from yeast (Velick, 1953), the latter after reaction with *p*-MB. There is some evidence that a mercuric dimer of ficin occurs (Liener, 1961) while carboxypeptidase forms very stable Hg^{++} complexes which still possess esteratic activity, although they no longer function as peptidases (Vallee *et al.*, 1961; Coleman and Vallee, 1961). There is thus sufficient evidence that many enzymes form well-characterized mercurial complexes and are quite stable in this state; we shall note other examples in the discussion of SH titrations of enzymes.

These complexes of enzymes with Hg^{++} offer strong support to the concept that completely selective reaction with SH groups can occur. However, if Hg^{++} is added in excess of that required for mercaptide formation, it is quite possible that other enzyme groups may be attacked. It is likely that other enzymes under the appropriate conditions can form dimers, or other polymers, with Hg^{++}, in which case the active centers may be made inaccessible even though the SH group is not within the confines of the center. The appearance of polymers will presumably depend strongly on the pH since, at pH's progressively removed from the isoelectric point, one might expect polymerization to be more and more reduced, due to the increasing charge on the enzymes.

Types of Inhibition Observed with the Mercurials

The concentration-inhibition curves for mercurials are generally sigmoid and rather steep, as would be expected of inhibitors combining tightly with enzyme groups. Indeed, when such curves are fairly flat, encompassing several pI units, one has the right to question if the inhibition is related to mercaptide formation, although it may well be. It should be emphasized that adequate kinetic studies can be made only in preparations of pure enzymes. The presence of impurities may distort the entire picture and the kinetics of inhibition.

One may classify the inhibitions classically into competitive, noncompetitive, uncompetitive, and mixed types, but the proper plotting procedures have seldom been used so that in the majority of cases we have little or no information. However, sufficient has been done to show that all these types of inhibition occur (Table 7-8). Competitive behavior has been observed in a surprisingly large number of instances. This is surprising at first if one assumes reaction with SH groups to be the primary mechanism of inhibition, because the tightness of the binding might be considered to prevent the exhibition of competition. Actually, most inhibitions by mercurials are probably competitive — either with substrate, coenzyme, or cofactor — in the fundamental sense of the word, but it is often difficult to demonstrate this by the usual analytical techniques which assume equilibrium conditions. It is easier to show that the presence of the substrate, coenzyme, or cofactor slows the development of the inhibition, although the equilibrium inhibition may not be detectably different (page 778). *Formally competitive behavior* might be expected to occur in the following circumstances. (1) The inhibitor acts by a non-SH reaction; the organic mercurials particularly possess groupings capable of interacting with active sites independently of the Hg atom, and such might be involved, for example, in the inhibition of D-amino acid oxidase by *p*-MB, the benzoate structure being of primary importance. (2) The binding of the mercurial to the SH groups may for some reason be weaker than usual and of a comparable magnitude to the affinity for the substrate. (3) The mercurial is bound much more tightly than the substrate but measurements are made before equilibrium is reached, as in the experiments showing protection of the enzyme by the substrate; when the inhibitions are determined soon after adding the mercurial in the presence of variable concentrations of the substrate, the data may provide formally competitive plots. One would expect this third explanation in certain examples given in Table 7-8, e.g., carbonic anhydrase, where $K_i/K_m = 3.87 \times 10^{-7}$ for *p*-MB (Chiba *et al.*, 1954 b). In the case of homogentisate oxidase, *p*-MB and MM inhibit competitively with respect to Fe^{++} but noncompetitively with respect to homogentisate, the mercurials being bound roughly 40–100 times as tightly as the Fe^{++} (Flamm and Crandall, 1963). Here, and in other instances where metal ion cofactors are involved, both cofactor and

TABLE 7-8

SOME EXAMPLES OF THE TYPES OF INHIBITION OBSERVED WITH THE MERCURIALS

Enzyme	Source	With respect to:	Reference
Noncompetitive			
Glutamate decarboxylase[a]	Mouse brain	L-Glutamate	Roberts and Simonsen (1963)
Lactate dehydrogenase	Human serum	Pyruvate	Hill (1956)
Malate dehydrogenase	Horse liver	Oxalacetate	Shull (1959)
Myokinase	Rabbit muscle	ADP	Callaghan and Weber (1959)
Uncompetitive			
Alkaline phosphatase[a]	Intestine	*p*-Nitrophenyl phosphate	Lazdunski and Ouellet (1962)
Competitive			
Acid phosphatase[a] (pH 5.2)	*Schistosoma mansoni*	*p*-Nitrophenyl phosphate	Nimmo-Smith and Standen (1963)
Aldehyde oxidase	Rabbit liver	N^1-Methylnicotinamide	Rajagopalan and Handler (1964)
Aldehyde oxidase	Pig liver	Acetaldehyde	Palmer (1962)
D-Amino acid oxidase	Pig kidney	Alanine	Frisell and Hellerman (1957)
		FAD	Yagi and Ozawa (1959)
β-Amylase	Wheat	Starch	Ghosh (1958)
Carbonic anhydrase	Spinach leaves	Bicarbonate	Chiba *et al.* (1954 b)
Cholinesterase	Horse serum	Acetylcholine	Robert *et al.* (1952)
α-Glucosidase	*Saccharomyces italicus*	*p*-Nitrophenyl-α-D-glucopyranoside	Halvorson and Ellias (1958)
β-Glucuronidase[b]	Rat preputial gland	Phenolphthalein-β-glucuronide	Fernley (1962)
Homogentisate oxidase	Calf liver	Fe^{++}	Flamm and Crandall (1963)
3-Hydroxyanthranilate oxidase	Beef liver	3-Hydroxyanthranilate	Mitchell *et al.* (1963)
Invertase	Yeast	Sucrose	Gemmill and Bowman (1950)

[a] These inhibitions are not of pure type but somewhat mixed; the dominant type is given.

[b] Inhibition by *p*-MB; results with Hg^{++} are more complex.

mercurial are bound to the same SH group. In most cases, increase in the substrate concentration does not reduce the inhibition once established, but Robert *et al.* (1952) claim that acetylcholine is able to displace Hg^{++} from horse serum cholinesterase, the affinity of the enzyme for the acetylcholine actually being greater than for the Hg^{++}; it is not known if mercaptide formation is involved. One suspects that the inhibition may sometimes appear to be competitive where actually the mercurial is reacting with the substrate, either exclusively or in addition to the enzyme (compare curves in Figs. I-5-1 and I-5-14), and such might be the case in the inhibition of β-amylase by *p*-MB and PM (Ghosh, 1958), although the extent of reaction of mercurials with starch is not known.

Noncompetitive inhibition may be observed when the mercurial reacts with groups, SH or other, adjacent to the active center, and thus suppresses the rate of breakdown of the ES complex, and when the affinity for the enzyme is not so high that mutual depletion kinetics hold. The interference with ES breakdown may be steric through the side chains introduced or secondarily by alteration of the protein structure. It must be remembered that mutual depletion systems usually indicate formally noncompetitive behavior if the common plotting procedures are used (compare Figs. I-5-3 and I-5-24), despite the fact that the inhibition may be fundamentally competitive. No pure instances of *uncompetitive or coupling inhibition* have been reported, but it is not unlikely that preferred reaction with the ES complex occurs. The inhibition of alkaline phosphatase is actually mixed (noncompetitive and uncompetitive), but *p*-MB reacts more readily with the ES complex ($K_i' = 0.163$ mM) than with E ($K_i = 2.5$ mM) (Lazdunski and Ouellet, 1962). There are some examples in which such reaction with the ES complex is possible, e.g., the inhibition of urease by Hg^{++} (Evert, 1952), of acid phosphatase by *p*-MB (Newmark and Wenger, 1960), and of succinate dehydrogenase by *p*-MB (Warringa and Giuditta, 1958). The inhibition of lactate dehydrogenase from *Propionibacterium pentosaceum* by *p*-MB is greater in the presence of lactate than when no substrate is present during incubation, and this was postulated to be due to the greater number of free SH groups, presumably arising through reduction by lactate (Molinari and Lara, 1960), and a similar situation may occur with glutathione reductase and NADPH, the presence of the reduced coenzyme increasing the inhibition markedly (Mapson and Isherwood, 1963).

Another approach to the classification of mercurial inhibitions, and perhaps the primary one, is the determination of the component — enzyme, substrate, coenzyme, or cofactor — with which the mercurial reacts. *Reaction with the apoenzyme* has generally been assumed above and with respect to the molecular mechanism might be divided into three types: (1) binding to an SH group at the active center, preventing complexing of the apoenzyme with any of the other components, (2) binding with an SH group

vicinal to the active center and interference with the catalysis sterically or electrostatically, and (3) secondary altering of the protein structure to disrupt the normal configuration of the active center. In the last case, which is probably fairly common (see page 787), the inhibition may be formally competitive (if the substrate stabilizes the enzyme structure), noncompetitive, or quite complex. *Reaction with the substrate* must often occur, especially when the substrate is protein, nucleic acid, nucleotide, or thiol, but in most cases this possibility seems to have been ignored. It is obvious for glutathione reductase and this complicates the analysis of the inhibition (Mapson and Isherwood, 1963), but it may also be an important mechanism when thioesters are involved, e.g., acetoacetyl-CoA in fatty acid synthesis (Stern, 1956) or malonyl semialdehyde pantetheine in propionate metabolism (Vagelos and Earl, 1959). The inhibition of NADPH: methemoglobin oxidoreductase by *p*-MB occurs when either the enzyme or the methemoglobin is incubated with the mercurial (Bide and Collier, 1964). Sometimes one finds indirect evidence for reaction with the substrate, as with 5′-adenylate deaminase (Lee, 1957). Here the inhibition by *p*-MPS is much greater when it is preincubated with adenylate and the reaction started by adding the enzyme than when preincubation is with the enzyme and reaction started by adding the substrate. *Reaction with coenzymes* is evident when lipoate or coenzyme A is involved, but may be more general than is usually supposed. A reaction of *p*-MB with NAD was detected spectrophotometrically by Palmer and Massey (1962) and this was considered to be significant in titrations of certain dehydrogenases. Hill (1956) had previously established a 1 : 1 complex of Hg^{++} with NADH, but had found no complex with *p*-MB. Onrust *et al.* (1954) considered the possibility that at least part of the inhibition of pyruvate oxidase by *p*-MB might be due to reaction with the sulfur of thiamine-diP, but excluded this when they found that thiamine-diP does not reverse the inhibition. However, Pershin and Shcherbakova (1958) observed that thiamine is able to reduce the bacteriostatic action of Hg^{++}, although this could be by a mechanism other than reaction of the thiamine with Hg^{++}. Kuratomi (1959), on the basis of preincubation experiments with components of the pyruvate oxidase system, postulated that *p*-MB can react with thiamine-diP. This problem remains to be settled and possibly is an important one. It would be interesting to know if mercurials can open the thiazole ring under physiological conditions (which is not likely) or react with the SH groups after ring opening, in which case the state of the thiamine-diP in the preparation would be important. Another possibility is that a complex is formed with groups other than the sulfur since opportunities for chelation exist.

It may be suggested that in all studies of enzyme inihibition, in which substrates or coenzymes capable of reacting with mercurials are involved, the appropriate preincubations with the inhibitor be carried out, as pre-

viously described (page I-569), since this technique will often provide information on complexes formed with components other than the apoenzyme.

Whatever the mechanism or formal type of inhibition by mercurials, it is certain that many systems must be represented by mutual depletion kinetics. This is clearly seen in many of the enzyme titrations (page 804), inhibition being produced by mercurials at roughly equimolar concentrations relative to the enzymes, but at this point the problem will be treated in a more general manner. Mutual depletion behavior implies that the inhibition will depend on the concentration of the enzyme. This is seen with yeast pyruvate decarboxylase in the work of Stoppani *et al.* (1953) (see accompanying tabulation), and even more markedly with pig heart suc-

Pyruvate decarboxylase (μg/ml)	% Inhibition by:	
	Hg^{++}	*p*-MB
7.8	85.0	95.0
15.7	—	75.0
30.5	43.0	33.0
61.0	15.9	7.0

cinate oxidase, which is inhibited 89% by 0.01 m*M* *p*-MB when the enzyme concentration is 0.15 mg/ml but only 59% by 0.76 m*M* *p*-MB when the enzyme concentration is 30 mg/ml (Stoppani and Brignone, 1957). Another example is muscle pyruvate oxidase (see accompanying tabulation) (Onrust

Enzyme extract (ml)	% Inhibition by *p*-MB 0.11 m*M*
0.4	82
0.8	56
1.2	34
1.5	33

et al., 1954). These few examples well illustrate the importance of this factor and very clearly demonstrate the quantitative meaninglessness of most reported inhibitions if the relative enzyme concentration is not known or stated. Impurities also may contribute to the depletion of the mercurial. The crude bacterial enzyme for converting histidinol to histidine is not inhibited by 0.02 m*M* *p*-MB, but the partially purified enzyme is inhibited 50% (Adams, 1954), and it is likely that the pure enzyme would be inhib-

ited even more strongly; such work points out the importance of enzyme purity for accurate studies of mercurial inhibition. The elevation of the pI_{50} from 0.0002 mM to 0.014 mM by serum for the inhibition of 3-phosphoglyceraldehyde dehydrogenase by *p*-MB is a further example (Weitzel and Schaeg, 1959).

When an enzyme is reported to be inhibited to a specified degree, say 50%, by a certain concentration of mercurial, exactly how is this to be interpreted? Is 50% of the enzyme combined with the mercurial in a completely inactive EI complex, or is all the enzyme combined with the mercurial and the EI complex possesses 50% of the original activity? If the ordinary equilibrium formulation is followed and it is assumed that the fractional activity of the EI complex is r, noncompetitive inhibition will be given by

$$i = \frac{(1 - r)\,(\mathrm{I})}{(\mathrm{I}) + K_i} \tag{7-4}$$

and

$$\frac{1}{i} = \frac{1}{(1 - r)} + \frac{K_i}{(1 - r)\,(\mathrm{I})} \tag{7-5}$$

so that a plot of $1/i$ against $1/(\mathrm{I})$ will give a straight line intersecting the $1/i$ axis at $1/(1 - r)$, or $1/i_{max}$. If mutual depletion occurs (zone C), a similar result is obtained, although the slope will be different. A simple plot of this type may help to decide between the two possibilities above. If the plot is not linear near the $1/i$ axis, one might suspect that another type of inhibition is occurring at higher inhibitor concentrations, or that secondary inactivation of the enzyme is a factor.

One example of the deviations from classic inhibition kinetics that may be seen with the mercurials is the inhibition of human plasma cholinesterase by Hg^{++} as analyzed by Goldstein and Doherty (1951). This slowly developing, pH- and temperature-dependent inhibition presents some interesting but often uninterpretable results. The $1/v$-$1/(\mathrm{S})$ plots exhibit two sorts of deviation (Fig. 7-11). The results from long incubation with low concentrations of Hg^{++} fall on reasonably straight lines (A and B), but the slopes are a good deal greater than expected for pure noncompetitive inhibition, as for mixed inhibition (Fig. I-5-6A) the interaction constant α being some finite value > 1. Of course, it may not actually be true mixed inhibition, the deviation being due to some other factor. The results from short incubations with high concentrations of Hg^{++} differ so much from any sort of classic behavior that it is impossible to interpret them (C and D). It was suggested that low and high concentrations of Hg^{++} inhibit by different mechanisms, possibly with different SH groups, the former with groups outside the active center causing secondary irreversible inactivation and the latter directly with groups in the active center. This would to some

extent explain why the inhibition is more competitive at high Hg^{++} and more noncompetitive at low Hg^{++}, but it does not explain the deviations discussed above. Curves C and D presumably do not represent equilibrium inhibitions and are more illustrative of protection of the enzyme by the substrate; it would seem that acetylcholine above 50 m*M* protects the enzyme almost completely against very short exposures to Hg^{++}, which is not too unreasonable considering the relatively high affinity of the enzyme for acetylcholine. Although the kinetics of protection and the application

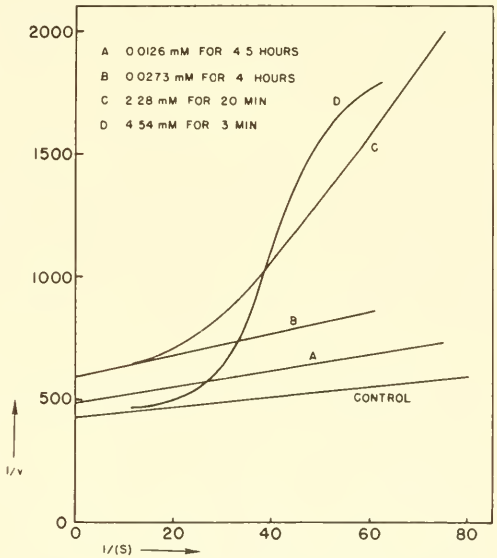


FIG. 7-11. Double reciprocal plots for the inhibition of human plasma cholinesterase by Hg^{++}, showing deviations from linearity at high Hg^{++} concentrations. (Modified from Goldstein and Doherty, 1951.)

to plotting procedures have never been worked out as far as I know — and it would be difficult to treat the phenomenon rigorously one might predict that curves with rather steep slopes in the $1/v$-1/(S) plot would be found, and that such curves would occasionally intersect the control curve to the right of the $1/v$ axis, i.e., the longer the incubation, the closer to equilibrium would the inhibition come, and the less competition or protection would be exerted by the substrate. One also wonders if the increased tilt of curves A and B might be due to the fact that these relatively high substrate concentrations protect the active center against structural changes brought about by reaction of the Hg^{++} at vicinal sites, since there are many examples

in which the substrate can slow down spontaneous or induced enzyme denaturation. However, neostigmine, which can protect cholinesterase against thermal denaturation, does not protect at all against Hg^{++}. There is actually some doubt as to whether the inhibition is related to SH groups, since *p*-MB and MM up to 1 m*M* do not inhibit even after 2 hr at 37°, or it might mean that the reacting SH groups are not at the active center and the inhibition by Hg^{++} is due to a dimerization or polymerization. If all enzymes subjected to mercurials were studied in as much detail as in this work, there would probably be many more interesting examples of deviations from classic theory; as long as one tests an enzyme under standard conditions with one concentration of a mercurial, as is done in most reports, interpretation presents no problems.

Protection of Enzymes against Mercurials

Enzymes may be protected against mercurials by (1) substrates, (2) coenzymes, (3) metal ion cofactors, (4) reversible inhibitors, and (5) thiols or other mercurial complexers. Various conclusions have been drawn from such experiments, mainly regarding the relation of SH groups to the binding of the protector, but there are many pitfalls; the discussion of protection with respect to iodoacetate (page 47 and Fig. 1-5 in Volume III) applies equally well to the mercurials. Protection may occur by two general mechanisms: reaction of the protector with the enzyme to block off the mercurial, or reaction of the protector with the mercurial. The latter mechanism applies to the thiols such as cysteine or glutathione, which have been widely used for this purpose, but, as has been pointed out several times, such protection does not provide much useful information, since in reality all one does is to reduce the effective mercurial concentration. It also applies to other complexers and perhaps is involved in the following: the protection of fumarate hydratase (Mello Ayres and Lara, 1962) and fumarase (Favelukes and Stoppani, 1958) by phosphate, of ascorbate oxidase by amino acids and RNA (Frieden and Maggiolo, 1957), of acid phosphatase by EDTA (Macdonald, 1961), of urease by ascorbate (Mapson, 1946), and of thyroxine delahogenase by FMN (Tata, 1960). However, in these cases it is often difficult to interpret the mechanism of the protection. We shall not be concerned with this type of protection, but only with those protectors presumably reacting with the enzyme.

Some examples of protection are summarized in Table 7-9 along with instances in which protection does not occur (or at least is not observed under the conditions used). The + sign does not indicate that complete protection can be achieved; indeed, in most cases only partial protection has been reported, and this is what we would expect. The degree of protection may depend on the concentrations of mercurial and protector; e.g., protection may be complete with low mercurial concentrations, whereas

the protector may be relatively ineffective against high concentrations, as in the effects of arginine on the inhibition of its oxidative decarboxylation (see accompanying tabulation) (Van Thoai and Olomucki, 1962). In most

p-MB	% Inhibition	
	p-MB alone	*p*-MB + arginine 10 m*M*
(m*M*)		
0.033	57	0
0.05	66	0
0.067	89	43
0.083	98	61

reports it is difficult to decide if the protection is simply due to a slowing of the rate of inhibition or to a true effect on the final equilibrium inhibition, since measurements are often made over arbitrary time intervals. It is evident that it is easier to slow down an inhibition than to modify its final level; enough substrate, coenzyme, or cofactor to saturate the enzyme substantially will quite markedly slow the reaction of the enzyme with the inhibitor, but the final inhibition need not be significantly changed, particularly with the mercurials which are usually bound tightly, if slowly. Most investigators have noted that although the protectors in Table 7-9 are effective when present during the development of the inhibition, they do not reverse the inhibition at all once it has reached a steady level, this apparently indicating that most of the protection results are fundamentally due to a slowing of the rate of inhibition.

Occasionally two components of the enzyme reaction, forming a ternary complex with the enzyme, protect more than each component alone. This is the situation with malate oxidative decarboxylase, the protections by malate and Mn^{++}, or malate and NADP, being additive; the protections by Mn^{++} and NADP are not (Rutter and Lardy, 1958). It may also be the case with liver alcohol dehalogenase, ethanol and NAD protecting more than either one alone (Yonetani and Theorell, 1962). In one situation, aspartate carbamyltransferase, neither substrate alone protects, but together they do so quite effectively (Reichard and Hanshoff, 1956). An example of protection by a reversible inhibitor is the reduction in the inhibition of succinate dehydrogenase by *p*-MB or Hg^{++} in the presence of oxalacetate (Stoppani and Brignone, 1957). Actually, an effective competitive inhibitor might be expected to protect better than the substrate.

The information derived from protection experiments is frequently not as reliable as commonly assumed, for reasons to be discussed in Chapter 1, Volume III. The fact that the action of a mercurial is reduced by a sub-

TABLE 7-9

EXAMPLES OF THE PROTECTION OF ENZYMES AGAINST MERCURIALS BY COMPONENTS OF THE ENZYME REACTION

Enzyme	Source	Mercurial	Substance	Protection	Reference
Adenosinetriphosphatase	Cockroach muscle	*p*-MB	Mg^{++}	+	Sacktor *et al.* (1953)
Alcohol dehydrogenase	Yeast	*p*-MB	Ethanol	−	Hoch and Vallee (1959)
			NAD	−	
	Horse liver	*p*-MPS	NAD	+	Yonetani and Theorell (1962)
Aldehyde dehydrogenase	Yeast (NADP-specific)	*p*-MB	NADP	+	Stoppani and Milstein (1957 a)
			NAD	−	
	Beef liver (NAD-specific)	*p*-MB	NAD	+	Stoppani and Milstein (1957 a)
			NADP	−	
Aldolase	Rabbit muscle	*p*-MB	Fructose-diP	−	Swenson and Boyer (1957)
Amino acid (neutral) decarboxylase	*Proteus vulgaris*	*p*-MB	Amino acids	+	Sutton and King (1959)
			Pyridoxal-P	−	
D-Amino acid oxidase	Lamb kidney	*p*-MB	FAD	+	Hellerman *et al.* (1946)
		p-MB	Alanine	+	Frisell and Hellerman (1957)
Amylomaltase	*E. coli*	*p*-MB	Maltose	−	Wiesmeyer and Cohn (1960)
Arginine decarboxyoxidase	*Streptomyces griseus*	*p*-MB	Arginine	+	Van Thoai and Olomucki (1962)
Aspartate carbamyltransferase	*E. coli*	*p*-MB	Aspartate	−	Reichard and Hanshoff (1956)

			Carbamyl-P	−	
			Both	+	
Betaine-aldehyde dehydrogenase	Rat liver	*p*-MB	Betaine-aldehyde	+	Rothschild and Barron (1954)
			NAD	+	
Butyryl-CoA dehydrogenase	Beef liver	*p*-MB	Butyryl-CoA	+	Mahler (1954)
			FAD	+	
Carbamyl-P synthetase	Frog liver	*p*-MB	Acetylglutamate	+	Marshall *et al.* (1961)
Carboxypeptidase	Beef pancreas	*p*-MB	Zn^{++}	+	Coombs *et al.* (1964)
Deoxycytidylate deaminase	Chick embryo	*p*-MB	dCTP	+	Maley and Maley (1964)
Estradiol-17β dehydrogenase	Human placenta	*p*-MB	Estradiol	+	Langer and Engel (1958)
			NAD	+	
Fatty acid synthetase	Pigeon liver	*p*-MB	Acetyl-CoA	+	Bressler and Wakil (1962)
β-Fructofuranosidase	Yeast	Hg^{++}	Sucrose	+	Myrbäck (1926)
	Neurospora crassa	*p*-MB	Sucrose	+	Metzenberg (1963)
Fumarase	Yeast	*p*-MB	Fumarate	+	Favelukes and Stoppani (1958)
			Malate	+	
Fumarate hydratase	*Propionibacterium pentosaceum*	*p*-MB	Fumarate	+	Mello Ayres and Lara (1962)
			Malate	−	
L-Galactono-γ-lactone dehydrogenase	Cauliflower florets	*p*-MB	L-Galactono-γ-lactone	+	Mapson and Breslow (1958)
Gluconokinase	Pig kidney	*p*-MB	Gluconate	−	Leder (1957)
			ATP	−	
Glucose-6-P dehydrogenase	Human erythrocytes	*p*-MB	NADP	+	Chung and Langdon (1963)

TABLE 7-9 (*continued*)

Enzyme	Source	Mercurial	Substance	Protection	Reference
β-Glucosidase	Yeast	*p*-MB	Substrates	+	Duerksen and Halvorson (1958)
L-Glutamate decarboxylase	Mouse brain	*p*-MB	Pyridoxal-P	—	Roberts and Simonsen (1963)
Glyoxylate reductase	Tobacco leaves	*p*-MB	Glyoxylate	+	Zelitch (1955)
			NADH	+	
Hexokinase	Brain	*p*-MB	Glucose	+	Sols and Crane (1954)
Hexokinase (Zn-dependent)	*Neurospora crassa*	*p*-MB	Zn^{++}	+	Medina and Nicholas (1957 a)
Homogentisicase	Beef liver	*p*-MB	Homogentisate	+	Tokuyama (1959)
			Fe^{++}	+	
3-Hydroxyanthranilate oxidase	Beef liver	*p*-MB	3-Hydroxy-anthranilate	+	Decker *et al.* (1961)
			Fe^{++}	+	Mitchell *et al.* (1963)
β-Hydroxyisobutyrate dehydrogenase	Pig kidney	*p*-MB	β-Hydroxyiso-butyrate	—	Robinson and Coon (1957)
			NAD	+	
Hydroxypyruvate reductase	*Aspergillus niger*	*p*-MB	Hydroxypyruvate	+	Behal and Hamilton (1962)
			NADH	+	
Isoictrate dehydrogenase	Beef heart	*p*-MPS	Isocitrate+Mn^{++}	+	Chen *et al.* (1964)
Lactate dehydrogenase	Beef heart	*p*-MB	Pyruvate	—	Takenaka and Schwert (1956)
			NADH	+	

	Beef brain	*p*-MB	NAD	+	Winer (1960)
	Rabbit muscle	*p*-MB	NAD	+	Dube *et al.* (1963)
Leucine decarboxylase	*Proteus vulgaris*	*p*-MB	Leucine	+	Sutton and King (1962)
			Pyridoxal-P	+	
Malate oxidative decarboxylase	Pigeon liver	*p*-MPS	Malate	+	Rutter and Lardy (1958)
			NADP	+	
			Mn^{++}	+	
NADH oxidase	*Streptococcus faecalis*	*p*-MB	FAD	+	Hoskins *et al.* (1962)
Nicotinamidase	*Torula cremoris*	*p*-MB	Nicotinamide	—	Joshi and Handler (1962)
Ornithine carbamyltransferase	Rat liver	*p*-MB	Ornithine	+	Reichard (1957)
			Carbamyl-P	+	
3-Phosphoglyceraldehyde dehydrogenase	Rabbit muscle	*p*-MB	2,6-Dichloro-phenolindophenol	+	Rafter and Colowick (1957)
Prolidase	Pig kidney	*p*-MB	Mn^{++}	—	Smith *et al.* (1954 a)
Pyruvate decarboxylase	Yeast	*p*-MB	Pyruvate	+	Stoppani *et al.* (1953)
Pyruvate oxidase	*Proteus vulgaris*	*p*-MB	Thiamine-diP	+	Baer (1948); Kuratomi (1959)
Succinate dehydrogenase	Rat liver	*p*-MB	NAD	—	Barker (1953)
	Beef heart	*p*-MB	Succinate	+	Singer *et al.* (1956 b)
	Pig heart	*p*-MB	Succinate	+	Stoppani and Brignone (1957)
Transaminase (tyrosine: α-ketoglutarate)	Yeast	*p*-MB	Pyridoxal-P	—	SentheShanmuganathan (1960)
Urocanase	Cat liver	*p*-MB	Urocanate	+	Miller and Waelsch (1957)
Xanthine oxidase	Chicken liver	*p*-MB	Hypoxanthine	—	Doisy *et al.* (1955)

strate, for example, does not necessarily imply that the substrate reacts with an SH group nor that the SH group is involved in the catalysis, although these may well be the case. A positive result is more valuable than a negative one. The failure to achieve protection may be due to an inadequate concentration of the protector, too low a relative affinity of the enzyme for the protector, or a long incubation wherein equilibrium is reached, and yet the substance examined may participate in the reaction and interact with the enzyme in the same way as effective protectors. Definite protection allows one to make the reasonable assumption that the mercurial binds somewhere in the region occupied by the protector. Potter and DuBois (1943) postulated an SH group to be located between the two cationic groups binding succinate to the dehydrogenase; protection against mercurials by succinate simply implies that succinate is able to shield this SH group from the mercurial, and not that the SH group is involved in the succinate binding or participates in the oxidation-reduction reaction. For steric reasons, the smaller the molecular sizes of the protector and the mercurial, the more certain can one be that a common SH group is involved in the binding of both.

Displacement of Coenzymes and Cofactors from Enzymes

Closely allied to protection experiments are those in which a mercurial is shown to dissociate an enzyme-coenzyme or enzyme-cofactor complex. It is now believed that several coenzymes and metal ion activators may be bound to apoenzymes through SH groups in part (Shifrin and Kaplan, 1960), and if this is so one would expect tightly bound SH reagents, such as the mercurials, to displace the coenzymes or activators. Certain coenzymes or cofactors, such as NAD, have been shown to react with thiols (van Eys and Kaplan, 1957 b), but in most cases the evidence for binding to enzyme SH groups is circumstantial. Certainly such displacement of necessary components of the enzyme reaction would be an important mechanism in the inhibition produced by mercurials, especially *in vivo* where the total binary or ternary complexes usually occur. One molecule of crystalline horse liver alcohol dehydrogenase binds 2 molecules of NADH at physiological pH and this is accompanied by a shift in the absorption spectrum of the NADH. Addition of *p*-MB was found by Theorell and Bonnichsen (1951), to reverse the spectral shift, and it was concluded that the bond between an enzyme SH group and the NADH pyridine ring is broken by the mercurial. However, some doubts have recently been cast on this simple interpretation. The liver alcohol dehydrogenase molecule has 28 SH groups as determined by *p*-MB titration; the presence of NADH does not reduce this number, although NADH protects the enzyme moderately (Witter, 1960). On a rather tenuous basis, Witter postulated that the function of the SH groups is to maintain the stable enzyme

structure rather than bind the NADH; disintegration of the structure brought about by *p*-MB would secondarily lead to release of the coenzyme. As the NADH is split from the apoenzyme by *p*-MB, rotatory dispersion titration indicates changes in optical rotation associated with denaturation, so that Li *et al.* (1962) likewise inclined to a theory involving structural changes as a basis for the displacement, since it is known that denaturation by heat or other agents releases the NADH. Yonetani and Theorell (1962) have used the very sensitive spectrofluorometric method for the measurement of NADH binding and dissociation.* They demonstrated that the enzyme configuration is stabilized by the NADH and additionally by the isobutyramide, although these can be associated directly with only a small fraction of the total number of SH groups, and suggested that denaturation may be initiated by local changes at the active centers and from there spread throughout the molecule. The SH groups may form a network of hydrogen bonds contributing to the stability of the tertiary structure, so that mercurials could create instability either locally or generally. All of this recent work shows that mercurials probably induce configurational changes in the enzyme, these being irreversible by the usual means, and they provide an alternative explanation for NADH release, but do not disprove the original hypothesis that a direct binding between NADH and SH groups occurs. The Zn^{++}-dependent alcohol dehydrogenase of yeast is inhibited by mercurials, and this was attributed to displacement of the Zn^{++} from SH groups (Wallenfels and Sund, 1957 a) on the basis that restoration of activity requires both glutathione and Zn^{++}. However, an investigation of the time course of the inhibition showed that glutathione alone is sufficient to reactivate if it is added soon after the mercurial, but the inhibition progressively becomes irreversible, at which time no Zn^{++} has been released (Snodgrass and Hoch, 1959). Zn^{++} is displaced progressively over a period of several hours, but the inhibition does not appear to be mediated through this displacement. Such a slow release, without correlation with the mercurial reaction or the inhibition, is probably due to structural changes in the enzyme.

The early work on NADH splitting from alcohol dehydrogenase was soon confirmed for 3-phosphoglyceraldehyde dehydrogenase (3-PGDH) by Velick (1953). The yeast enzyme requires 2 SH groups for full activity and inhibition by *p*-MPS increases until 2 equivalents of the mercurial are added. In contrast to the alcohol dehydrogenase, this inhibition is readily revers-

* Liver alcohol dehydrogenase forms a very stable ternary complex with NADH and *iso*butyramide, and this complex is strongly fluorescent. The enzyme may be titrated in the presence of 100 m*M* *iso*butyramide with NADH, measuring the fluorescence increase at 410 mμ, and then back-titrated with *p*-MB or *p*-MPS as the NADH is released from the apoenzyme. This will probably be a very valuable technique for the study of coenzyme binding.

ible with cysteine. Muscle 3-PGDH behaves similarly but, after charcoal treatment, reacts with 3 equivalents of *p*-MPS. If the enzyme is ultracentrifuged it carries down most of the NAD but the inhibited enzyme does not, most of the NAD being free in the medium. The correlations between these various events are shown in Fig. 7-12. The change in spectral absorption at 340 μm associated with NAD binding disappears progressively with added *p*-MPS, again indicating coenzyme release. Velick was careful not to assume that these results necessarily point to a binding of the NAD by the SH groups, but stated that the *p*-MPS can sterically or electrostatically

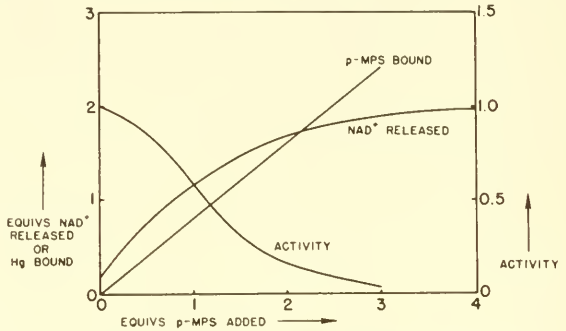


FIG. 7-12. Dissociation of NAD from 3-phosphoglyceraldehyde dehydrogenase by *p*-MPS, and the simultaneous loss of activity. (From Velick, 1953.)

interfere with the coenzyme binding if the SH groups are close enough to the active center. Fluorometric titration of the 3-PGDH-$(NADH)_3$ complex from rabbit muscle with *p*-MB demonstrates NADH dissociation, but the kinetics indicate that each *p*-MB bound weakens the coenzyme binding at the other sites, so that perhaps structural changes in the protein occur (Velick, 1958). The same number of equivalents of *p*-MB is required to release the NADH from 3-PGDH-$(NADH)_1$, 3-PGDH-$(NADH)_2$, and 3-PGDH-$(NADH)_3$. If the displacement were due to direct competition with the NADH for SH groups, one would expect the *p*-MB to attack the unoccupied SH groups first, with no release of NADH, but this is not the case, the release beginning immediately, as if the reaction of any SH group altered the enzyme structure throughout. These structural changes, if they occur, must be readily reversible.

Results with a few other enzymes will be discussed briefly. Reports on coenzyme displacement from lactate dehydrogenase have not been entirely consistent. Apparently the state of the enzyme and particularly the source are important factors. Kaplan and Ciotti (1954) found that *p*-MB releases NAD from the liver enzyme, this being associated with a fall in absorption at 300 mμ, but Chance (1954) could detect no release from heart lactate

dehydrogenase at 5°, an observation confirmed by Velick (1958). However, Winer *et al.* (1959) find a slow dissociation of NADH from heart lactate dehydrogenase, complete release occurring after 1 hr at 26° and pH 7 with 0.135 m*M* *p*-MB. The L(+)-lactate dehydrogenase of yeast (cytochrome b_2) possesses a flavin prosthetic group and this is readily dissociated by *p*-MPS (Armstrong *et al.*, 1960, 1963). The binding of NADH and NADPH to cytochrome b_5 aporeductase is blocked by *p*-MB (Strittmatter, 1961 b) and there is some evidence that pyridoxal-P may be split from L-threonine dehydrase by the same mercurial (Nishimura and Greenberg, 1961). The evidence for the displacement of Fe^{++} from homogentisate oxidase by *p*-MB has already been discussed, and the reactions of inhibition and reactivation (Crandall, 1955) may be written:

$$\text{Inhibition:} \quad \text{E—S—Fe}^{+} + \text{R—Hg—X} \rightarrow \text{E—S—Hg—R} + \text{Fe}^{++} + \text{X}^{-}$$

$$\text{Reactivation:} \quad \begin{cases} \text{E—S—Hg—R} + \text{GSH} \rightarrow \text{E—SH} + \text{GS—Hg—R} \\ \text{E—SH} + \text{Fe}^{++} \rightarrow \text{E—S—Fe}^{+} + \text{H}^{+} \end{cases}$$

The nonheme Fe of succinate dehydrogenase is lost more rapidly by dialysis after treatment with *p*-MPS, and this may be related to the marked spectral changes observed upon reaction with the mercurial (Massey, 1958). The iron of the photosynthetic pyridine nucleotide reductase is released as Fe^{+++} by *p*-MB with proportional loss of activity (Katoh and Takamiya, 1963). The inhibition of aminopeptidase by EDTA is made irreversible by simultaneous treatment with *p*-MB and it was concluded that the Mn^{++} is bound to an SH group (Bryce and Rabin, 1964). A final type of experiment will be mentioned. Mn^{++} activates the hydroxylamine reductase of *P. aeruginosa* and this activation is prevented by *p*-MB, "suggesting that SH groups may be involved in binding the metal to the enzyme" (Walker and Nicholas, 1961). It seems to me that such conclusions are unjustified, inasmuch as any mechanism of inhibition would presumably abolish activation by Mn^{++}, whether it affected the binding or not.

The results on coenzyme displacement may be summarized by stating that the same difficulties are encountered as in protection experiments. There are three general mechanisms by which a mercurial could dissociate an enzyme-coenzyme complex: (1) compete with the coenzyme for the SH group, (2) sterically or electrostatically interfere with coenzyme binding by reacting at an adjacent site, and (3) alter the enzyme configuration in such a way as to disrupt secondarily the coenzyme binding. In no case have these mechanisms been distinguished.

Changes in Enzyme Structure Brought About by Mercurials

Evidence has accumulated during the past several years that mercurials occasionally initiate configurational changes in enzymes; certain aspects of

this have been discussed in the previous section and we shall now inquire what further evidence on this important problem has come to light. One of the more obvious reasons for suspecting denaturation is the progressive development of irreversibility during contact with the mercurial, such as has been reported for cholinesterase (Goldstein and Doherty, 1951), prostatic phosphomonoesterase (Tsuboi and Hudson, 1955 a), muscle aldolase (Swenson and Boyer, 1957), and muscle 3-PGDH (Elödi, 1960) — to mention only a few instances, in most cases reversal being attempted with glutathione or dimercaprol. Of course, one might attribute failure to reverse to very tight binding to the enzyme, but the progressive increase in the irreversibility points more to structural changes. The question often remains as to whether these changes are responsible for the inhibition or are superimposed upon it, i.e., inhibition followed by inactivation.

Elödi (1960) investigated the changes in several properties of pig muscle 3-PGDH treated with *p*-MB, and found significant deviations in the optical rotation and the intrinsic viscosity, the latter increasing linearly with the equivalents of mercurial added. The following phases were postulated: (1) an initial reversible binding and inhibition, (2) a progressive disintegration of the secondary structure of the enzyme as a result of the blocking of SH groups, this probably involving an unfolding of the polypeptide helices, and (3) polymerization and precipitation consequent to the freeing of groups which form intermolecular bridges. The simultaneous changes in the activity, NADH binding, and rotatory dispersion of yeast alcohol dehydrogenase treated with *p*-MB led Wallenfels and Müller-Hill (1964) to postulate that modifications of the secondary and tertiary protein structure occur when the SH groups are blocked. Reaction of 10 SH groups on muscle aldolase with *p*-MB does not reduce the activity but the susceptibility to tryptic digestion is increased (Szabolcsi and Biszku, 1961). Untreated aldolase or treated enzyme in the presence of fructose-diP is not digested by trypsin; thus the substrate apparently protects the active center, and perhaps the entire molecule, from hydrolysis. It is thought that reaction of the first 7 free SH groups labilizes the tertiary structure of the enzyme, and from then on a progressive denaturation occurs. Addition of substrate may restore to some degree the normal structure. The inhibition that occurs later or with excess *p*-MB does not seem to be directly related to mercaptide formation but dependent on the structural changes when they have proceeded past a certain point. Another interesting approach was made by Massey (1958) in showing that the chelation of the nonheme iron of succinate dehydrogenase by *o*-phenanthroline is accelerated by treatment with *p*-MPS, this being interpreted as a structural change exposing the iron. The SH groups of yeast hexokinase can be titrated with *p*-MB in the presence of glucose without loss of activity, but spontaneous denaturation quickly follows (Fasella and Hammes, 1963). Glucose-6-P does not prevent

loss of activity during the titration. These results indicate that the SH groups are not directly involved in the catalysis, but function to stabilize the enzyme in the active configuration.

Another type of structural change is depolymerization of the enzyme into subunits following mercurialization. Muscle phosphorylase is progressively inhibited by *p*-MB until around 18 equivalents of the mercurial are combined, and this is accompanied by the appearance of a new molecular species in the ultracentrifuge, the sedimentation constant being lower than that for either phosphorylase a or b (Madsen and Cori, 1955):

Phosphorylase a:	S = 13.2
Phosphorylase b:	S = 8.2
Inactive enzyme:	S = 5.6

Both phosphorylase a and b form this new species with *p*-MB and it was suggested that the former is split into 4 subunits, the latter into 2 subunits. Light scattering studies are consistent with this interpretation (Madsen, 1956). The inhibition develops more rapidly than the depolymerization, however, so the relationship between them is not clear. Removal of the *p*-MB with cysteine restores both activity and the normal dimer or tetramer (Madsen and Cori, 1956). The extent of the conversion of the phosphorylase tetramer to the monomer is proportional to the number of SH groups reacted and an all-or-none dissociation of the units is likely (Madsen and Gurd, 1956). The sedimentation constant of yeast alcohol dehydrogenase is reduced from 7.2 to 3.3 by *p*-MB, this being secondary to the inhibition of the enzyme, so that here dissociation into subunits apparently occurs (Snodgrass *et al.*, 1960). Reaction of myosin ATPase with MM also causes the appearance of a small subunit, but this is not related to the binding to the SH groups responsible for the activity (Kominz, 1961). In addition there is some aggregation to a faster sedimenting species and this is perhaps correlated with reaction of SH groups at the active center. The inhibition of rabbit muscle enolase by *p*-MB was considered to be secondary to denaturation and not directly due to SH group reaction, on the basis of the variation of activity with the equivalents of mercurial present and the appearance of turbidity (Malmström, 1962). The sedimentation constant of liver glutamate dehydrogenase is reduced by MM and PM and again the most likely explanation is a splitting into subunits (Rogers *et al.*, 1962, 1963; Greville and Mildvan, 1962). The relationship of the disaggregation to the unique changes in enzyme activity is not clear.

There is no doubt that mercurials can induce structural changes in certain enzymes, and cause aggregation or fractionation into subunits in others, but the significance for primary inhibition has not been clarified. Is the inhibition due to the blocking of functional SH groups or secondarily to the structural changes? Does the denaturation result from general SH group

reaction or can it originate solely by mercaptide formation at the active center? The results taken all in all tend to signify that inhibition usually occurs upon the initial reaction of the SH groups at or near the active center, this being reversible, and that slower structural alterations proceed as a result of either the mercurial already combined or the continued reaction with more mercurial (perhaps with the less available SH groups), these changes becoming more and more irreversible, a progressive inactivation being superimposed on the primary inhibition. Lability may also come about by a displacement of coenzyme or cofactors, since these undoubtedly help maintain configurational integrity, especially in the abnormal state in which isolated enzymes find themselves. This does not imply that all enzymes behave in this fashion; it is quite possible that in some the structural changes may be primary and the sole cause of the inhibition. Certain enzymes suffer only the primary inhibition and the stability is not reduced by the mercurial, and indeed stability may be increased, as we have observed with papain and pinguinain. The requirement to solve the problems of the relation between inhibition and inactivation, and between both processes and the types of SH group reacted, is for more detailed studies correlating the time courses of as many of these changes as possible as they occur after introduction of the mercurial. Another approach might be to do occasional experiments at low temperatures, where inactivation or denaturation would occur very slowly, in this way possibly separating the primary inhibition from these other changes. Finally, it might be suggested that every effort to create conditions favoring stability of the enzymes be made. One gets the impression that often so little attention is paid to the proper pH, ion concentrations, buffers, and other factors, that the enzyme as studied is in a relatively unstable state and hypersusceptible to any inhibitors subjecting the normal protein configuration to even minor stress.

Effects of pH, Ions, and Buffers on Mercurial Inhibition

The effects of pH on OH^- complexes with mercurials (page 736), on mercaptide formation (page 749), and on reactions of proteins with mercurials (page 760) have been discussed. The results and the factors which may be involved can be summarized as follows: (1) pH affects the ionization of the SH groups or the competition between mercurial and H^+ for the S^- group, (2) pH alters the concentration of OH^- and hence the amount of mercurial complexed with this anion, (3) pH influences the protein charge possibly attracting or repelling charged mercurials, (4) pH determines the rate of secondary inactivation or denaturation, (5) pH affects the aggregation state of protein-mercurial complexes (e.g., the degree of dimerization of mercaptalbumin complexes with Hg^{++}), (6) pH affects both the rate and the number of SH groups reacted, and (7) generally there is an increased rate of protein reaction with mercurials as the pH is reduced.

The effects of pH on enzyme inhibition by the mercurials are even more complex and one would not anticipate consistent behavior, a prediction that is borne out in the following discussion.

Some pH effects on mercurial inhibition are shown in Table 7-10. In 7 cases the inhibition is greatest at low pH and in 6 cases at high pH, and this certainly indicates that more than one factor must be involved. Indeed, many of the inhibition-pH curves are complex (Figs. 7-13 and 7-14) and

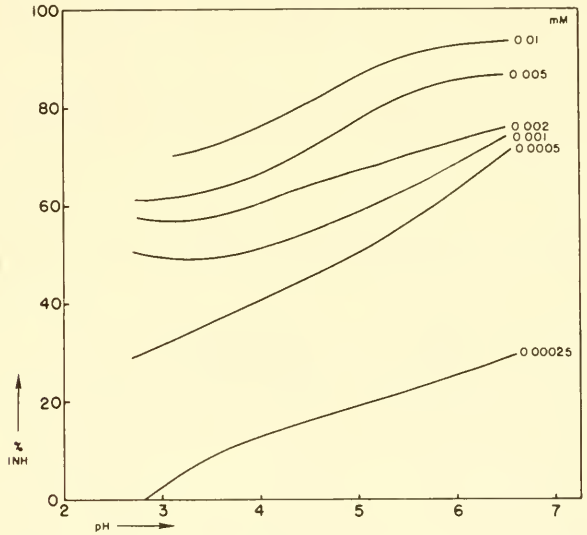


FIG. 7-13. Effects of pH on the inhibitions of β-fructofuranosidase by $HgCl_2$ and $Hg(NO_3)_2$. (Data from Myrbäck, 1926.)

often biphasic (β-glucuronidase, β-fructofuranosidase, ATPase, and ribonuclease). In some cases marked stimulation is found within a certain pH range, inhibition occurring outside this range. This implies that the activity-pH curves and the pH_{opt} are shifted by the mercurials, usually to lower pH's, as for yeast proteinase (Lenney, 1956) and ascites cell ribonuclease (Ellem and Colter, 1961; Colter *et al.*, 1961). It is difficult to interpret such shifts in pH_{opt}, but if the pH_{opt} is related to the ionization of two or more groups on enzyme and substrate (see page I-660), a shift implies some modification of the enzyme groups in or near the active center with a resultant alteration of the interaction of the substrate with the enzyme. Dixon's method of plotting K_m and K_i against pH (page I-683) was applied to β-glucuronidase by Fernley (1962), and the curves are shown in Fig. 7-15. The pK_i curve is suggestive of two ionizing groups on the enzyme with pK_a's around 4.4 and 6.3 if it could be simply interpreted, or possibly the

TABLE 7-10

EFFECTS OF pH ON INHIBITION OF ENZYMES BY MERCURIALS

Enzyme	Source	Mercurial	Concentration (mM)	pH	% Inhibition[a]	Reference
Adenosinetriphosphatase	Rabbit muscle	*p*-MB	0.04 μmole/ mg protein	5.5	63	Stracher and Chan (1961)
				6.5	+ 17	
				7.5	+260	
				8.5	8	
				9.5	65	
Alcohol dehydrogenase	Yeast	*p*-MB	0.00016	7.6	50	Wallenfels and Sund (1957 a)
			0.00018	8.6	50	
		Hg^{++}	0.000064	7.6	50	
			0.00025	8.6	50	
			0.0001	7.6	100	
				8.6	0	
α-Amylase[b]	*Bacillus subtilis*	*p*-MB	Satd	4.7	58	Di Carlo and Redfern (1947)
				6.0	0	
Bromelain	Pineapple	*p*-MB	0.1	5.3	90	Ota *et al.* (1961)
				7.0	73	
β-Fructofuranosidase	Yeast	Hg^{++}	0.0005	2.8	30	Myrbäck (1926)
				3.4	36	
				4.6	46	

				5.5	57	
				6.5	68	
β-Glucuronidase	Rat preputial gland	Hg^{++}	0.05	3.5	46	Fernley (1962)
				4.0	21	
				4.5	40	
				5.0	86	
				5.5	89	
		PM	0.4	4.0	59	
				4.5	48	
				5.0	48	
				5.5	53	
L-Glutamate dehydrogenase	Beef liver	PM	0.5 μmole/ mg protein	<7.9	Ihn	Mildvan and Greville (1962)
				8.5	+150	
NADH:quinone oxidoreductase	Pig liver	Mersalyl	0.07	7.4	50	Frimmer (1960)
			0.25	8.0	50	
Penicillinase	*Aerobacter cloacae*	*p*-MB	0.014	6.0	50	J. T. Smith (1963 b)
			0.06		100	
			0.002	7.4	50	
			0.01		100	
Proteinase	*Clostridium perfringens*	Hg^{++}	1	5.0	89	Maschmann (1937 b)
				7.0	3	
Proteinase[c]	Yeast	*p*-MB	1	4.3	0	Lenney (1956)
				5.0	55	
				6.0	67	
				7.0	84	
				7.5	100	

TABLE 7-10 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	pH	% Inhibition[a]	Reference
Ribonuclease	Mouse ascites tumors	*p*-MB	0.4	5.0	+ 36	Colter *et al.* (1961)
				6.0	+232	
				7.0	+535	
				8.0	+ 50	
				8.5	42	
				9.0	79	
Tyrosinase	Potato	Hg^{++}	0.9	4.5	19	Sasaki (1940)
				5.6	46	
				6.3	75	
				7.1	87	
Urease	Jack beans	Hg^{++}	0.0185	4.94	41	Jacoby (1933)
				5.96	38	
				7.0	15	
				8.03	22	
				9.18	21	

[a] Stimulation indicated by + sign.

[b] Incubated at pH 6 at 37.5° and at pH 4.7 at 5°.

[c] Enzyme incubated with *p*-MB at pH 5 for 60 min and then tested at pH's indicated.

group with pK_a near 4.4 could be the benzoate group of *p*-MB, particularly as no inflection is shown here in the K_m curve. However, as Fernley clearly pointed out, one must proceed with caution because of the many complexes possible with the mercurial, and particularly the buffer effects. It seems unlikely that the decrease in K_i above pH 5 is due to complexing with OH^- ions entirely, but it might be the result of reactions with the buffer; the similar inflection for K_m makes it reasonable that an enzyme group is involved. The results with PM are quite different than with Hg^{++} or *p*-MB (Fig. 7-14);

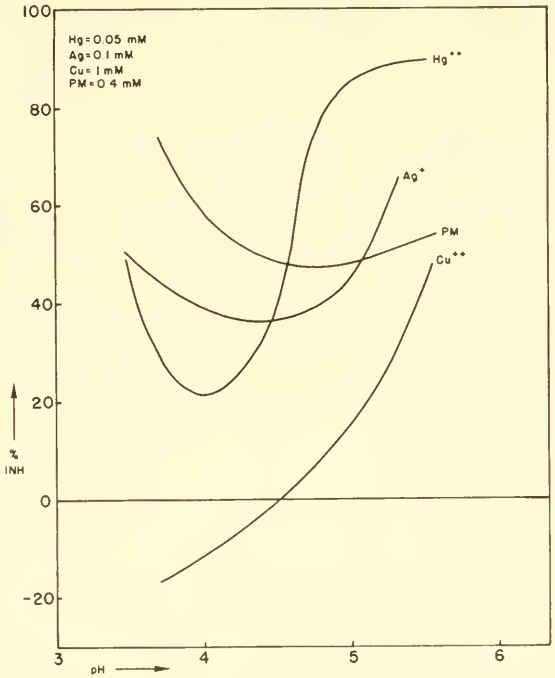


FIG. 7-14. Effects of pH on the inhibitions of β-glucuronidase by heavy metal ions. (From Fernley, 1962.)

PM is generally less inhibitory than *p*-MB, does not increase K_m whereas *p*-MB does, facilitates the formation of the ES_2 complex (favoring substrate inhibition) whereas *p*-MB does not, and increases the pH_{opt} whereas Hg^{++} and *p*-MB decrease it. The marked effect of PM on substrate inhibition is shown in Fig. 7-16 but no explanation is available. There is certainly a need for more accurate comparisons of the different mercurials, not only with respect to pH effects but generally. The different curves obtained with Hg^{++} and PM acting on pancreatic amylase (Fig. 7-17) are intriguing and one feels that an explanation of such phenomena might well aid in our under-

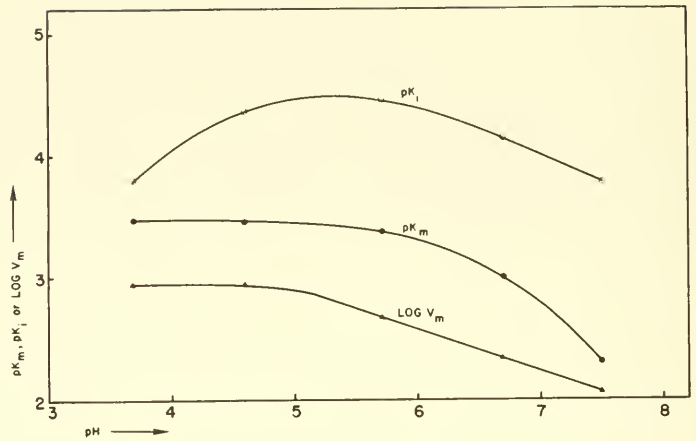


FIG. 7-15. Effects of pH on the K_m and V_m for β-glucuronidase, and the K_i for the inhibition by *p*-MB. (From Fernley, 1962.)

standing of the mechanisms of enzyme inhibition. The pH changes may affect the reactivities of different SH groups on an enzyme to different degrees. Xanthine oxidase has two types of SH group, one reacting relatively

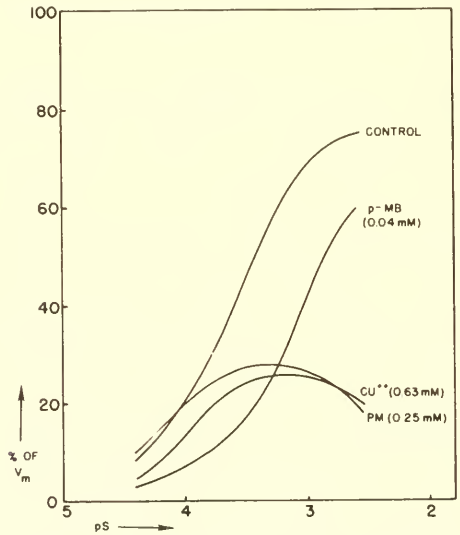


FIG. 7-16. Effects of substrate concentration on the inhibition of β-glucuronidase by *p*-MB, PM, and Cu^{++} at pH 5.9. (From Fernley, 1962.)

rapidly and unrelated to the enzyme activity and the other reacting slowly to produce inactivation; altering the pH from 5.2 to 7.2 modifies the rates of reaction of these groups with *p*-MB in quite different ways (Gilbert, 1963).

Increase of any ligand capable of complexing the mercurials should reduce the inhibition, but this has been studied very little. Fernley (1962) noted that raising the Cl^- concentration suppresses the inhibition of β-glucuronidase by Hg^{++}. It is very difficult in such cases to separate a direct complexing action from an ionic strength effect. Green and Neurath (1953)

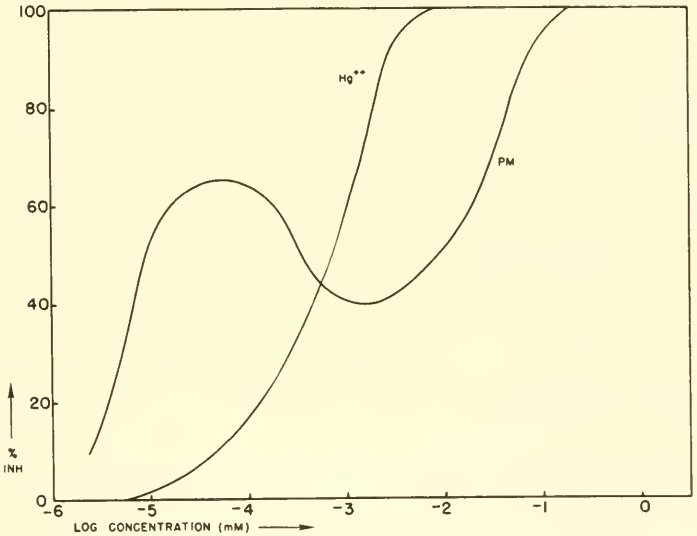


FIG. 7-17. Concentration-inhibition curves for the actions of Hg^{++} and PM on pancreatic amylase. (From Owens, 1953 b.)

varied the ionic strength with NaCl, $SrCl_2$, and $(NH_4)_2SO_4$ and found the inhibition of trypsin by Hg^{++} to be suppressed at high ionic strengths (Fig. 7-18). Since all the salts had essentially the same effect, they assumed that this is not due to specific ions, but certainly part of the reduction must be due to increasing formation of the Hg^{++} complexes with Cl^-, NH_4^+, and $SO_4^=$. The nature of the inhibition of trypsin is not clear since free SH groups are generally not considered to play a role in the active center. The inhibition of pancreatic amylase by Hg^{++} and PM was postulated by Owens (1953 a) to involve phosphate ion, and the unusual configuration of the PM inhibition curve was attributed, at least in part, to the formation of phosphate complexes, although it is strange that Hg^{++} is not similarly affected. It is also not clear to me why the curve should assume this shape, particularly why increase in PM from 0.1 to 1 m*M* should bring about a

lessening of the inhibition. The inhibition of NADH dehydrogenase by *p*-MPS is weaker in phosphate than in THAM buffer, and this could again be due to complexes with phosphate (Minakami *et al.*, 1963). Despite the paucity of experimental information, the effects of ions and buffers should be more frequently taken into account in the use of the mercurials. In addition to the ionic effects just described, certain specific actions have been noted,

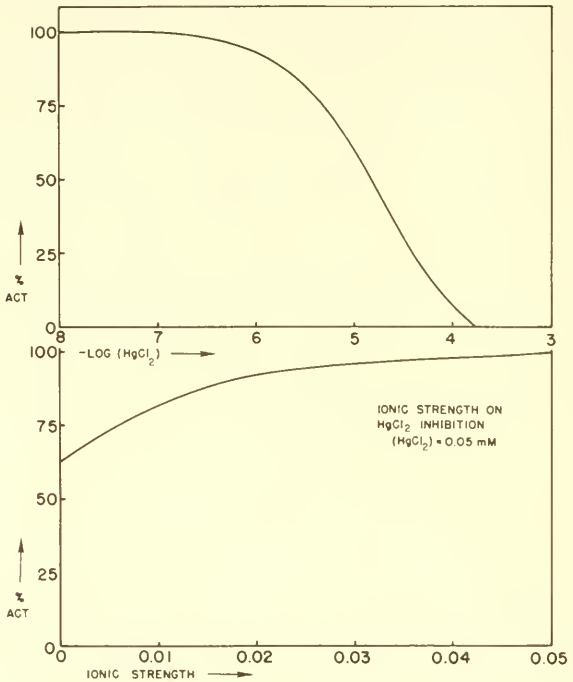


FIG. 7-18. Inhibition of trypsin by Hg^{++} and the effect of ionic strength. (From Green and Neurath, 1953.)

especially with ATPase (Novikoff *et al.*, 1952; Lardy and Wellman, 1953; Sacktor *et al.*, 1953), where the concentrations of Mg^{++} and Ca^{++} determine to some extent the susceptibility of the mitochondrial enzyme to mercurials, generally Mg^{++} lessening the inhibition, although here one may be dealing with more than one enzyme.

Titration of Enzyme SH Groups

The primary purpose of a titration with a mercurial is to determine the number of reactive SH groups which an enzyme possesses, either normally or after being treated in various ways (e.g., denatured with urea, guanidine,

or high temperature), and in this respect the problem is no different from that of protein titration (page 762). We are interested here not so much in the number of SH groups on an enzyme, but how these SH groups relate to the catalytic activity and the mechanisms of mercurial inhibition, and thus in following simultaneously the loss of SH groups and the development of the inhibition as more and more mercurial is added. Let us assume that we have a solution of a pure enzyme and we add to this a solution of *p*-MB, or other mercurial, so that the molar ratio of mercurial to enzyme is slowly increased, and further assume that we allow time for the reaction to come to equilibrium. We measure the number of SH groups reacted (spectrophotometrically, polarographically, argentimetrically, or otherwise) and the enzyme activity. There are only a few fundamental relationships between mercaptidization and inhibition that could emerge, and these are illustrated in Fig. 7-19. These are extreme situations, of course, and intermediate behavior would more often be expected. Let us consider what each result may mean and how valid certain interpretations may be.

Case A: The inhibition runs parallel to the SH groups reacted.

(1) The only SH groups that react are at the active center and mercaptide formation abolishes the enzyme activity; titration to 100% inhibition will give the number of SH groups at the active center.

(2) There are n equireactive SH groups on the enzyme, but only a certain fraction of these is at the active center or involved in the catalysis; the titration will not provide the actual number at the active center.

(3) The SH group or groups are not at the active center, but reaction of them leads to inactivation of the enzyme by some means; titration will not provide useful information.

It is impossible to distinguish between these possibilities by simple titration nor can one determine accurately the number of SH groups at the active center. Let us assume that an enzyme has 10 reactive SH groups totally but that complete inhibition occurs when only 3 are reacted. It has sometimes been concluded that 3 SH groups are necessary for the enzyme activity. This is not a valid conclusion. If 1 of the 3 groups were related in some way to the activity, one would obtain the same data. In other words, the number of equivalents of mercurial added, or the number of SH groups reacted, to achieve 100% inhibition does not provide directly the number of SH groups involved in the catalysis.

Case B: SH groups are reacted but inhibition does not occur.

The conclusion here is obvious: the reactive SH groups are not involved, directly or indirectly, in the enzyme activity. It is, of course, possible that there are SH groups at the active center, perhaps even functional, but that they do not react with the mercurial under the experimental conditions.

Case C: *Inhibition develops only after so many SH groups have reacted.*

(1) The most reactive SH groups are not related to the activity of the enzyme, but the less readily available ones are.

(2) None of the SH groups is directly related to the activity, but when a sufficient number are reacted the enzyme is structurally altered so that inhibition appears.

These situations may be distinguished sometimes by determining the reversibility of the inhibition, but occasionally denaturation is reversible.

Case D: *Inhibition develops completely before all the SH groups react.*

(1) The most reactive SH groups are at the active center and when they

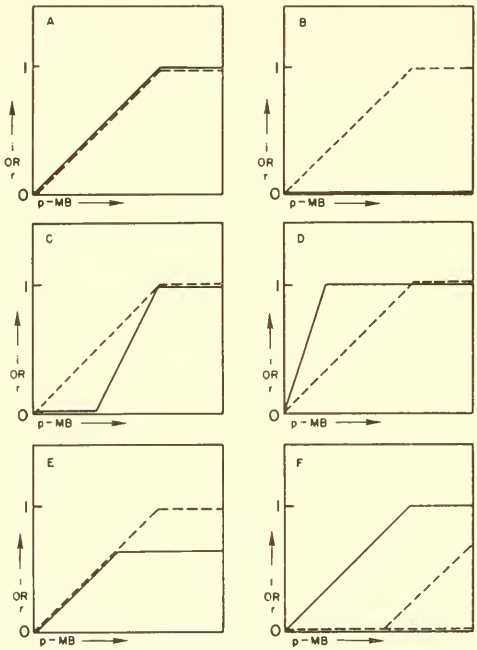


FIG. 7-19. Illustrations of the various relationships between the reaction of enzyme SH groups with mercurials and the inhibition of enzyme activity. These are all extreme cases and intermediate situations can also occur. *p*-MB has been designated but any mercurial may be used. The solid lines show the inhibition and the dashed lines the reaction with SH groups; i is the fractional inhibition and r is the fraction of the total SH groups reacted.

are all combined with the mercurial the enzyme is completely inhibited, the less readily reacting SH groups being now available.

(2) Only a small number of SH groups are normally reactive, but following mercaptide formation the enzyme unfolds and exposes other SH groups, the groups reacted after total inhibition being secondarily released.

As discussed under Case A, the titration to 100% inhibition does not necessarily provide the number of SH groups involved in the catalysis. Reversal experiments run in parallel to the titration may give some information as to which mechanism is involved.

Case E: *Inhibition parallels the SH group reaction but levels off.*

One must assume that complete combination of the enzyme with the mercurial does not produce complete inhibition, which could easily be the case if the SH groups reacted are sufficiently far from the active center so that the introduced side chain would modify only the catalysis. A control without the enzyme is, of course, mandatory to eliminate nonenzymic components to the rate.

Case F: *Inhibition develops without the reaction of SH groups.*

(1) The mercurial, which was assumed to be specific for SH groups, is not, and is inhibiting by another mechanism.

(2) Reaction of SH groups leads to structural changes by which more SH groups appear; lack of reaction might be concluded from titrations in which residual SH groups are determined, not by direct spectrophotometric methods.

(3) The method for the estimation of SH group disappearance may be faulty, i.e., those groups reacted by the mercurial may be resistant to the titrating agent.

There are some instances in which non-SH enzymes are inhibited by mercurials, but in general a result of this type should suggest a re-examination of the methods used.

Let us now examine a simple system in greater detail. An enzyme having 2 SH groups per molecule, one of these groups involved in the catalysis and one not, is treated with increasing amounts of a mercurial; how will the relative reactivities of the SH groups affect the results? Representing the mercurial by M, the two equilibria may be written:

$$M^+ + S_1^- \rightleftarrows MS_1 \qquad K_1 = \frac{(M^+)\,(S_1^-)}{(MS_1)} \tag{7-6}$$

$$M^+ + S_2^- \rightleftarrows MS_2 \qquad K_2 = \frac{(M^+)\,(S_2^-)}{(MS_2)} \tag{7-7}$$

If we assume that the mercurial is so tightly bound that free mercurial in the medium may be neglected, the conservation equations take the form:

$$(M_t) = (MS_1) + (MS_2) \tag{7-8}$$

$$(S_1^-)_t = (S_1^-) + (MS_1) \tag{7-9}$$

$$(S_2^-)_t = (S_2^-) + (MS_2) \tag{7-10}$$

The fractions of each S^- group combined with mercurial will be represented by f_1 and f_2, and it is easy to show that these are related by:

$$\frac{f_1 K_1}{1 - f_1} = \frac{f_2 K_2}{1 - f_2} \tag{7-11}$$

The ionization of the SH groups has purposefully been assumed to be complete in order to simplify the expressions. If S_1^- is assumed to be necessary for enzyme activity, $i = f_1 = (MS_1)/(S_1^-)_t$, while the reaction of S_2^- is without effect on the activity. Finally, we shall designate by r the fraction of the total SH groups reacted with the mercurial, this being determined by titration. In a specific case where $(S_1^-)_t = (S_2^-)_t = 10^{-5}\ M$, if we vary the ratio K_2/K_1 — i.e., the relative affinities of the S^- groups for the mercurial — the curves in Fig. 7-20 are obtained, r always being linear to complete reaction while i can follow any of the curves between $K_2/K_1 = 0$ and ∞. When $K_2/K_1 < 1$, the situation corresponds to case C in Fig. 7-19, and when $K_2/K_1 > 1$, it corresponds to case D. One thing we immediately note is that the affinities of the mercurial for the different S^- groups must be quite different if the i curves are to deviate from the r line appreciably; i.e., unless K_2/K_1 is much greater or much less than 1, it will be difficult to demonstrate that only one of the S^- groups is necessary for the enzyme activity. This treatment can be readily extended to enzymes with more than 2 S^- groups, in which case:

$$\frac{f_1 K_1}{1 - f_1} = \frac{f_2 K_2}{1 - f_2} = \ldots = \frac{f_n K_n}{1 - f_n} \tag{7-12}$$

and to situations in which more than one S^- group are involved in the catalysis. One can also plot i against r to obtain curves characteristic of the various situations described above.

A few examples of the different types of behavior are summarized here, as far as it is possible to evaluate the data published.

Type A

Lactate dehydrogenase — beef heart (Millar and Schwert, 1963)
Malate dehydrogenase — pig heart (Wolfe and Neilands, 1956; Pfleiderer *et al.*, 1962)
3-Phoshoglyceraldehyde dehydrogenase — yeast (Velick, 1953)
Pyrophosphatase — pig brain (Seal and Binkley, 1957)
Succinate dehydrogenase — rat liver (Hirade and Hayaishi, 1953)

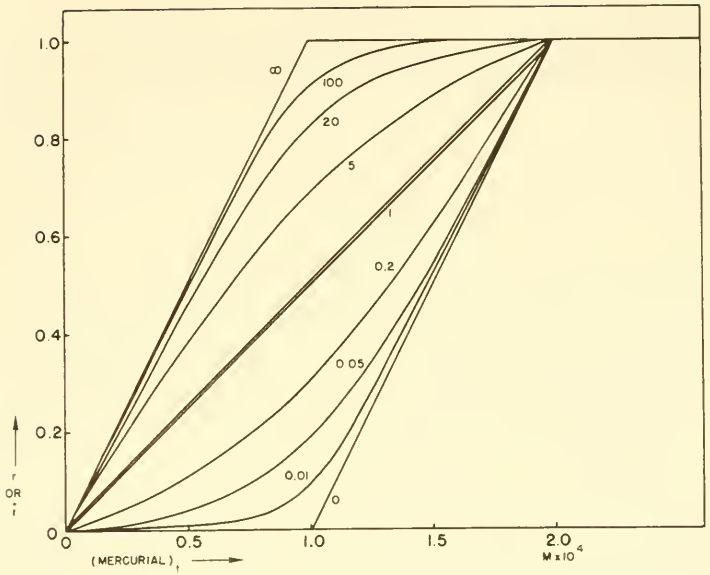


FIG. 7-20. Theoretical curves showing the relationship between reaction of enzyme SH groups and inhibition of the enzyme activity for an enzyme containing two SH groups, only one of which is involved in the catalytic activity. $(SH_1)_t = (SH_2)_t = 0.01$ mM and K_2/K_1 varied as indicated by the numbers on the curves; r is the fraction of SH groups reacted and is represented by the straight line. It is assumed that free mercurial is zero.

Type B

Catalase — beef liver (Schütte and Nürnberger, 1959)
Enolase — rabbit muscle (Holt and Wold, 1961)
Hexokinase — yeast (Fasella and Hammes, 1963)

Type C

ATPase — moysin (Singer and Barron, 1944; Bárány, 1959; Gilmour and Gellert, 1961)
Alcohol dehydrogenase — yeast (Barron and Levine, 1952)
Aldolase — rabbit muscle (Swenson and Boyer, 1957; Szabolcsi and Biszku, 1961)
β-Amylase — barley (Rowe and Weill, 1962)
Malate dehydrogenase — pig heart (Thorne and Kaplan, 1963)
Phosphorylase — rabbit muscle (Madsen and Cori, 1955, 1956)
Rhodanese — beef liver (Sörbo, 1963)
Urease — jack bean (Hellerman *et al.*, 1943)
Xanthine oxidase — milk (Gilbert, 1963)

Type D

Aldehyde oxidase — rabbit liver (Rajagopalan *et al.*, 1962)

Carbamyl-P synthetase — frog liver (Marshall *et al.*, 1961)
Cytochrome c reductase — calf liver (P. Strittmatter, 1959)
Malate dehydrogenase — beef heart (mitochondria) (Siegel and Englard, 1962).

Type E

Lactate dehydrogenase — pig muscle (Jécsai and Elödi, 1963)
Phosphoglucomutase — rabbit muscle (Milstein, 1961)
Phosphorylase — potato (Lee, 1960 b).

No clear-cut example of type F has been reported, but presumably the inhibition of D-amino acid oxidase by *p*-MB, which is competitive with respect to the benzoate portion of the inhibitor, would fall into this category. Certain enzymes have been shown to have a single SH group at the active center and necessary for activity, namely, papain (Kimmel and Smith, 1957; Finkle and Smith, 1958; Sanner and Pihl, 1963), ficin (Liener, 1961), and glycerol-P dehydrogenase (van Eys *et al.*, 1959). They seem generally to belong to type A.

A few selected titration curves may further illustrate the relations between SH reaction and inhibition. The titration of 3-phosphoglyceraldehyde dehydrogenase with *p*-MPS has been discussed (Fig. 7-12) and is seen to follow type A behavior (deviating toward type C), although the release of NAD is not exactly parallel to the disappearance of SH groups. Yeast alcohol dehydrogenase contains 10–12 SH groups per molecule but some inhibition occurs when only one is reacted, although the curve (Fig. 7-21) shows the inhibition at first to lag behind; it is difficult to know if this is type C or D. ATPase presents a more complex situation (Fig. 7-22) since reaction of the first 4 SH groups seems to produce only some stimulation of the activity, reaction of the next 2 SH groups causing complete inhibition. Other properties of myosin, e.g., the ability to complex with actin and the viscosity response of actomyosin to ATP, are more directly dependent on the reaction of the first SH groups. ITPase activity conforms more to type A behavior. The relationship of ATPase activity to SH reaction depends on the state of the enzyme (Fig. 7-23), no initial stimulation being observed when EDTA is the activator instead of Ca^{++}. The titration of muscle phosphorylase a gives partial type C behavior, but over most of the range there is a linear relationship between SH reaction and inhibition (Fig. 7-24). Since the inhibition may be completely reversed by cysteine, it is unlikely that a secondary inactivation is involved. Microsomal cytochrome c reductase demonstrates typical type D behavior, one SH group being closely related to the enzyme activity, as shown by the extrapolation of the inhibition curve to complete inhibition (Fig. 7-25), from which it may be estimated that K_2/K_1 is around 25–50.

Accurate, reliable, and directly interpretable titrations of enzymes are not easy to perform in some cases. Some of the possible difficulties which may arise will be summarized. (1) There is a failure to reach equilibrium,

i.e., reaction of the mercurial is not complete at the time chosen for the readings. Some enzyme SH groups react almost instantaneously with mercurials and others require 30–60 min at least (see page 809). Kinetic studies should always accompany any titration; to decide arbitrarily that n minutes of incubation with the mercurial at each concentration is adequate is not a satisfactory procedure. (2) The enzyme may be altered structurally by the mercaptide formation so that new SH groups are progressively ex-

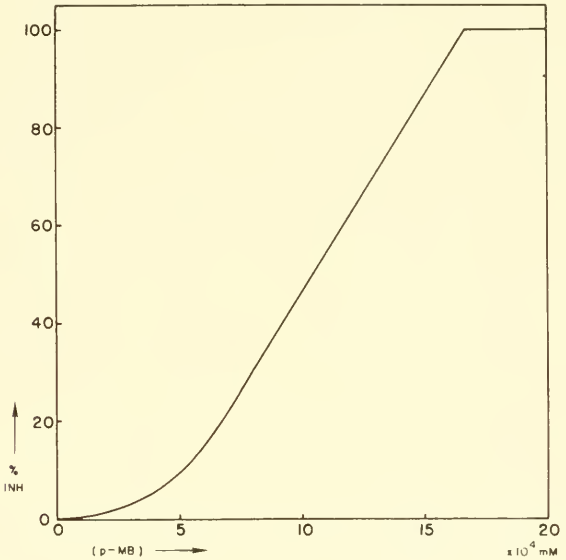


FIG. 7-21. Titration of yeast alcohol dehydrogenase with p-MB, showing the nonlinearity at low concentrations of the mercurial. (From Barron and Levine, 1952.)

posed, in which case there is no clear end-point and the results do not correspond to the original native enzyme. Sometimes the stability of the enzyme can be increased by creating a more physiological environment. (3) The enzyme SH groups may be oxidized during the titration, reducing the number of titratable SH groups. Use of oxygen-free solutions and a nitrogen atmosphere often eliminates this problem. (4) The mercurial may react with other groups or other components of the enzyme system, e.g., in the spectrophotometric titration with p-MB or p-MPS, causing absorption changes unrelated to SH groups. (5) The mercurial may do something that secondarily alters the ultraviolet absorption, e.g., split off a coenzyme, as demonstrated for NADH: lipoamide oxidoreductase (Palmer and Massey, 1962). (6) The presence of substances, especially buffers, reacting with the

mercurial may alter the rate and extent of reaction with the SH groups. The titrations of yeast alcohol dehydrogenase by *p*-MB in phosphate and in THAM buffers at pH 7.5 are quite different (Hoch and Vallee, 1960). It is probably advisable to reduce the buffer concentration as far as possible. (7) The number of reactive SH groups on an enzyme and the titration of these groups vary with several experimental conditions, such as pH, temperature, and absence or presence of substrate, and the question often arises

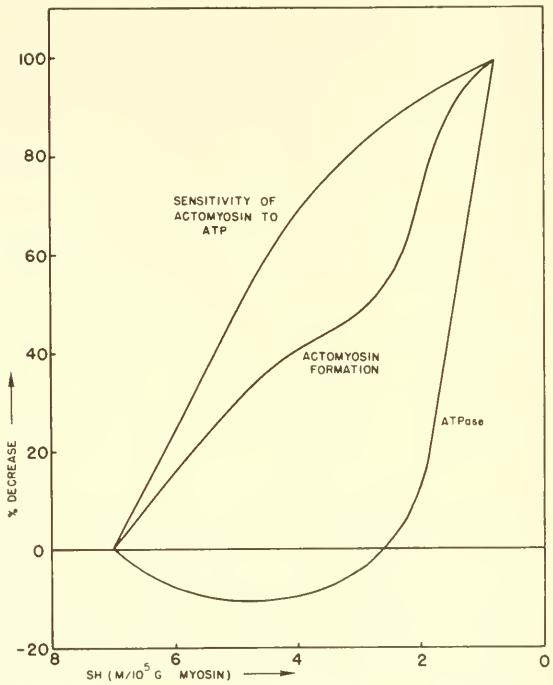


FIG. 7-22. Titration of myosin with mersalyl, showing the effects on ATPase activity, the ability to form actomyosin, and the sensitivity of the actomyosin to ATP (measured by viscosity changes). (From Bárány, 1959.)

as to what conditions are optimal. Titrations are often done at unphysiological pH's because reaction is faster or more complete, but it must be remembered that the results do not necessarily apply to the enzyme under normal conditions. Boyer and Segal (1954) showed definite difference in the titration of 3-phosphoglyceraldehyde dehydrogenase spectrophotometrically at pH 4.6 and 7, and this is probably a general phenomenon. The effect of temperature is well illustrated by the study of yeast hexokinase,

the SH groups at the active center becoming unavailable for reaction below 30° (Barnard and Ramel, 1962). The presence of substrate may either facilitate reaction of SH groups — as with xanthine oxidase (Fridovich and Handler, 1958) and myosin ATPase (Gilmour and Gellert, 1961) — or protect certain SH groups. The question as to which pH, temperature, and medium should be used, or whether substrate or coenzyme should be present during the incubation, can only be answered generally by stating that

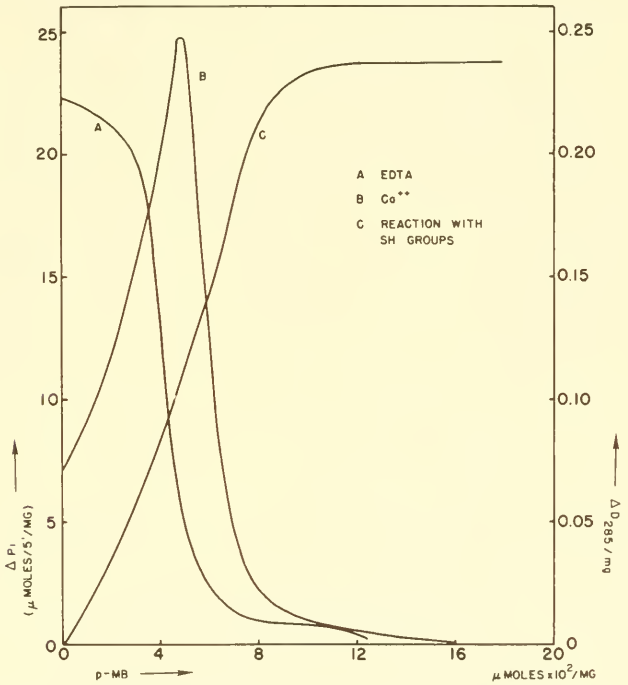


FIG. 7-23. Titration of myosin ATPase with *p*-MB, showing the different responses of the EDTA-treated and Ca^{++}-activated activity. (From Kiellye and Bradley, 1956.)

whenever possible one should strive for physiological conditions. It is necessary, of course, to vary these factors in many instances in order to study the behavior of the SH groups, but the variation should be from a standard set of conditions designed to provide information relevant to the enzyme in a normal state.

It is frequently difficult to determine with certainty the total number of free SH groups in a native enzyme under standard conditions and especially to relate certain SH groups to the catalytic activity. Thorne and Kaplan (1963) titrated pig heart malate dehydrogenase with *p*-MB, allowing 1 hr

for reaction at 25°, and could obtain no reliable end-point. As the molar ratio of I : E is increased there is no marked effect on the activity, except for a slight stimulation, until after a value of 5 is exceeded, and then there is a progressive loss of activity as the ratio is elevated, nearly complete inhibition occurring at a value of 21.6. It is impossible to interpret these data in terms of relating SH groups to activity. Indeed, it is likely that the enzyme is structurally altered so that SH groups normally not accessible are secondarily unmasked, since good titrations can be determined with the urea-

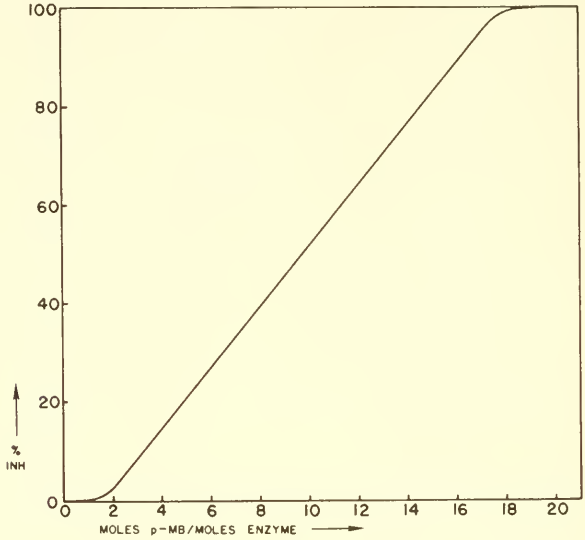


FIG. 7-24. Titration of muscle phosphorylase a with *p*-MB. (From Madsen and Cori, 1955.)

denatured enzyme. The aspartate: α-ketoglutarate transaminase from pig heart contains a total of 7 SH groups; when 2 moles of *p*-MB per mole of enzyme are added, this reacting with 1–2 SH groups, the activity is reduced by 50% (Turano *et al.*, 1963). Such data again are uninterpretable and it is impossible to conclude that SH groups are related in any way to the catalysis. Di Sabato and Kaplan (1963) titrated the lactate dehydrogenases from a variety of sources with both Hg^{++} and *p*-MB. The total number of SH groups per mole of enzyme varied from 17 to 27 but generally inactivation occurred when 4 moles of mercurial were bound for each mole of enzyme. It is likely that no major configurational changes occur because no alterations of fluorescence, sedimentation constant, or rotatory dispersion and no immunological changes could be detected, and furthermore cysteine could essentially completely reverse the inhibition. They felt that certain SH groups are part of the active site rather than being vicinal, since statis-

tically it is unlikely that in all the dehydrogenases such a distribution would occur. On the other hand, Jécsai and Elödi (1963) claimed that pig muscle lactate dehydrogenase in the native state does not react with *p*-MB, but that at pH 10 the blocking of 20 SH groups leads to 50% inactivation. They concluded that in this particular enzyme the SH groups are not at all involved in the catalysis. These examples only illustrate some of the problems which arise in enzyme titrations and emphasize that a program for relating SH groups to enzyme activity cannot be undertaken lightly.

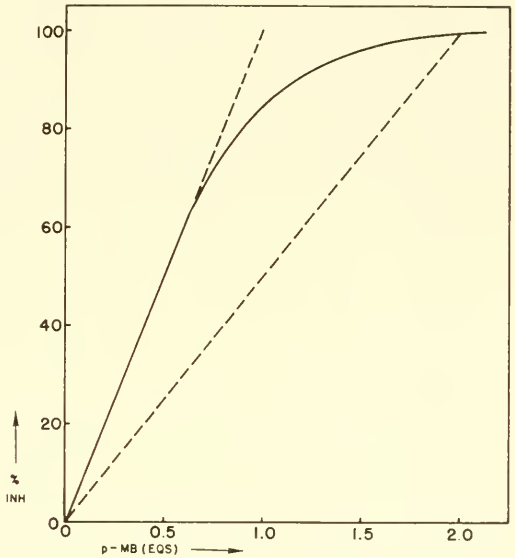


FIG. 7-25. Titration of microsomal cytochrome c reductase with *p*-MB. The solid curve gives the development of the inhibition as equivalents of mercurial are added. The dashed line continuing from the initial linear portion of the curve shows that one SH group is required for activity; the other dashed curve shows the assumed reaction with SH groups (it was not experimentally determined). It may be estimated that K_2/K_1 is near 25–50 if there are two SH groups. (From P. Strittmatter, 1959.)

Kinetics of Mercaptide Formation and Development of Inhibition

Apparently SH groups range in reactivity all the way from those which combine with mercurials so rapidly that the rates are difficult to measure, to those which are completely blocked and do not react at all. It is thus

not surprising that one finds a great deal of variation in the rates at which enzyme SH groups react and at which inhibition occurs. In some cases the inhibition has been said to appear instantaneously, or to reach full magnitude within 1–2 min; such is the inhibition of succinate dehydrogenase (Fig. I-12-12) (Slater, 1949), bromelain (Murachi and Neurath, 1960), pyruvate decarboxylase (Stoppani *et al.*, 1953), and leucine decarboxylase (Sutton and King, 1962). Then there are enzymes which require about 5 min for maximal inhibition to develop; examples are catalase (Cook *et al.*, 1946), transaminases (Grein and Pfleiderer, 1958; Segal *et al.*, 1962) and phosphoglucomutase (Milstein, 1961), although in the last instance only 2 of the 3 SH groups react so rapidly. It is interesting to note that Nygaard (1955) has reported marked differences in rates between the mercurials, lactate dehydrogenase being very rapidly inhibited by Hg^{++} but only slowly by *p*-MB. The next group of enzymes seems to require about 15–20 min for complete inhibition: 3-phosphoglyceraldehyde dehydrogenase (Boyer and Segal, 1954) and enolase (Malmström, 1962) may be cited. These are, of course, arbitrary categories and if one knew the rates of reaction for many

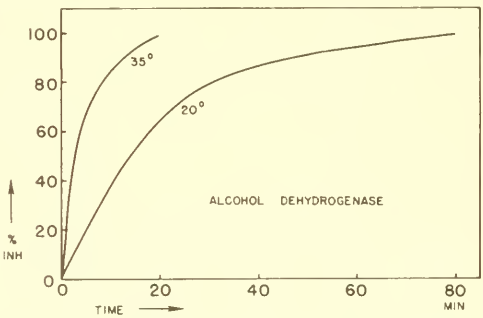


FIG. 7-26. Rates of inhibition of liver alcohol dehydrogenase by *p*-MB, at pH 7.6 and two different temperatures. ADH = 1.78×10^{-8} *M*, *p*-MB = 10^{-6} *M*, and NAD = 3×10^{-4} *M*. (From Wallenfels *et al.*, 1959.)

enzymes, there would be a continuous distribution, and furthermore the rate in any particular case will depend on a number of factors, so that the values given above and below must be taken as applying only to the experimental conditions imposed on each enzyme.

More interesting are those enzymes which react slowly enough with mercurials for the kinetics to be investigated. Some typical curves for *p*-MB are given in Figs. 7-26 and 7-27, and similar rate curves have been previously presented for cholinesterase (Fig. I-12-8) and lactate dehydrogenase (Fig. I-12-11). The results in Figs. 7-26 and 7-27 have been exponentially plotted

in Fig. 7-28 to indicate more clearly the relative rates of inhibition (see Eq. I-12-14 and Figs. I-12-3 and I-12-9), the slopes being proportional to the bimolecular rate constants. One notes that most of the curves deviate from linearity, frequently at high inhibitions; this is probably an expression of the different relative reactivities of the SH groups on a single enzyme, some reacting initially at a rapid rate and others reacting more slowly. The rate constants have been calculated for some enzymes, e.g., 18.8 liters/mole/sec for glutamate decarboxylase (Shukuya and Schwert, 1960), 51 liters/mole/sec for muscle phosphorylase (Madsen and Cori, 1956), and 61.4 liters/mole/sec for heart lactate dehydrogenase (Takenaka and Schwert, 1956), in all cases *p*-MB being the inhibitor. The effects of temperature and mercurial concentration on the rates of inhibition are well illustrated for alcohol dehydrogenase (Fig. 7-26) and β-fructofuranosidase (Fig. 7-27), respectively. Glutamate decarboxylase presents an interesting phenomenon, in that exposure of the enzyme to low temperatures appears to liberate additional SH groups (Fig. 7-27).

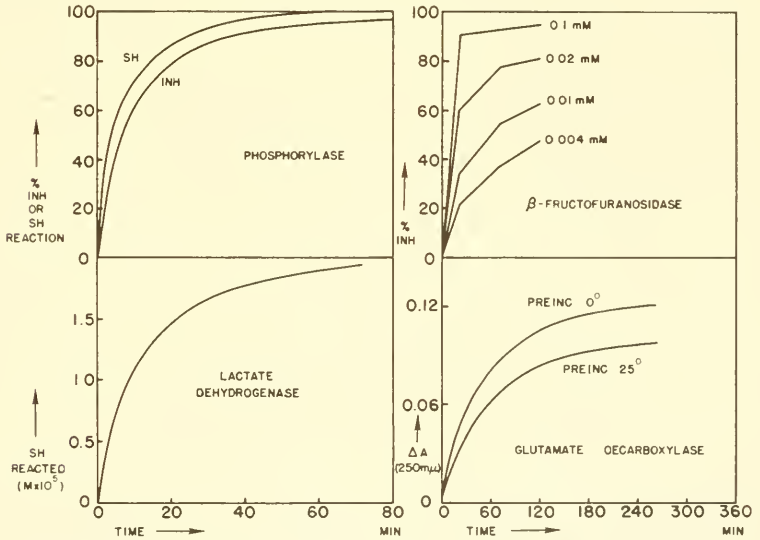


FIG. 7-27. Rate titrations of various enzymes with *p*-MB. The reaction of SH groups was determined by absorption changes at 250 mμ. Phosphorylase a from rabbit muscle: *p*-MB = 0.04 m*M*, pH 6.7, and 21° (Madsen and Cori, 1956.) Lactate dehydrogenase from heart: *p*-MB = 0.00448 *M*m, pH 6.8, and 25° (Takenaka and Schwert, 1956.) β-Fructofuranosidase from *Neurospora*: *p*-MB concentrations given in the graph, pH 6.8, and 0° (Metzenberg, 1963). Glutamate decarboxylase from *E. coli*: preincubation with *p*-MB for 4 hr at either 0° or 25°, and reaction run at 25° and pH 6.5 (Shukuya and Schwert, 1960).

Progressive inhibition or inactivation of enzymes by mercurials is very common and takes a variety of forms. Epididymal α-mannosidase is inhibited 62% by 0.01 mM Hg^{++} without preincubation with the inhibitor; the inhibition is 67% at 30 min, 77% at 60 min, and 88% at 120 min (Conchie and Hay, 1959). This is one of the numerous examples in which an enzyme is rapidly inhibited to a certain level, further increase in inhibi-

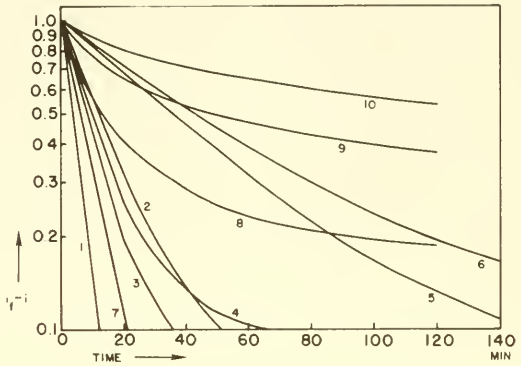


FIG. 7-28. Logarithmic plots of $i_f - i$ for the enzymes in Figs. 7-26 and 7-27, either loss of activity or reaction of SH groups being used as a measure of the reaction with p-MB. These curves have been estimated from the published curves and hence are not strictly accurate, but indicate the relative rates for the more slowly reacting SH groups. 1, Alcohol dehydrogenase (35°); 2, Alcohol dehydrogenase (20°); 3, phosphorylase; 4, lactate dehydrogenase; 5, glutamate decarboxylase (25°); 6, glutamate decarboxylase (0°); 7, β-fructofuranosidase (0.1 mM); 8, β-fructofuranosidase (0.02 mM); 9, β-fructofuranosidase (0.01 mM); 10, β-fructofuranosidase (0.04 mM.)

tion being slow. Such behavior is not surprising when one measures SH reaction, since one assumes generally the occurrence of SH groups of different reactivities; thus when 3-phosphoglyceraldehyde dehydrogenase is titrated with p-MB, 11 SH groups react immediately, but 3 more require at least 40 min (Koeppe *et al.*, 1956), and aldolase behaves very similarly, 7 SH groups being blocked rapidly and 3–4 more groups taking 40 min for reaction (Szabolcsi and Biszku, 1961). But the interpretation of inhibition following such a time course is not so clear. If a certain level of inhibition is reached rapidly and then the rate falls off markedly, one must assume that the enzyme is not completely inhibited when its rapidly reacting SH

groups are combined with mercurial (assuming that there is sufficient mercurial to react with all these groups). The more slowly developing inhibition could be due to reaction of less readily available SH groups or to a secondary inactivation following the initial mercaptide formation. Quite different results are obtained with amylase, mersalyl at 1 m*M* not inhibiting at all during the first hour, but slowly inhibiting until there is 40% depression after 48 hr (Muus *et al.*, 1956), or with bromelain, *p*-MB inhibiting only 25% after 4 hr and 80% after 20 hr at 0.1 m*M* (Ota *et al.*, 1961). Many different time courses of inhibition are observed and it is likely that the major factors involved are (1) the relative reactivities of the SH groups, (2) the relationship between the SH groups and the catalytic activity, and (3) the tendency for structural changes leading to inactivation to occur. However, it is quite clear that many enzymes react quite slowly with mercurials and require 2–4 hr (and occasionally more) to complete the process. Such enzymes are difficult to titrate, since one does not know how many of the SH groups finally reacted were originally present, and, when one is adding increasing amounts of mercurial to correlate mercaptide formation and inhibition, it is not easy to decide on the optimal preincubation interval.

In the previous section the correlation between inhibition and SH reaction by mercurials was considered in terms of variable quantities of mercurial. Another approach to relate these phenomena is to determine their changes with time at a particular mercurial concentration. If the SH groups which are combined initially are necessary for enzyme activity, one would expect inhibition to parallel blocking of these groups; if the most readily reacting SH groups are not related to activity, or the enzyme undergoes progressive inactivation, the inhibition may lag behind mercaptide formation. Madsen and Cori (1956) observed that inhibition of phosphorylase by *p*-MB developed more slowly than the change in absorbance at 250 mμ (Fig. 7-27), so that when 50% of the reactive SH groups had been blocked the inhibition was only 14%. If the SH blocking itself is not responsible directly for the inhibition, but initiates an unfolding of the enzyme, the rate of inhibition may be more dependent on the rate of configurational change. In the case of phosphorylase, we have seen that splitting into subunits occurs during reaction with *p*-MB, so the rate at which this occurs may have something to do with the inhibition rate. Inasmuch as cysteine reverses the inhibition completely, marked structural alterations would not be very likely.

Another phenomenon which must be taken into account in kinetic studies is the spontaneous recovery of enzyme activity in the presence of the mercurial, first observed, I believe, by von Euler and Svanberg (1920) in studies of the inhibition of yeast β-fructofuranosidase by Hg^{++}. Reisberg (1954) reported that the inhibition of choline acetylase by *p*-MB is less at 30 min

than at 10 min. Other more recently observed examples of this include epididymal β-galactosidase with low concentrations (0.0002 mM) of Hg^{++} (Conchie and Hay, 1959), xanthine oxidase with 0.44 mM p-MB, which inhibits NADH oxidation 50% initially but less and less as the reaction proceeds (Westerfeld *et al.*, 1959), and leucine decarboxylase with 0.005 mM p-MB (Sutton and King, 1962). The most marked spontaneous recovery is seen with pig heart lactate dehydrogenase, the rate and degree of reactivation being dependent on the molar ratio of p-MB to enzyme (Fig. 7-29)

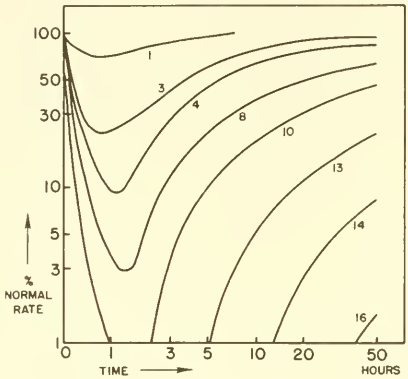


FIG. 7-29. Effects of p-MB on pig heart lactate dehydrogenase, showing the initial inhibition and the spontaneous reactivation. The numbers on the curves are the molar ratios of p-MB to LDH. (From Gruber *et al.*, 1962.)

(Gruber *et al.*, 1962). The most common explanation for such recovery is a slow migration of the mercurial from those groups initially attacked to other groups not involved in the enzyme activity. The rates at which various SH groups react with a mercurial are not necessarily related to the affinities of the groups for the mercurial. Groups which bind the mercurial very tightly may be masked and react very slowly, as fairly conclusively demonstrated for myosin ATPase by Gilmour and Gellert (1961). Another factor which may be of importance when the inhibition decreases during the period when the enzyme activity is measured, as was the case with leucine decarboxylase, is the displacement of the mercurial by the substrate. Leucine was shown to protect the enzyme against p-MB and it could eventually overcome the inhibition somewhat, especially since its concentration was some 1000 times greater than the mercurial. A substrate might also be able to restore toward a normal configuration a slightly luxated active center, substrates being known to stabilize the active forms of certain enzymes.

This phenomenon of spontaneous recovery must be even more common in cellular preparations than with pure enzymes, because there is much greater opportunity for redistribution of the mercurial.

Stimulation of Enzymes by Mercurials

Mercurials is common with other heavy metals and SH reagents frequently increase enzyme activity, especially at low concentration, the action-concentration curves being biphasic. Polis and Meyerhof (1947) first observed the stimulation of Ca^{++}-activated myosin ATPase by PM, a 30–40% elevation of the rate occurring with concentrations between 0.005 and 0.12 m*M*, and this has been confirmed in several more recent reports, the degree of stimulation, however, varying greatly with the experimental conditions. Many different types of enzyme exhibit this phenomenon (Table 7-11) but the mechanisms involved have only rarely been clarified. Let us briefly consider some possible mechanisms and what relevant evidence is available.

(A) *The mercurial inactivates a naturally occurring inhibitor.* If an inhibitor is isolated with the enzyme and is suppressing the activity, and if this inhibitor is an SH protein (as many natural inhibitors seem to be), a mercurial by reacting preferentially with the inhibitor may release the enzyme from its inhibition. J. S. Roth (1953 a, 1956, 1958) has been a proponent of this theory with respect to the activation of rat liver homogenate ribonuclease by *p*-MB or PM, and has found a natural inhibitor with which the mercurials react at concentrations having no direct effect on ribonuclease. The degree of stimulation varies with the tissue from which the ribonuclease is obtained — all the way from 0% with pancreas, 57% with brain, 104% with muscle, 284% with liver, to 1500% with ascites carcinoma — and this may be due to the different amounts of inhibitor present (Ellem and Colter, 1961). Indirect evidence often points to such a mechanism for other enzymes. Phillips and Langdon (1962) found that *p*-MB stimulates microsomal NADPH:cytochrome c reductase but only inhibits the purified enzyme. Of course, the activation could also be due to some effect of the mercurial on the microsomal structure. It has also been noted occasionally that stimulation occurs, and is relatively constant, over a wide range of mercurial concentration, inhibition appearing rather suddenly when this range has been exceeded, and this indicates some component with which the mercurial reacts readily and completely.

(B) *The mercurial reacts with the substrate to labilize it.* Ledoux (1953) initially attempted to explain the stimulation of ribonuclease by *p*-MB as due to a reaction with RNA, this favoring in some manner the enzymic hydrolysis, and detected spectral changes upon mixing RNA and *p*-MB (see page 741). Although mercurials do complex with nucleic acids, it is doubtful if this is a major factor in the activation. The proper preincubation

TABLE 7-11

EXAMPLES OF ENZYME STIMULATION BY MERCURIALS

Enzyme	Source	Mercurial	Concentration (mM)	% Stimulation[a]	Reference
Adenosinetriphosphatase	Rat liver mitochondria	*p*-MB	0.1	130	Myers and Slater (1957 b)
	Myofibrillar	PM	1.25	115	Chappell and Perry (1955)
	Myosin (with Ca^{++})	*p*-MB	0.05 μmole/mg	250	Kielley and Bradley (1956)
			0.04 μmole/mg	54	Tonomura and Kitagawa (1957)
			0.05 μmole/mg	590	Blum (1960)
			0.0007	25	Gilmour and Griffiths (1957)
Amine oxidase	Rat liver	Hg^{++}	0.0005	15	Lagnado and Sourkes (1956)
			0.005	42	
Amino acid-activating enzyme	Rabbit liver	*p*-MB	1	13	Nohara and Ogata (1959)
Arylsulfatase	*Charonia lampas* liver	*p*-MB	0.5	15	Takahashi (1960 a)
Arylsulfatase b	Beef cornea	*p*-MB	0.2	20	Wortman (1962)
Aspartate trans-carbamylase	*E. coli*	*p*-MB	0.1	50	Gerhart and Pardee (1961)
C-esterase	Pig kidney	PM	0.4	100	Bergmann *et al.* (1957)
Dihydrofolate reductase	Chicken liver	*p*-MB	0.08	300	Kaufman (1964)
Esterase	Pig liver microsomes	*p*-MB	0.1	22	Krisch (1963)
Glucosamine-6-P deaminase	Human brain	Hg^{++}	0.5	40	Pattabiraman and Bachhawat (1961)
Glutamate dehydrogenase	Calf liver	MM	0.006	132	Hellerman *et al.* (1958)
	Chicken liver	MM	0.008	422	
	Beef liver	PM	0.5 μmole/mg	150	Mildvan and Greville (1962)
		MM	0.068 μmole/mg	400	Rogers *et al.* (1962)
		PM	0.49 μmole/mg	300	

Glycerate dehydrogenase	Beef liver	*p*-MB	0.00003	250	Heinz *et al.* (1962)
Glycerate-2,3-diphosphatase	Rabbit erythrocytes	Hg^{++}	0.1–0.4	230	Rapoport and Luebering (1951)
	Rabbit muscle	Hg^{++}	0.08	80	Rapoport *et al.* (1955)
	Rabbit muscle	Hg^{++}	0.1	633	Hashimoto *et al.* (1960)
Glycerate-2-phosphatase	Rabbit erythrocytes	Hg^{++}	0.1	590	Rapoport and Luebering (1951)
Glycerate-3-phosphatase	Rabbit erythrocytes	Hg^{++}	0.1	3800	Rapoport and Luebering (1951)
NADH : quinone oxidoreductase	Yeast	*p*-MB	0.023	100	Misaka and Nakanishi (1963)
NADPH : cytochrome c oxidoreductase	Pig liver	*p*-MB	0.0008	8	Williams and Kamin (1962)
	Rat liver	*p*-MB	0.16	50	Phillips and Langdon (1962)
Nucleotide incorporation enzyme	Rabbit muscle	*p*-MB	0.1	75	Anthony *et al.* (1963)
Phosphatase (acid)	*Lupinus albus*	*p*-MB	0.037	115	Newmark and Wenger (1960)
Phosphatase (alkaline)	*E. coli*	*p*-MB	15	20	Heppel *et al.* (1962)
3-Phosphoglyceraldehyde dehydrogenase	Pig muscle	*p*-MB	I/E = 2	10–20	Szabolcsi *et al.* (1960)
Phosphorylphosphatase	Pig kidney	Hg^{++}	0.004	30	Augustinsson and Heimbürger (1955)
Pyridoxol dehydrogenase	Yeast	*p*-MPS	0.01	25	Holzer and Schneider (1961)
Ribonuclease	Ehrlich ascites tumor	*p*-MB	0.4	535	Colter *et al.* (1961)
Ribonuclease T2	Takadiastase	*p*-MB	0.1	25	Naoi-Tada *et al.* (1959)
Succinate oxidase	Oyster eggs	PM	0.01	10	Cleland (1949)
Tryptophan peroxidase	Rat liver	*p*-MB	0.5	100	Koike and Okui (1964)
UDP-glucuronyltransferase	Mouse liver	*p*-MB	0.02	20	Storey (1964)

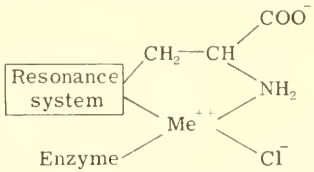
[a] In most cases the degree of stimulation varies with several factors and the values given are for maximal effect, although in many cases the stimulation would probably have been greater if the conditions had been varied.

procedures might easily solve this problem. There is no other instance where this mechanism has been postulated, but it is a possibility that must be borne in mind.

(C) *The mercurial reacts with a SH group to create a more favorable electric field at the active center.* Since the binding of the substrate and the breakdown of the ES complex are influenced by the electric field arising from charged groups vicinal to the active center, it is possible that the binding of a charged mercurial could so alter this field as to facilitate the catalysis. There is no evidence for this and it would be difficult to obtain.

(D) *The mercurial increases the active form of the enzyme.* Certain enzymes within the cell or as extracted may be in an inactive form, possibly with the active center not in the proper configuration. Reaction with a mercurial might so alter the protein structure as to release the activity, much as some enzymes can be activated by heat. This would probably apply only to true activations, i.e., where the initial state of the enzyme is inactive, as in the work of Hilz and Klempien (1959) on ascites tumor ribonuclease, Hg^{++} at 0.005 and 0.05 m*M* increasing the enzyme rate from 0 to 12 and 24 μmoles TCA-soluble phosphate/hr, respectively, but here it may be only that the enzyme is completely inactivated by a natural inhibitor.

(E) *The Hg^{++} ion may replace a normal metal ion activator.* Carboxypeptidase which is normally activated by Zn^{++} can be activated with respect to the esterase activity with Hg^{++} (Coleman and Vallee, 1961). This situation is probably very uncommon. Rapoport *et al.* (1955) reported that Hg^{++} stimulates glycerate-2,3-diphosphatase only in the presence of certain organic nitrogen substances, and the activation was assumed to be due to the complex formed. Substances which are necessary for the Hg^{++} effect include nonenzyme proteins and certain amino acids, of which histidine is the most effective (Sauer and Rapoport, 1959). It was concluded that in addition to an SH group, activity requires the presence of some metal ion and some complexer bound in a cylic resonance system:



with the enzyme. In this case, Hg^{++} would simply function as a metal ion for linking the resonating system to the enzyme.

(F) *The mercurial may disrupt water structure.* The structure of water around the active center may be such as to retard somewhat the access of

the substrate. Reaction of a mercurial with a vicinal SH group could by introducing a new side chain break down this water structure.

(G) *The mercurial reduces the binding of an inhibitory product.* L-Glutamate dehydrogenase is stimulated by PM at pH 8.5 and this is reduced by substrate, NAD, and glutarate, a competitive inhibitor (Greville and Mildvan, 1962). Thus PM must combine at or near the active center. The product of the reaction, α-ketoglutarate, is inhibitory. PM increases the K_i for α-ketoglutarate 8-fold and for glutarate more than 20-fold. Part of the stimulation by PM can be due to reduction of the effects of α-ketoglutarate. It would seem that such behavior would be reflected in the rate curves, little stimulation being expected initially.

(H) *The mercurial dissociates the enzyme into active subunits.* Some of the active centers may be more accessible when the enzyme is disaggregated, and it is known that mercurials can sometimes split enzymes into subunits (page 788). Greville and Mildvan (1962) observed that PM dissociates glutamate dehydrogenase, and Rogers *et al.* (1962) also noted effects on the sedimentation properties. The possibility of such dissociation playing a role in the mercurial activation was studied by Rogers *et al.* (1963), who found no change in molecular weight upon treatment with MM when the enzyme is in high concentration. However, when low enzyme concentrations were used, a disaggregation sensitive to the mercurial was detected, but it is not certain if this is related directly to the stimulation.

(I) *The mercurial reacts primarily with an inhibitory SH groups.* The stimulation of ATPase by mercurials has generally been explained since the report of Kielley and Bradley (1956) in terms of differently located SH groups around the active center. An SH group, for example, might bind a group on ATP and interfere with the optimal orientation on the enzyme. This SH group has been postulated to react with the 6-amino group of ATP; when mercaptide formation occurs, this discouraging action on ATP is abolished (Gilmour, 1960; Greville and Tapley, 1960). In essence, the mercurial prevents the excessive occupancy of the active center by disoriented ATP, allowing ATP to proceed directly to hydrolysis. A somewhat different view has been voiced by Blum (1960): ATP induces a configurational change in the active center, this involving the SH groups, and mercurials at low concentrations tend to prevent this change. ITP does not so alter the structure and its hydrolysis is inhibited only by mercurials. Mercurials would thus maintain the active center in the configuration binding ITP, a state conducive to rapid hydrolysis of ATP.

(J) *The mercurial inhibits a second enzyme which suppresses the reaction rate.* A number of possibilities for stimulation were discussed in Chapter I-7. In a monolinear chain:

$$A \xrightarrow{E_1} B \xrightarrow{E_2} C$$

inhibition of the E_2 will increase the steady-state level of B or its rate of formation. If the formation of B is being measured and an enzyme destroying it is present, inhibition of this enzyme will appear to stimulate E_1. Likewise in a divergent chain, inhibition of one branch may increase the rate of the other branch; whether stimulation will be observed will depend on what is measured. In the incorporation of nucleotides into amino acid transfer RNA, the presence of any enzyme attacking the nucleotides will reduce the incorporation, and the inhibition of such an enzyme will appear to stimulate the incorporation. Stimulation was indeed observed with *p*-MB by Starr and Goldthwait (1963), who thought that this might be due to a contaminating phosphodiesterase, but Anthony *et al.* (1963) obtained evidence that such an enzyme did not occur at significant levels in their preparation.

The stimulation of many enzymes by mercurials is strongly pH-dependent. The activating effect of Hg^{++} on glycerate-2,3-diphosphatase becomes progressively less as the pH is increased beyond 7 (Rapoport *et al.*, 1955), and this is true also for ascites cell ribonuclease, although here the activation disappears as the pH is decreased to 5 (Colter *et al.*, 1961). In the latter case, a 50% stimulation changes to a 42% inhibition as the pH rises from 8 to 8.5. Liver mitochondrial ATPase stimulation by *p*-MB is optimal around a pH of 9 and falls off rapidly on both sides (Myers and Slater, 1957 b), and the optimal pH for activation of myosin ATPase is 7.8, little stimulation being observed at pH 5.7 or 10 (Tonomura and Furuya, 1960). Such pH effects may be important in working out the mechanisms of the stimulation but have not so far been studied in enough detail to contribute evidence. Temperature can apparently also play a role, since *p*-MB stimulates myosin ATPase at 25° but only inhibits at 0° (Fig. 7-30) (Gilmour and Griffiths, 1957). It is also evident in this figure that the DNP-activated ATPase is not further stimulated by *p*-MB. The EDTA-activated ATPase is likewise not stimulated by *p*-MB, whereas in the presence of Ca^{++} the stimulation is marked (Fig. 7-23). Finally, the effect of mercurial concentration is occasionally very striking, as in the case of myosin ATPase (Fig. 7-23), maximal stimulation by *p*-MB being observed when about half the SH groups are reacted. Another example of variation of stimulation with mercurial concentration is shown in Fig. III-1-1 for ribonuclease, although here the form of the curve may depend primarily on the nature and amount of the natural inhibitor, as well as the susceptibility of the enzyme itself.

The problem of enzyme stimulation by mercurials, or other inhibitors, is a very interesting one, and probably quite important in understanding not only the mechanisms whereby the mercurials can affect enzymes but some of the many instances of stimulation of metabolism, which will be mentioned later in this chapter.

Reversal of Mercurial Inhibition

The general treatment of the reversal of enzyme inhibition by substances binding the inhibitor (page I-615) will now be extended to those situations often encountered in studies of the mercurials. The most common reversors are thiols, e.g., cysteine, glutathione, mercaptoethanol, mercaptoacetate, and dimercaprol (BAL), and the reduction in the inhibition can be considered as a transfer of the mercurial from enzyme SH groups to the reversor (R) SH groups. In many reactivation experiments the concentration of free mercurial is very small and the reversal reaction can be written as:

$$\begin{array}{ccccccc} \mathrm{EI} & + & \mathrm{R} & \leftrightharpoons & \mathrm{E} & + & \mathrm{RI} \\ (\mathrm{E}_t) - x & & (\mathrm{R}_t) - x & & x & & x \end{array} \tag{7-13}$$

If the enzyme is initially completely inhibited, $(\mathrm{EI}) = (\mathrm{E}_t)$, and the final equilibrium concentrations are as written under the equation. The equilibrium is characterized by the following constants:

$$\frac{(\mathrm{EI})\,(\mathrm{R})}{(\mathrm{E})\,(\mathrm{RI})} = \frac{[(\mathrm{E}_t) - x]\,[(\mathrm{R}_t) - x]}{x^2} = K = \frac{K_r}{K_i} \tag{7-14}$$

$$\frac{(\mathrm{E})\,(\mathrm{I})}{(\mathrm{EI})} = K_i\,; \qquad \frac{(\mathrm{R})\,(\mathrm{I})}{(\mathrm{RI})} = K_r$$

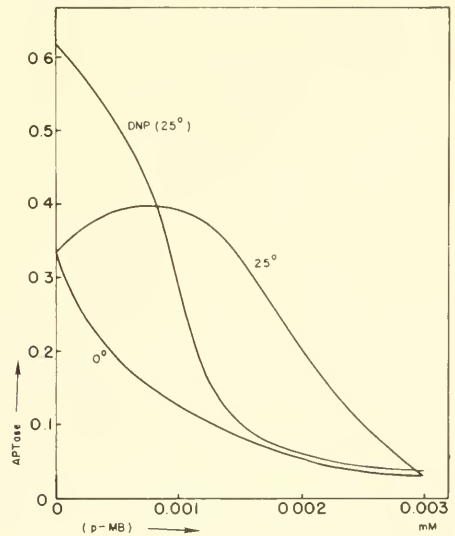


FIG. 7-30. Effects of *p*-MB on myosin ATPase at 0° and 25°, and in the presence of 2,4-dinitrophenol. The ATPase activity is given as μmoles P_i/mg/min. (From Gilmour and Griffiths, 1957.)

The value of x, the concentration of active enzyme regenerated, can be calculated from the quadratic equation:

$$x^2(1 - K) - x\,[(R_t) + (E_t)] + (E_t)\,(R_t) = 0 \tag{7-15}$$

if the total concentrations of enzyme and reactor are known. The value of the final inhibition is given simply by:

$$i_f = 1 - \frac{x}{(E_t)} \tag{7-16}$$

If both enzyme and reversor SH groups ionize similarly, we need not include the effect of (H^+), and since the mercurial is assumed to be bound to either the enzyme or the reversor, we need not consider (X^-), the concentration of mercurial-complexing ligands in the medium. The situation described here is that in which an enzyme is titrated to complete inhibition and the reversor then added. If any irreversible inactivation has occurred, the experimentally measured inhibition will be greater than in Eq. 7-16.

In most reactivation experiments, (R_t) is much greater than (E_t), and thus the reversor concentration is not reduced significantly by the binding of the mercurial. The equation for the determination of x is simplified to:

$$x^2K + x(R_t) - (E_t)\,(R_t) = 0 \tag{7-17}$$

It is interesting to specify the conditions required to reverse the inhibition to a determined value i_f. Substitution of $(E_t)\,(1 - i_f)$ for x in the solution to the quadratic equation leads to

$$\frac{K_r}{K_i} = \frac{i_f}{(1 - i_f)^2}\,\frac{(R_t)}{(E_t)} \tag{7-18}$$

$$\frac{(R_t)}{(E_t)} = \frac{(1 - i_f)^2}{i_f}\,\frac{K_r}{K_i} \tag{7-19}$$

Equation 7-18 gives the ratio of the dissociation constants of the two mercaptides so that the inhibition will be reduced to i_f when the enzyme and reversor concentrations are given, while Eq. 7-19 shows how much reversor must be present relative to the enzyme to achieve a reactivation to i_f when the dissociation constants are known. In many experiments the reversor is added at a concentration of 1–10 mM and thus $(R_t)/(E_t)$ is often 10^3 and 10^6, since enzyme concentrations commonly run from 10^{-6} to 10^{-2} mM. If we assume that $K_r = K_i$, which is a reasonable approximation in many cases, the values of $(R_t)/(E_t)$ shown in the following tabulation must be used to reduce the inhibition to the levels indicated. Thus one would expect that the concentrations of reversor often used would completely abolish the inhibition, so that if the enzyme activity is experimentally not restored

to normal values it is likely that (1) some inactivation has occurred, (2) insufficient time has been provided for the reversal, (3) the binding of the mercurial to the enzyme is much tighter than to the reversor ($K_r \gg K_i$),

Final % inhibition	$(R_t)/(E_t)$
50	0.5
20	3.2
10	8.1
5	18
1	98

or (4) some secondary factor has complicated the situation, e.g., failure to add a displaced cofactor (page I-625) or the oxidation of enzyme SH groups by the oxidized thiol reversor (page I-625). If the complete reversibility of the inhibition has been established, and if (E_t) and K_r are known, it is possible to calculate the value of K_i from experiments in which low concentrations of reversor are added and the i_f determined.

It is relatively easy to treat equilibrium conditions quantitatively, but the problems encountered in considering the rates of reversal are at the present mainly insoluble. Investigators have been chiefly interested in whether reversal occurs or not and since extremely few rate studies have been made, there are not adequate data upon which to base accurate analyses. If the reversal occurs in two steps — the dissociation of EI and the combination of I with R — the individual reactions are not as simple as with most inhibitions. For example, at physiological pH and with most media used, the dissociation of EI must be written as:

$$EI + H^+ + X^- \rightarrow EH + IX \tag{7-20}$$

where X^- represents any complexing anion in the medium. Likewise, the second step must usually be described by:

$$IX + RH \rightarrow RI + X^- + H^+ \tag{7-21}$$

Now it is not known if the reactions occur in this manner, or whether I can be directly transferred from E to R without forming the IX complex, either on the surface of the enzyme or in solution. A study of the effects of (X^-) on the reversal rate would help to solve this problem. It is also not known if the reactive form of the reversor is RH or R^-, but from the effects of pH on the rates of reaction of mercurials with simple thiols it appears that at least the form R^- reacts much more rapidly than RH, as would be anticipated. In any event, the pH must be an important factor

in the rates of reversal, although it may not markedly influence the equilibrium conditions.

Despite the lack of data on reversal rates, one obtains the impression that reversal is often a good deal faster than the development of inhibition. Unfortunately those workers who measured the rates of inhibition the most carefully usually did not examine the reversibility or, if they did, remarked only that it occurs, without giving data on the time required; and in other cases no reversal was observed. We are thus in the surprising position of having essentially no information in any one case of mercurial inhibition as to the relative rates of inhibition and reversal. Certainly in some instances the reversal is very rapid, as with 3-phosphoglyceraldehyde dehydrogenase (Velick, 1953) and hexokinase (Sols and Crane, 1954) treated with cysteine after inhibition by *p*-MB, as previously discussed (page I-623 and Fig. I-13-8). However, in these cases the rates of inhibition are not known, although they are probably quite fast; in both, the enzyme and *p*-MB were preincubated for an arbitrary time (for hexokinase 15 min at 0°). It would be difficult to compare the rates of reversal by dialysis and reversor — it seems never to have been done experimentally — but dialysis is not a very efficient method due to the fact that only a small fraction of the dissociated inhibitor at any time is able to pass out through the membrane, most recombining with enzyme, while the presence of a high concentration of reversor throughout the medium ensures that free inhibitor is rapidly bound. Therefore, one does not know in reversal which step is limiting, or if the reversor can facilitate the dissociation of the mercurial from the enzyme. It is impossible to predict the relative rates of inhibition and reversal theoretically because we do not know the exact reactions involved, particularly the influence of H^+ and complexing ions. There is only one way to solve these problems: to do a few critical and accurate experiments.

The information to be derived from simple reactivation experiments, especially those in which a high concentration of reversor is used, is not as much or as reliable as many seem to have believed (see page I-624). If complete reversibility is obtained, this shows that no serious inactivation of the enzyme has occurred; it does not imply that structural changes have not been induced by the mercurial. This information is usually important and must be obtained in any quantitative kinetic studies, but this is all that this type of reversal experiment will provide. Failure to recover the activity can be explained in a variety of ways, previously enumerated (page 651). More careful reversal studies, especially determination of rates, the effects of pH and ligand concentration, and the degree of reactivation with low reversor concentrations, would undoubtedly be more informative.

Several hundred studies on mercurial inhibition have included statements relative to reversibility with thiols. It would serve very little useful purpose to list these results. Summarizing all of them one finds that complete

reversal was obtained in 59%, partial reversal in 30%, and no reversal in 11%. This shows at least that in most cases the enzyme is not seriously altered or denatured by severe mercurial inhibition, providing the contact with the inhibitor is not prolonged. On the other hand, there are the enzymes such as papain which are more stable when complexed with Hg^{++}. Some of the instances where partial or no reversal was found are undoubtedly due to inactivation, but some to the other factors mentioned above. In a few studies, some interesting sidelights were noted and we shall content ourselves with discussing some of these.

Two more instances of very rapid, almost instantaneous reversal of mercurial inhibition may be noted. Yeast alcohol dehydrogenase is immediately inhibited by *p*-MB and likewise rapidly reactivated by glutathione (Snodgrass *et al.*, 1960). The enzyme at 4.5×10^{-9} *M* is completely inhibited by 2.5×10^{-7} *M* Hg^{++}; glutathione reverses this inhibition rapidly, but partially — after 30-sec exposure to Hg^{++} 54% and after 10-min exposure only 17%. This failure to reverse is not due to displaced Zn^{++}, since Zn^{++} was found not to be released from the enzyme so rapidly and the addition of Zn^{++} does not improve the reversal. Heart lactate dehydrogenase is very rapidly reactivated from *p*-MB inhibition by cysteine, and in this case the rate of inhibition is relatively slow, about 15 min being required for maximal inhibition (Neilands, 1954). This is one of the few instances in which reversal definitely seems to occur faster than inhibition. The effectiveness of reversors in comparison with other methods for reactivation is seen with succinate dehydrogenase. If the enzyme inhibited by *p*-MB is dialyzed for 3.5 hr there is no reactivation, but addition of glutathione reverses completely (Singer *et al.*, 1956 b). Also no reversal was observed following dilution of the inhibited enzyme, whereas thiols reactivate partially (Slater, 1949). The rate of reactivation of erythrocyte pyruvate kinase by 100 m*M* glutathione after 1-min contact of the enzyme with 0.1 m*M* *p*-MB is fairly slow (see accompanying tabulation); however, if contact with the inhibitor is 10 min,

Time (min)	% Reversal
10	18
15	47
25	59

only 28% reversal is seen after 25-min exposure to glutathione (Solvonuk and Collier, 1955). There is no obvious explanation why the reversal rates with some enzymes are so fast and in others so slow, especially as this is not correlated at all with the affinities of the enzymes for the mercurials.

Low concentrations of reversor in a definite molar ratio to enzyme or mercurial have seldom been used, but in the examples we have the reversal is only partial. Succinate oxidase preparation from pigeon muscle is inhibited 88% after 15–min incubation with 0.03 mM Hg^{++}; reversal by dimercaprol for 30 min depends on the amount of the dithiol added (see accompanying tabulation) (Barron and Kalnitsky, 1947). Myocardial succin-

Dimercaprol : Hg^{++}	% Reversal
3.3	0
5	33
10	63

ate oxidase inhibited completely by *p*-MB can be 31% reactivated by glutathione at a molar ratio to the mercurial of 1 : 1 and 65% at a ratio of 5 : 1 (Slater, 1949). Treatment of 3-phosphoglyceraldehyde dehydrogenase with *p*-MB leads to changes in the optical rotation; if the exposure to the mercurial is only 1 min, cysteine at a molar ratio of 6 : 1 reverses these changes around 75%, but the structural changes become progressively more irreversible (Elödi, 1960). To reverse the inhibition of glutamate dehydrogenase by *p*-MB maximally (80%) it requires around 60 times as much glutathione as mercurial* (Olson and Anfinsen, 1953).

* These experiments were done by varying the *p*-MB concentration from 0.003 to 3.3 mM and keeping the reversor, glutathione, at 10 mM, so that the more normal conclusion is simply that the reversibility is less, the higher the mercurial concentration, a phenomenon commonly observed with other enzymes. The plotting is ambiguous; the final enzyme activity is plotted as % of the uninhibited enzyme, but the results with inhibitor alone are not given, although they can be estimated from another figure, so the degree of reversal is not immediately apparent. For example, when *p*-MB is 0.11 mM the inhibition is given elsewhere as 50%; after 10 mM glutathione (molar ratio 90 : 1) the enzyme activity is given as approaching 80%, which I would call 60% reversal on one basis or 30% on another. However, this is stated to be 70% reversal in the text. No incubation times were mentioned so possibly the failure to achieve more reversal is due to an insufficient time with the reversor. Other than the work on reversal, this investigation is an excellent, detailed, and quantitative study on an enzyme, and thus illustrates a rather common phenomenon — the lackadaisical and disoriented approach to inhibition reversal. In 90% of the reports in which reversal is determined, apparently an arbitrary (but high) concentration of reversor is added, and the enzyme activity is measured after an arbitrary interval, so the conclusions should usually be taken as arbitrary. If these remarks, and others scattered throughout the book, can stimulate the performance of more accurate and interpretable reversal experiments, my aim will be achieved.

Unusual results on the effects of thiols on the inhibition of β-fructofuranosidase by Hg^{++} were observed by Gemmill and Bowman (1960), in that dimercaprol reverses the inhibition to varying degrees, whereas both cysteine and glutathione increase the inhibition (Table 7-12). The thiols themselves, even at the highest concentrations used, do not significantly affect the enzyme activity, so the additional inhibition cannot be attributed to an excess of the thiol. Several explanations are possible: (1) The cysteine and glutathione reduce enzyme disulfide groups to SH groups and increase the binding of the Hg^{++} to the enzyme; (2) the Hg^{++} reacts with both enzyme SH groups and these thiols to form E—S—Hg—S—R complexes which are less active than the simple E—S—Hg^{+} complexes; or (3) the R—S—HgX or R—S—Hg—S—R mercaptides formed are inhibitory by a mechanism unrelated to enzyme SH groups. Whatever the explanation applicable here, one would expect that these situations would occasionally be important in the reversal of other enzyme inhibitions, particularly when the bifunctional Hg^{++} is used, and possibly failure to achieve reversal in some cases may be due to these reactions.

Complexities arise in some reversal studies and a few will be mentioned briefly. When the aspartate: α-ketoglutarate transaminase from pig heart is treated with *p*-MB there is inactivation but the pyridoxal-P remains bound to the apoenzyme (Turano *et al.*, 1964). Incubation for 10 hr at pH 6.4 and 4° with 0.35 m*M* *p*-MB, however, releases the coenzyme. The addition of pyridoxal-P restores the activity in the sense that addition of glutamate is followed by transamination to form pyridoxamine-P, but the further transamination to oxalacetate is lost. In this instance, reversal thus occurs for only part of the normal reaction. Carboxypeptidase A is activated by Zn^{++} and apparently the Zn^{++} is bound to the SH groups of a single cysteine residue and the α-amino group of a terminal asparagine (Coombs *et al.*, 1964). Zn^{++} protects the enzyme from inhibition by *p*-MB, but if the apocarboxypeptidase is inactivated by *p*-MB, addition of Zn^{++} does not restore the activity.

Spontaneous reversal of enzyme inhibition by *p*-MB occurs with 3-phosphoglyceraldehyde dehydrogenase of muscle (Szabolcsi *et al.*, 1960), and has occasionally been observed in other systems, particularly homogenates. This has usually been attributed to redistribution of the mercurial from the rapidly reacting SH groups to others which bind the mercurials more tightly, but in this case it was thought that intermolecular rearrangements, whereby eventually some of the enzyme is inactive and some completely active, are responsible. This should not be too uncommon a phenomenon if the proper mercurial concentrations are used.

TABLE 7-12

EFFECTS OF THIOLS ON THE INHIBITION OF β-FRUCTOFURANOSIDASE BY Hg^{++} [a]

Dimercaprol			Cysteine			Glutathione		
Thiol (mM)	% Inhibition with:		Thiol (mM)	% Inhibition with:		Thiol (mM)	% Inhibition with:	
	Hg^{++}	Hg^{++} + thiol		Hg^{++}	Hg^{++} + thiol		Hg^{++}	Hg^{++} + thiol
0.0004	57	57	0.00065	58	60	0.00065	56	63
0.0012	59	14	0.0013	58	62	0.0013	56	65
0.019	58	10	0.013	58	76	0.013	56	69
0.045	58	11	0.029	55	84	0.23	56	94
0.23	61	4	0.23	57	78	—	—	—

[a] The concentration of Hg^{++} was 0.00013 mM in all cases and incubation with the inhibitor was 22–24 min before mixing with the sucrose-reversor solution. (From Gemmill and Bowman, 1950.)

Survey of Enzyme Inhibitions

Some enzyme inhibitions produced by the mercurials are given in Table 7-13. These represent only about a fifth of the enzymes studied. It is a difficult matter to decide which results should be in the table. I have tried to include principally two sorts of enzyme, those that are "important" and those that are inhibited "potently." But which enzymes are important? Every enzyme is important for a particular pathway, or a certain organism, or the investigator who studies it. The following groups of enzymes have generally been chosen: those in the glycolytic Embden-Meyerhof or pentose-P pathways, the tricarboxylate cycle, electron transport systems, phosphate transfer, and central amino acid metabolism; those often involved in cell function (such as choline acetylase, cholinesterase, carbonic anhydrase, etc.); and certain classic SH enzymes (such as urease). We now must consider how the word "potently" should be defined. I have arbitrarily chosen enzymes inhibited significantly (usually 50% or more) by concentrations of mercurial of 0.01 m*M* or less, since such enzymes could usually be considered as having reactive SH groups at or near the active center. It is evident that a certain enzyme differs often very markedly in susceptibility depending on the tissue or species from which it is obtained, so that one cannot speak of the sensitivity of NADH oxidase, for example, in quantitative terms without specifying which NADH oxidase is meant. At least most will agree I think that the enzymes included in Table 7-13 are important in common metabolic pathways and/or are inhibited quite potently.*

Another problem in presenting the inhibitions in Table 7-13 is that the degree of inhibition by mercurials depends on a number of factors to a greater extent than with most other inhibitors. Particularly important are the pH (mainly because of competition of H^+ with the mercurial for the S^- group), the temperature (because of both the high temperature coefficients of mercurial reactions and the possibility of secondary thermal inactivation), the composition of the medium (principally because of competition of anions with the S^- group for the mercurials), the period of the incubation with the inhibitor (since in many cases the inhibition does not attain a constant level), and the presence of impurities (most of which can complex with the mercurials and reduce their effectiveness). There are too many of these variables to include in the table. The purposes of the table are to provide very roughly some information on the relative sensitivities of the more important enzymes, and to present an experimental basis for the appreciation of the lack of specificity of the mercurials when used in complex cellular preparations.

In addition to these problems, it is likely that many of the most notable

* If a reader wishes to obtain information on the inhibition of an enzyme not given in the table, I shall be happy to try to supply this upon receiving a written request.

TABLE 7-13

INHIBITION OF SELECTED ENZYMES BY MERCURIALS

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
Acetate kinase	*E. coli*	Hg^{++}	0.0001	50	Rose *et al.* (1954)
		p-MB	0.0003	50	
Acetoacetate decarboxylase	*Clostridium acetobutylicum*	Hg^{++}	0.01	100	Seeley (1955)
Acetyl-CoA carboxylase	Wheat germ	Hg^{++}	0.01	100	Hatch and Stumpf (1961)
		p-MB	0.01	90	
Acetyl-CoA kinase	*Aspergillus niger*	*p*-MB	0.4	26	Shah and Ramakrishnan (1963)
			0.8	63	
			1.6	100	
Aconitase	*E. coli*	*p*-MB	0.5	35	Rahatekar and Rao (1963)
	Bacillus subtilis			24	
	Pseudomonas fluorescens			20	
	Micrococcus lysodeikticus			14	
	Aspergillus niger			0	
	Streptomyces griseus			0	
	Yeast			0	
	Germinating peas			2	
Aconitase	Pigeon muscle	Hg^{++}	0.05	38	Krebs and Eggleston (1944)
			0.5	99	

Acylase	Pig kidney	Hg^{++}	0.004	50	Bell and Mounter (1958)
		PM	0.0063	50	
Adenosinase	*Vibrio cholerae*	Hg^{++}	0.07	100	Agarwala *et al.* (1954)
Adenylate deaminase	Peas	Hg^{++}	0.01	100	Turner and Turner (1961)
		p-MB	0.2	100	
Adenylosuccinate lyase	Yeast	*p*-MPS	0.00035	70	Cohen and Bridger (1964)
			0.006	100	
		Hg^{++}	0.000046	23	
Alanine dehydrogenase	*Mycobacterium tuberculosis*	*p*-MB	0.0003	13	Goldman (1959 b)
			0.001	52	
			0.01	100	
	Bacillus cereus	Hg^{++}	0.1	37	O'Connor and Halvorson (1960)
		p-MB	0.01	61	
		p-MPS	0.01	58	
Alcohol dehydrogenase	*Pseudomonas aeruginosa*	*p*-MB	1	100	Azoulay and Heydeman (1963)
	Leuconostoc mesenteroides	*p*-MB	0.1	100	DeMoss (1955)
	Yeast	*p*-MB	0.001	44	Barron and Levine (1952)
			0.0015	88	
	Yeast	*p*-MB	0.00016	50	Wallenfels and Sund (1957 a)
			0.001	100	
	Yeast	Hg^{++}	0.00025	100	Snodgrass *et al.* (1960)
	Rice	*p*-MB	0.1	100	App and Meiss (1958)
	Horse liver	*p*-MB	0.006	0	Snyder *et al.* (1963)
			0.06	72	
			0.6	100	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
Aldehyde dehydrogenase	*Pseudomonas aeruginosa*	*p*-MB	0.01	25	Heydeman and Azoulay (1963)
			0.1	100	
	Acetobacter suboxydans	Hg^{++}	0.02	52	King and Cheldelin (1956)
			0.2	98	
		p-MB	0.01	38	
			0.02	70	
			0.1	97	
	Acetobacter sp.	*p*-MB	0.6	44	Nakayama (1961 a)
	Yeast (K^+-activated)	*p*-MB	0.1	50	Black (1951)
	Yeast (NADP-specific)	*p*-MB	0.008	61	Stoppani and Milstein (1957 a)
	Rabbit liver	*p*-MB	0.033	10	Hurwitz (1955 a)
			0.049	51	
			0.067	88	
	Pig liver	*p*-MB	0.08	8	Stoppani and Milstein (1957 a)
	Pig liver	*p*-MB	0.0002	5	Mahler *et al.* (1954)
			0.001	70	
			0.002	85	
	Monkey liver	*p*-MB	0.01	35	Lakshmanan *et al.* (1964)
			0.1	80	
Aldolase	*Mycobacterium* sp.	*p*-MB	0.004	50	Bastarrachea *et al.* (1961)
	Spirochaeta recurrentis	*p*-MB	0.8	100	P. J. C. Smith (1960 a)
	Peas	Hg^{++}	0.1	0	Stumpf (1948)

		PM	0.1	0	
	Rabbit muscle	Hg^{++}	0.02	16	Herbert *et al.* (1940)
	Rabbit muscle	*p*-MB	0.03	88	Swenson and Boyer (1957)
	Rabbit muscle	*p*-MB	0.1	50	Tsen and Collier (1959)
D-Allose kinase	*Aerobacter aerogenes*	Hg^{++}	0.01	100	Gibbins and Simpson (1963)
		p-MB	0.01	99	
Aminopeptidase	Beef lens	Hg^{++}	0.001	50	Wolff and Resnick (1963)
		p-MB	0.0005	50	
α-Amylase	*Bacillus subtilis*	Hg^{++}	0.001	0	Di Carlo and Redfern (1947)
			0.1	16	
			1	56	
	Bacillus stearothermophilus	*p*-MB	10	0	Manning and Campbell (1961)
β-Amylase	*Vicia faba*	Hg^{++}	1	100	Nadkarni and Sohonie (1963)
		p-MB	0.0005	100	
	Wheat	*p*-MB	0.001	33	Ghosh (1958)
		PM	0.001	73	
	Barley	*p*-MB	0.2	100	Weill and Caldwell (1945 b)
	Barley	*p*-MB	0.1	91	Tsen and Collier (1959)
	Sweet potato	Hg^{++}	0.01	100	Englard *et al.* (1951)
		p-MB	0.0005	38	
			0.001	62	
			0.005	91	
Arginine deiminase	*Streptococcus faecalis*	*p*-MB	0.0001	16	Kihara and Snell (1957)
			0.001	94	
Arginine kinase	Crayfish muscle	*p*-MB	0.00001	27	Morrison *et al.* (1957)
			0.0001	100	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
Arylesterase	Human serum	Hg^{++}	0.0007	50	Erdös *et al.* (1960)
		p-MB	0.01	97	
L-Asparaginase	Guinea pig serum	Hg^{++}	0.01	70	Tower *et al.* (1963)
			0.1	83	
		p-MPS	0.1	40	
L-Asparagine deamidase	*Bacillus coagulans*	*p*-MB	0.001	42	Manning and Campbell (1957)
Aspartase	*Bacillus cadaveris*	Hg^{++}	0.01	100	Emery (1963)
		p-MB	0.02	100	
Aspartate transcarbamylase	Rat liver	Hg^{++}	0.067	20	Bresnick (1963)
Carbamyl-ADP phosphotransferase	*Serratia marcescens*	*p*-MB	0.002	60	Glasziou (1956)
Carbon dioxide-activating enzyme	Pig heart	Hg^{++}	0.005	100	Kupiecki and Coon (1959)
		p-MB	0.006	100	
Carbonic anhydrase	Beet leaves	Hg^{++}	0.1	100	Sibly and Wood (1951)
		p-MB	0.0022	24	
			0.0043	40	
			0.022	90	

	Spinach leaves	Hg^{++}	0.1	100	Chiba *et al.* (1954 a)
		p-MB	0.5	100	
Catalase	Barley malt	Hg^{++}	0.00077	5	Charmandarjan and Tjutjunnikowa
			0.003	14	(1930)
			0.049	29	
			0.39	50	
			3.1	83	
	Horse liver	Hg^{++}	0.001	23	Blaschko (1935)
			0.01	51	
			0.1	98	
	Beef liver	PM	0.045	36	Cook *et al.* (1946)
	Beef liver	PM	0.0033	10	Sohler *et al.* (1952)
			0.083	25	
			0.33	37	
Cholesterol esterase	Rat liver	*p*-MB	0.4	95	Deykin and Goodman (1962)
Choline acetylase	Squid head ganglion	*p*-MB	0.004	12	Reisberg (1954)
			0.002	40	
			0.01	69	
			0.04	96	
Cholinesterase	*Electrophorus electricus*	*p*-MB	0.5	35	Hargreaves (1955)
	Cobra venom	Hg^{++}	3.3	19	Chaudhuri (1950 a)
			5	40	
			10	69	
	Horse serum	Hg^{++}	0.0037	4	Frommel *et al.* (1944)
			0.037	15	
			0.37	26	
			3.7	45	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
	Horse serum	Hg^{++}	2	50	Robert *et al.* (1952)
	Human serum	Hg^{++}	0.22	50	Robert *et al.* (1952)
	Human serum	Hg^{++}	1	40	Wilde and Kekwick (1964)
		p-MPS	1	7	
	Human serum	Hg^{++}	0.114	76	Goldstein and Doherty (1951)
	Human serum	Hg^{++}	0.16	50	Mounter and Whittaker (1953)
		p-MB	2.5	50	
	Human erythrocytes	Hg^{++}	2.5	50	Mounter and Whittaker (1953)
		p-MB	6.3	50	
	Human erythrocytes	*p*-MB	10	0	Markwardt (1953 a)
Citrate synthetase (condensing enzyme)	Pig heart	*p*-MB	0.2	27	Eggerer and Remberger (1963)
			0.4	95	
Creatinase	*Pseudomonas ovalis*	*p*-MB	0.04	100	Appleyard and Woods (1956)
Creatine kinase	Rabbit erythrocytes	Hg^{++}	0.01	17	Solvonuk *et al.* (1956)
			0.1	100	
		p-MB	0.1	14	
			1	100	
	Rabbit muscle	*p*-MB	0.01	100	Kuby *et al.* (1954)
	Sheep muscle	*p*-MB	0.00001	6	Ennor and Rosenberg (1954)
			0.0001	29	
			0.001	100	

Crotonase	Beef liver	*p*-MB	0.001	25	Stern and del Campillo (1956)
			0.01	51	
			0.1	74	
Cytochrome oxidase	*Penicillium chrysogenum*	*p*-MB	0.1	0	Sih *et al.* (1958)
	Arum spadix	*p*-MB	0.05	0	Simon (1957)
	Micrococcus sp.	*p*-MB	0.1	50	Hori (1963)
Deoxycytidylate deaminase	Chick embryo	*p*-MB	0.0002	62	Maley and Maley (1964)
			0.0006	100	
Deoxyribose-P aldolase	Rat liver	*p*-MB	0.0025	13	Roscoe and Nelson (1964)
			0.01	60	
			0.1	100	
Dipeptidase	Pig kidney	Hg^{++}	0.01	40	Traniello and Vescia (1964)
			0.02	100	
		p-MB	0.01	19	
			0.02	50	
			0.1	99	
Enolase	Potato	Hg^{++}	2.7	90	Boser (1959)
		p-MB	0.0027	50	
	Rabbit muscle	*p*-MB	0.7	50	Boser (1959)
	Rabbit muscle	*p*-MB	0.031	St 15	Malmström (1962)
Fructokinase	Beef liver	*p*-MB	0.015	50	Ponz and Llinás (1963)
Fructose diphosphatase	Spinach leaves	*p*-MB	0.2	75	Racker and Schroeder (1958)
	Liver	PM	0.1	100	Walsh and Walsh (1948)
β-Fructofuranosidase	Yeast	Hg^{++}	0.0058	56	von Euler and Svanberg (1920)
			0.014	77	
			0.029	90	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
	Yeast	Hg^{++}	0.00025	55	Myrbäck (1926)
			0.0005	80	
	Yeast	Hg^{++}	0.0000001	10	Gemmill and Bowman (1950)
			0.000001	25	
			0.00001	40	
			0.0001	55	
			0.001	75	
	Neurospora crassa	*p*-MB	0.004	49	Metzenberg (1963)
			0.01	63	
			0.02	81	
			0.1	96	
Fumarase	Yeast	*p*-MB	0.02	87	Favelukes and Stoppani (1958)
			0.03	96	
α-Galactosidase	*Diplococcus pneumoniae*	*p*-MB	0.1	80	Li *et al.* (1963)
β-Galactosidase	Rat epididymis	Hg^{++}	0.0002	16	Conchie and Hay (1959)
			0.0004	100	
Gluconate-6-P dehydrogenase	*Streptomyces olivaceus*	*p*-MB	0.06	5	Maitra and Roy (1959)
	Pea plant	*p*-MB	1	100	Gibbs *et al.* (1955)
	Rat liver	*p*-MB	0.1	35	Glock and McLean (1953)
			0.25	83	
			0.35	90	

Gluconokinase	Pig kidney	*p*-MB	0.001	0	Leder (1957)
			0.01	59	
Glucose dehydrogenase	*Bacterium anitratum*	*p*-MB	3.6	0	Hauge (1960)
	Rat liver	*p*-MB	0.01	0	Metzger *et al.* (1964)
Glucose-6-P dehydrogenase	*Leuconostoc mesenteroides*	Hg^{++}	1	13	Ciferri and Blakley (1959)
			5	100	
		p-MB	1	88	
	Streptomyces olivaceus	*p*-MB	0.06	20	Maitra and Roy (1959)
	Tobacco leaves	*p*-MB	0.1	50	Clayton (1959)
	Rat liver	*p*-MB	0.25	91	Glock and McLean (1953)
	Human erythrocytes	*p*-MB	0.01	100	Kirkman (1962)
	Human erythrocytes	*p*-MB	0.1	77	Chung and Langdon (1963)
Glucose-P isomerase	*Trichinella spiralis*	*p*-MB	6	0	Mancilla and Agosin (1960)
			10	15	
	Phaseolus radiatus	Hg^{++}	2	13	Ramasarma and Giri (1956)
	Human erythrocytes	Hg^{++}	1	100	Tsuboi *et al.* (1958)
		p-MB	2	0	
β-Glucosidase	*Blaberus craniifer* (cockroach)	Hg^{++}	0.83	65	Fisher (1964)
		p-MB	0.002	40	
			0.04	62	
β-Glucuronidase	*E. coli*	*p*-MB	0.0009	28	Doyle *et al.* (1955)
			0.009	68	
			0.017	73	
			0.09	86	
			0.17	91	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
D-Glutamate oxidase	*Octopus vulgaris*	*p*-MB	0.001	27	Rocca and Ghiretti (1958)
			0.01	74	
			0.1	100	
L-Glutamate dehydrogenase	*Pasteurella tularensis*	Hg^{++}	0.005	28	Rendina and Mills (1957)
			0.01	32	
			0.05	56	
		p-MB	0.06	65	
			0.6	75	
		p-MPS	0.042	49	
			0.17	57	
			0.42	63	
	Fusarium oxysporum (NADP-dependent)	*p*-MB	0.5	66	Sanwal (1961)
			1	89	
	Puccinia helianthi	*p*-MB	1.3	60	J. E. Smith (1963)
	Corn leaves (NAD-dependent)	*p*-MB	1	13	Bulen (1956)
			2.8	35	
	Frog liver (NADP-dependent)	*p*-MPS	0.16	0	DeGroot and Cohen (1962)
			0.62	40	
			2.5	95	
	Pig liver (NAD-dependent)	*p*-MB	0.5	100	Singer and Barron (1945)
	Beef liver (NAD-dependent)	Hg^{++}	0.001	2	Olson and Anfinsen (1953)
			0.004	50	
			0.01	93	

		p-MB	0.01	19	
			0.11	50	
			1	85	
Glutaminase	Guinea pig liver	Hg^{++}	0.5	73	Guha (1962)
		p-MB	0.5	75	
Glutamine synthetase	Peas	*p*-MB	0.001	19	Varner and Webster (1955)
			0.01	99	
Glutamic semialdehyde reductase	Rat liver	Hg^{++}	0.01	19	Smith and Greenberg (1957)
			0.02	40	
			0.04	82	
		p-MB	0.006	12	
			0.02	87	
			0.04	94	
Glutathione reductase	Rat liver	Hg^{++}	0.000067	15	Mize and Langdon (1962)
			0.00033	100	
		p-MB	0.0001	St 32	
			0.0033	60	
			0.017	89	
Glycerate dehydrogenase	Spinach leaves	*p*-MB	0.001	30	Holzer and Holldorf (1957)
			0.005	55	
			0.01	91	
		PM	0.001	38	
			0.005	83	
			0.01	87	
Glycerate kinase	*Candida mycoderma*	*p*-MB	0.0012	50	Bergmeyer *et al.* (1961)
			0.01	100	
	Brassica campestris	*p*-MB	0.011	89	Ozaki and Wetter (1960)

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
α-Glycerolphosphate dehydrogenase	*Trypanosoma rhodesiense*	*p*-MB	0.001	46	Grant and Sargent (1961)
	Rabbit muscle	*p*-MB	0.01	93	van Eys *et al.* (1959)
α,β-Glycosylphosphatase	*Phaseolus aureus*	Hg^{++}	0.05	96	Felenbok and Neufeld (1962)
		p-MB	0.002	40	
Glycylglycine dipeptidase	Yeast	*p*-MB	0.001	57	Nishi (1958)
			0.01	84	
Glyoxylate reductase	Tobacco leaves	*p*-MB	0.00013	50	Zelitch (1955)
Guanosine phosphorylase	Human erythrocytes	Hg^{++}	0.001	87	Tsuboi and Hudson (1957)
			0.01	100	
L-Gulonate dehydrogenase	Pig kidney	*p*-MB	0.01	100	Bublitz and York (1961)
Hexokinase	*Acetobacter suboxydans*	PM	0.1	100	Fewster (1957)
	Bacillus sp.	Hg^{++}	1	95	Sato *et al.* (1956)
		p-MB	0.01	95	
	Neurospora crassa	*p*-MB	1	89	Medina and Nicholas (1957 a)
	Wheat germ	Hg^{++}	1	32	Saltman (1953)
		p-MB	0.01	18	
			0.1	24	
	Schistosoma mansoni	*p*-MB	0.01	52	Bueding and Mackinnon (1955)
			0.02	84	

	Echinococcus granulosus	*p*-MB	1.4	38	Agosin and Aravena (1959)
			2.5	81	
	Rat brain	*p*-MB	0.0017	23	Sols and Crane (1954)
			0.0042	78	
			0.0084	93	
Homoserine deaminase	Rat liver	Hg^{++}	0.05	100	Matsuo and Greenberg (1959)
		p-MB	0.01	96	
L-α-Hydroxyacid oxidase	Pig kidney	*p*-MB	0.001	35	Robinson *et al.* (1962)
3-Hydroxyanthranilate oxidase	Beef liver	*p*-MB	0.005	68	Decker *et al.* (1961)
β-Hydroxybutyrate dehydrogenase	Beef heart	Hg^{++}	0.004	50	Sekuzu *et al.* (1963)
		p-MPS	0.001	50	
γ-Hydroxy-α-ketoglutarate synthetase	Rat liver	Hg^{++}	2	100	Kuratomi and Fukunaga (1963)
Imidazole-*N*-methyl-transferase	Guinea pig brain	*p*-MB	0.01	91	Brown *et al.* (1959)
Iron oxidase	*Ferrobacillus ferrooxidans*	*p*-MB	0.01	54	Blaylock and Nason (1963)
			0.1	73	
Isoamylase	Yeast	Hg^{++}	0.1	98	Gunja *et al.* (1961)
		p-MB	0.01	59	
		PM	0.01	55	
Isocitrate dehydrogenase	*Mycobacterium tuberculosis*	*p*-MB	0.002	45	Goldman (1956 a)
			0.02	100	
	Trypanosoma cruzi	*p*-MB	0.1	60	Agosin and Weinbach (1956)
			1	100	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
	Pig heart	*p*-MB	0.02	88	Lotspeich and Peters (1951)
			0.04	100	
	Pig heart	*p*-MB	0.000025	50	Siebert *et al.* (1957)
	Beef heart	*p*-MPS	0.0067	100	Chen *et al.* (1964)
α-Ketoglutarate oxidase	Mosquitoes	*p*-MB	0.01	42	Gonda *et al.* (1957)
			0.1	96	
	Pig heart	*p*-MB	0.07	50	Sanadi *et al.* (1952)
			0.2	100	
	Rat liver	*p*-MB	0.055	50	Hirade and Hayashi (1953)
α-Ketoglutarate reductase	*Achromobacter* sp.	*p*-MB	0.1	63	Beppu (1961)
α-Ketoisocaproate decarboxylase	*Proteus vulgaris*	*p*-MB	0.01	69	Sasaki (1962)
			0.05	94	
Lactate dehydrogenase	*Propionibacterium pentosaceum*	*p*-MB	1	19	Molinari and Lara (1960)
			1.7	47	
			8.3	74	
	Yeast	*p*-MB	0.005	0	Yamashita (1960)
			0.01	35	
			0.013	53	
			0.033	100	

	Yeast	*p*-MPS	0.0005	60	Gregolin and Singer (1963)
			5	60	
	Chick embryo liver	*p*-MB	0.03	0	Solomon (1958)
			10	70	
	Rabbit muscle	*p*-MB	0.1	100	Tsen and Collier (1959)
	Rabbit muscle	*p*-MB	0.16	23	Dube *et al.* (1963)
			0.4	44	
			0.64	61	
	Beef heart	*p*-MB	0.1	92	Neilands (1954)
	Beef brain	*p*-MB	0.1	50	Winer (1960)
	Human serum	Hg^{++}	0.0009	50	Hill (1956)
	Rabbit muscle	Hg^{++}	0.0000035	50	Hill (1956)
Lactonase-I	Beef liver	Hg^{++}	0.001	71	Yamada (1959)
		p-MB	0.0075	100	
L-Leucine decarboxylase	*Bacillus cereus*	*p*-MB	0.001	77	Sanwal and Zink (1961)
			0.01	100	
Lombricine kinase	Earthworms	*p*-MB	0.00005	20	Gaffney *et al.* (1964)
			0.0001	100	
Luciferase	*Achromobacter fischeri*	*p*-MB	0.004	50	Green and McElroy (1955)
	Achromobacter fischeri	*p*-MB	0.002	80	Cormier *et al.* (1956)
	Cypridina	Hg^{++}	0.5	30	Shimomura *et al.* (1961)
Lysine decarboxylase	*E. coli*	Hg^{++}	0.01	98	Taylor and Gale (1945)
Malate dehydrogenase	*Pseudomonas ovalis*	PM	0.1	100	Francis *et al.* (1963)
	Mycobacterium avium	Hg^{++}	0.01	100	Kimura and Tobari (1963)

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
	Chicken liver	*p*-MB	0.03	0	Solomon (1958)
			10	90	
	Pigeon heart	*p*-MB	0.2	100	Barron and Singer (1945)
	Pig heart	*p*-MB	0.01	12	Thorne and Kaplan (1963)
			0.015	50	
			0.025	94	
	Horse liver	*p*-MB	0.05	35	Shull (1959)
	Beef brain	*p*-MB	0.05	50	Winer (1960)
	Human erythrocytes	*p*-MPS	0.33	10	Shrago and Falcone (1963 b)
Malate dehydrogenase (decarboxylating)	Flax rust uredospores	Hg^{++}	0.005	19	Johnson and Frear (1963)
			1	44	
		p-MB	0.01	0	
		p-MPS	0.01	13	
	Pigeon liver	Hg^{++}	0.005	94	Rutter and Lardy (1958)
		p-MB	0.0002	52	
			0.001	100	
	Beef lens	PM	0.1	10	van Heyningen and Pirie (1953)
			1	87	
Malate : vitamin K reductase	*Mycobacterium phlei*	*p*-MB	0.5	90	Asano and Brodie (1963)

Malonyl-CoA-CO_2 exchange enzyme	*Clostridium kluyveri*	*p*-MB	0.003	25	Vagelos and Alberts (1960)
			0.01	60	
			0.1	100	
Mevalonate dehydrogenase	Rat liver	*p*-MB	0.01	70	Nakamura and Greenberg (1961)
			0.1	100	
Monoamine oxidase	Beef liver	*p*-MB	0.005	0	Barbato and Abood (1963)
			0.01	25	
			0.012	50	
			0.1	90	
			1.2	100	
Myokinase	Rabbit muscle	*p*-MB	0.01	65	Noda (1958)
	Rabbit muscle	*p*-MB	0.000015	50	Callaghan and Weber (1959)
NAD glycohydrolase	*Mycobacterium tuberculosis*	Hg^{++}	0.001	7	Gopinathan *et al.* (1964)
			0.01	100	
		p-MB	0.001	23	
			0.01	100	
NADH oxidase	*Azotobacter vinelandii*	*p*-MB	0.025	94	Repaske and Josten (1958)
	Lactobacillus casei	*p*-MB	0.5	95	P. Strittmatter (1959)
	Clostridium perfringens	*p*-MB	0.0017	44	Dolin (1959)
			0.017	94	
	Streptococcus faecalis	*p*-MB	0.001	100	Hoskins *et al.* (1962)
	Xanthomonas phaseoli	*p*-MB	1	58	Hochster and Nozzolillo (1960)
	Rhodospirillum rubrum	*p*-MB	0.5	100	White and Vernon (1958)
		PM	1	100	
	Lettuce seeds	*p*-MB	0.5	33	Mayer (1959)

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
	Potatoes	*p*-MB	0.1	0	Hackett (1958)
			1	100	
	Cucumbers	*p*-MB	0.02	50	Beevers (1954)
	Tetrahymena pyriformis	*p*-MB	0.0003	0	Eichel (1956 a)
			0.003	82	
	Rat liver microsomes	*p*-MB	0.01	100	Avi-Dor *et al.* (1958)
	Rat heart sarcosomes	*p*-MB	0.01	33	Avi-Dor *et al.* (1958)
	Pig liver	Hg^{++}	0.1	100	Endahl and Kochakian (1961)
		p-MB	0.1	97	
	Pig heart	Hg^{++}	0.005	12	Huennekens *et al.* (1955)
			0.02	100	
		p-MB	0.02	40	
			0.1	100	
	Beef heart	*p*-MB	0.1	100	Mackler (1961)
NADH:CoQ oxidoreductase	Beef heart	*p*-MPS	0.006	100	Hatefi *et al.* (1961 a)
NADH:cytochrome c oxidoreductase	*Bacillus cereus*	*p*-MB	0.01	49	Doi and Halvorson (1961)
	E. coli	*p*-MB	0.01	81	Brodie (1952)
	Proteus vulgaris	Hg^{++}	0.1	94	Feldman and O'Kane (1960)
		p-MB	0.0033	100	

	Pig liver	Hg^{++}	1	100	Garfinkel (1957)
		p-MB	0.01	97	
		p-MPS	0.01	97	
		PM	0.1	98	
	Beef liver	*p*-MB	0.1	100	Penn and Mackler (1958)
	Rat muscle	*p*-MB	0.1	60	Lehman and Nason (1956)
			0.5	100	
	Pig heart	*p*-MB	0.1	100	Vernon *et al.* (1952)
	Beef heart	*p*-MPS	0.01	60	Hatefi *et al.* (1961 b)
			0.05	92	
	Beef heart	*p*-MB	0.1	68	Kaufman and Kaplan (1961)
			1	85	
	Beef heart	*p*-MB	0.0005	27	King and Howard (1962)
			0.001	56	
NADH : DCPIP oxidoreductase	Rat liver	*p*-MB	0.1	45	Ernster *et al.* (1962)
			1	85	
	Pig liver	*p*-MB	0.01	85	Garfinkel (1957)
		p-MPS	0.01	85	
		PM	0.1	87	
	Beef heart	*p*-MB	0.0005	31	King and Howard (1962)
			0.001	40	
			0.01	55	
NADH : ferricyanide oxidoreductase	*Streptococcus faecalis*	Hg^{++}	0.01	21	Dolin (1957)
		p-MB	0.33	20	
	Pig liver	*p*-MB	0.02	50	Mahler *et al.* (1958)
			0.2	100	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
NADH : menadione oxidoreductase	Pig liver	*p*-MB	0.01	90	Raw *et al.* (1961)
	Rat brain	*p*-MB	0.03	50	Giuditta and Strecker (1960)
NADH : nitrate oxidoreductase	*Rhizobium japonicum*	*p*-MB	0.02	76	Cheniae and Evans (1959)
			0.05	84	
NADH : quinone oxidoreductase	Pig liver	*p*-MB	0.17	50	Frimmer (1960)
		Mersalyl	0.07	50	
NADH : tetrazolium oxidoreductase	Rat liver	*p*-MB	0.8	95	Slater (1959)
	Rat brain	*p*-MB	0.05	50	Hess and Pearse (1963)
NADP glycohydrolase	Pig spleen	Hg^{++}	0.1	55	Dickerman *et al.* (1962)
			1	80	
		p-MB	0.1	0	
		p-MPS	5	13	
NADPH : cytochrome c oxidoreductase	*Neurospora crassa*	*p*-MB	0.0001	9	Kinsky and McElroy (1958)
			0.01	100	
	Spinach chloroplasts	*p*-MB	0.8	57	Marrè and Servettaz (1958)
			5	91	
	Pig liver	*p*-MB	0.0016	43	Williams and Kamin (1962)
			0.002	87	
			0.003	97	

	Beef heart	Hg^{++}	1	50	Lang and Nason (1959)
		p-MB	1	0	
	Beef heart	*p*-MB	1	12	Kaufman and Kaplan (1961)
NADPH : menadione oxidoreductase	Spinach	*p*-MB	0.1	0	Lazzarini and San Pietro (1964)
			1	16	
	Phaseolus aureus	*p*-MB	0.1	26	Shichi and Hackett (1962)
NADPH : methemoglobin oxidoreductase	Human erythrocytes	*p*-MB	0.01	20	Huennekens *et al.* (1957 a)
	Rabbit erythrocytes	*p*-MB	0.5	1	Bide and Collier (1964)
			1.65	45	
NADPH : nitrite oxidoreductase	*E. coli*	*p*-MB	0.001	83	Lazzarini and Atkinson (1961)
NADPH oxidase	Rabbit liver	Hg^{++}	0.01	2	Gillette *et al.* (1957)
			0.2	78	
	Human erythrocytes	*p*-MB	0.03	60	Shrago and Falcone (1963 a)
NADPH : trichloro-indophenol oxidoreductase	Spinach leaves	Hg^{++}	0.023	50	Avron and Jagendorf (1956)
		p-MB	0.13	57	
Neuraminidase	Chick embryo chorioallantois	*p*-MB	0.0005	0	Ada (1963)
			0.005	30	
			0.05	93	
		MM	0.005	0	
			0.05	87	
Ornithine carbamyltransferase	Rat liver	*p*-MB	0.0001	56	Reichard (1957)
			0.0005	82	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
Oxalosuccinate decarboxylase	Pig heart	*p*-MB	0.00004	50	Siebert *et al.* (1957)
Phenylalanine deaminase	Barley	Hg^{++}	0.01	100	Koukol and Conn (1961)
		p-MPS	0.01	100	
Phosphoenolpyruvate carboxylase	*Nocardia corallina*	*p*-MB	0.025	100	Baugh *et al.* (1960)
	Yeast	*p*-MB	0.1	98	Cannata and Stoppani (1963 b)
	Sweet orange leaves	Hg^{++}	0.02	39	Wallace and Mueller (1961)
Phosphoenolpyruvate carboxytransphosphorylase	*Propionibacterium shermanii*	*p*-MB	0.01	50	Siu and Wood (1962)
Phosphofructokinase	Peas	Hg^{++}	5	96	Axelrod *et al.* (1952)
3-Phosphoglyceraldehyde dehydrogenase	Rabbit muscle	*p*-MB	0.024	36	Barron and Dickman (1949)
			0.048	79	
	Rabbit muscle	*p*-MB	0.0002	50	Weitzel and Schaeg (1959)
	Rabbit muscle	*p*-MB	0.025	50	Holzer (1956)
		PM	0.02	50	
Phosphoglycerate mutase	Rabbit muscle	Hg^{++}	0.1	100	Cowgill and Pizer (1956)
		p-MB	0.05	100	
Phosphomevalonate kinase	Guinea pig liver	*p*-MB	0.1	100	Hellig and Popják (1961)
Phosphorylase	Potatoes	Hg^{++}	0.1	65	Lee (1960 a)
	Potatoes	*p*-MB	2.5	51	Hughes and Spragg (1958)

	Lobster muscle	*p*-MB	0.01	100	Cowgill (1959)
	Rabbit muscle	*p*-MB	0.001	50	Madsen and Gurd (1956)
Phosphoribosyl-PP amidotransferase	Chicken liver	*p*-MB	0.025	45	Hartman (1963 a)
Phosphoserine phosphatase	Chicken liver	*p*-MB	0.0011	0	Neuhaus and Byrne (1959)
			0.011	85	
			0.11	100	
Propionyl-CoA carboxylase	Beef liver	*p*-MB	0.0001	0	Halenz *et al.* (1962)
			0.001	42	
			0.01	100	
	Pig heart	*p*-MB	0.01	100	Kaziro *et al.* (1962)
Protein disulfide reductase	Peas	Hg^{++}	0.002	92	Hatch and Turner (1960)
		p-MB	0.054	88	
Pyrocatechase	*Pseudomonas fluorescens*	Hg^{++}	0.01	96	Hayaishi *et al.* (1957)
		p-MB	0.01	77	
Pyruvate carboxylase	Chicken liver	Hg^{++}	0.001	41	Keech and Utter (1963)
			0.01	87	
		PM	0.01	81	
Pyruvate decarboxylase	*Acetobacter suboxydans*	Hg^{++}	0.2	95	King and Cheldelin (1954)
		p-MB	0.2	95	
	Zymosarcina ventriculi	Hg^{++}	0.2	100	Bauchop and Dawes (1959)
		p-MB	0.36	100	
	Yeast	*p*-MB	0.0014	50	Stoppani *et al.* (1953)
	Yeast	Hg^{++}	0.00235	20	Kuhn and Beinert (1947)
			0.0094	82	
		p-MB	0.00235	15	
			0.0094	89	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
Pyruvate dehydrogenase	*Clostridium pasteurianum*	*p*-MB	0.1	100	Westlake *et al.* (1961)
	Mycobacterium tuberculosis	*p*-MB	1	0	Goldman (1959)
Pyruvate kinase	Yeast	*p*-MB	0.01	100	Washio and Mano (1960)
	Human erythrocytes	Hg^{++}	0.01	60	Solvonuk and Collier (1955)
			0.1	94	
		p-MB	0.01	0	
			0.1	100	
Pyruvate oxidase	*Proteus vulgaris*	*p*-MB	0.3	100	Baer (1948)
	Proteus vulgaris	Hg^{++}	0.01	100	Moyed and O'Kane (1956)
	Pigeon muscle	*p*-MB	0.11	82	Onrust *et al.* (1954)
	Pigeon brain	Hg^{++}	0.1	25	Thompson and Whittaker (1947)
			0.2	65	
			0.3	85	
	Dog heart	*p*-MB	0.1	52	Kuratomi (1959)
Riboflavin kinase	*Phaseolus radiatus*	Hg^{++}	0.01	52	Giri *et al.* (1958)
Ribose isomerase	Wheat germ	*p*-MB	0.5	96	Pubols *et al.* (1963)
Ribose-5-P isomerase	*Echinococcus granulosus*	*p*-MB	0.00052	24	Agosin and Aravena (1960)
			0.001	61	
			0.0052	95	

	Spinach leaves	*p*-MB	0.22	20	Tabachnick *et al.* (1958)
			0.44	45	
	Human erythrocytes	*p*-MB	0.1	95	Urivetzky and Tsuboi (1963)
	Erythrocytes	Mersalyl	0.02	85	Bruns *et al.* (1958)
	Rabbit muscle	*p*-MB	0.1	100	Dickens and Williamson (1956)
Ribulose-5-P kinase	Spinach leaves	Hg^{++}	0.00002	33	Hurwitz *et al.* (1956)
			0.00005	64	
			0.0005	100	
		p-MB	0.00015	77	
			0.00036	87	
			0.007	100	
Succinate dehydrogenase	*Micrococcus lactilyticus*	*p*-MB	0.03 μmole/mg	52	Warringa and Giuditta (1958)
			0.15 μmole/mg	77	
			0.90 μmole/mg	100	
	Mycobacterium avium	*p*-MB	0.75	90	Knsunose *et al.* (1956)
	Proteus vulgaris	*p*-MB	1	100	Feldman and O'Kane (1960)
	Rhizobium japonicum	*p*-MB	0.02	95	Cheniae and Evans (1959)
			0.05	100	
	Arum maculatum spadix	*p*-MB	0.05	97	Simon (1957)
	Phaseolus vulgaris	*p*-MB	0.01	75	Hiatt (1961)
	Potatoes	PM	0.001	33	Millerd (1951)
			0.01	33	
			0.1	95	
	Oyster eggs	PM	0.1	35	Cleland (1949)
			1	97	
	Pigeon muscle	Hg^{++}	0.012	50	Barron and Kalnitsky (1947)

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
	Pigeon muscle	*p*-MB	0.05	74	Barron and Singer (1945)
	Rat liver	*p*-MB	0.01	30	Potter and DuBois (1943)
	Rat liver	Hg^{++}	0.0033	13	Jacobs *et al.* (1956)
	Rat liver	Hg^{++}	5	95	Nachlas *et al.* (1960)
		p-MB	0.5	93	
	Rat kidney	*p*-MB	0.001	50	Handley and Lavik (1950)
	Rat heart	*p*-MB	0.04	50	Kreke *et al.* (1949)
	Rat heart	Meralluride	0.015	50	Ruskin and Ruskin (1953)
			0.04	90	
	Beef heart	*p*-MB	0.001	91	Singer *et al.* (1956 b)
	Beef heart	*p*-MB	0.0005	41	Massey and Singer (1957)
			0.001	78	
Succinate semialdehyde dehydrogenase	*Pseudomonas aeruginosa*	Hg^{++}	0.1	80	K. Nakamura (1960)
		p-MB	0.01	93	
	Monkey brain	*p*-MPS	0.001	50	Albers and Koval (1961)
Threonine dehydrogenase	Bullfrog liver	*p*-MB	0.01	100	Hartshorne and Greenberg (1964)
Transaminases					
Alanine:α-ketoglutarate	Rat liver	*p*-MB	0.001	9	Segal *et al.* (1962)
			0.0025	45	
			0.005	82	
			0.01	93	

	Pig heart	*p*-MB	1	49	Singer and Barron (1945)
	Pig heart	*p*-MB	0.055	100	Grein and Pfleiderer (1958)
	Rat brain	*p*-MPS	1	74	Fonnum *et al.* (1964)
γ-Aminobutyrate:α-ketoglutarate	Beef brain	*p*-MB	0.7	100	Baxter and Roberts (1958)
δ-Aminovalerate:α-ketoglutarate	*Pseudomonas* sp.	*p*-MB	0.1	74	Ichihara *et al.* (1960)
Asparagine:pyruvate	Rat liver	*p*-MB	0.1	50	T'ing-sêng (1961 b)
Aspartate:α-ketoglutarate	Cauliflower	*p*-MB	0.5	50	Ellis and Davies (1961)
	Rat liver	*p*-MB	0.1	27	Goldstone and Adams (1962)
			0.5	77	
	Pig heart	*p*-MB	0.05	99	Polyanovskii (1962)
Glutamine:pyruvate	Rat liver	*p*-MB	0.01	10	T'ing-sêng (1961 a)
Glycine:α-ketoglutarate	Rat liver	*p*-MB	0.5	0	Nakada (1964)
Tryptophan:α-ketoglutarate	Rat brain	*p*-MPS	1	66	Fonnum *et al.* (1964)
Tyrosine:α-ketoglutarate	Yeast	*p*-MB	0.00283	56	SentheShanmuganathan (1960)
	Rat liver	*p*-MPS	0.0016	50	Kenney (1959)
Transketolase	Pig liver	Hg^{++}	0.005	7	Simpson (1960)
			0.2	40	
			0.8	72	
		p-MPS	0.025	6	
			0.25	16	

TABLE 7-13 (*continued*)

Enzyme	Source	Mercurial	Concentration (m*M*)	% Inhibition	Reference
Triphosphoinositide phosphomonoesterase	Beef brain	Hg^{++}	0.041	98	Dawson and Thompson (1964)
		p-MB	0.041	74	
			0.41	81	
			1.6	100	
		PM	0.042	60	
			0.17	76	
Triphosphoinositide phosphodiesterase	Beef brain	Hg^{++}	0.05	88	Thompson and Dawson (1964)
			0.1	90	
		p-MB	0.1	62	
			0.5	78	
		PM	0.01	77	
			0.05	85	
Tryptophanase	*E. coli*	Hg^{++}	0.001	20	Happold (1950)
			0.01	95	
	E. coli	*p*-MB	0.05	100	Wada *et al.* (1958)
UDP-galactose-4-epimerase	Calf liver	*p*-MB	0.001	30	Maxwell (1957)
			0.002	50	
			0.004	56	
			0.01	94	
UDP-glucose dehydrogenase	Pea seedlings	*p*-MB	0.001	55	Strominger and Mapson (1957)

	Calf liver	*p*-MB	0.0001	27	Maxwell *et al.* (1956)
			0.0002	43	
			0.0003	69	
			0.0004	72	
			0.0005	93	
UDP-glucuronyltransferase	Mouse liver	*p*-MB	0.4	100	Storey (1964)
		PM	0.4	100	
Urease	*Blastomyces dermatitidis*	Hg^{++}	0.00016	90	Taylor and Johnson (1962)
			0.0008	93	
			0.004	97	
	Jack beans	Hg^{++}	0.0016	15	Mapson (1946)
			0.0032	33	
			0.0064	65	
	Jack beans	*p*-MB	0.1	96	Tsen and Collier (1959)
Uricase	Housefly	Hg^{++}	0.002	28	Nelson (1964)
Uroporphyrinogen decarboxylase	Rabbit reticulocytes	*p*-MB	0.7	98	Mauzerall and Granick (1958)
Xanthine dehydrogenase	Chicken liver	*p*-MB	0.0007	90	Doisy *et al.* (1955)
Xylanase	*Pericularia oryzae*	*p*-MB	0.01	100	Sumizu *et al.* (1961)
Xylulokinase	*Aerobacter aerogenes*	Hg^{++}	0.002	38	Bhuyan and Simpson (1962)
			0.02	51	
			0.2	85	
		p-MB	0.001	48	
			0.1	68	
			2.5	85	
		p-MPS	1	74	

inhibitions constitute mutual depletion systems and are in zones B or C (see page I-66). The inhibition is independent of K_i in zone C — i.e., $i = (I_t)/(E_t)$ — and so the degree of inhibition indicates, not the affinity of the enzyme for the mercurial, but only the concentration of enzyme (or of other substances binding the mercurials). When we see in the table that 0.001 mM p-MB inhibits an enzyme 50%, does this mean that K_i is approximately 0.001 mM, or that the enzyme concentration is 0.002 mM (assuming pure enzyme)? It is actually more likely to be the latter, and this is one reason why the values in the table should not be taken too seriously. These questions will be considered in the following section.

It is rather difficult to find many enzymes which are insensitive to the mercurials. The following enzymes might be considered as relatively resistant ($< 10\%$ inhibition at 1 mM or above): adenylate kinase, most alkaline phosphatases, α-amylase, potato apyrase, cellobiase, coagulases, coproporphyrinogen oxidase, dinitrophenol reductase, elastase, β-glycerophosphatase, glycolate oxidase, kynurenine formamidase, certain lipases, maltase, neuraminidase, nitrite reductase, oxalate decarboxylase, pepsin, peroxidases, phospholipases, many proteases and peptidases (especially bacterial, fungal, and venom), some pyrophosphatases, most RNases and DNases, certain sulfatases, thiamine diphosphatase, and uricase. These certainly do not constitute as a whole a very extensive or particularly important group of enzymes. Actually, the majority of enzymes are inhibited in an intermediate fashion between these and the examples in Table 7-13, i.e., 50–100% by concentrations in the range 0.05–1 mM, although some of these would undoubtedly exhibit a much greater sensitivity if examined under appropriate conditions (in a pure form, at physiological temperatures and pH, and in the absence of high concentrations of ligands).*

Comparison of Mercurials

Since the introduction of p-MB some 30 years ago, it and the related p-MPS have been used for enzyme inhibition more and more frequently at the expense of Hg^{++}. It is interesting, therefore, to look into the results which have been obtained with the inorganic and organic mercurials. Of the total of 160 reports on enzyme inhibition using both Hg^{++} and p-MB, 25% do not allow an accurate comparison, due mainly to different concen-

* A word should perhaps be said against the common practice of reporting only an inhibition of 100% with a single mercurial concentration, particularly if this is relatively high, since such results are not very meaningful and the enzymes cannot be accurately classified as to sensitivity. For example, to state that 1 mM p-MB inhibits 100% is bad on two counts: One does not know the true sensitivity of the enzyme, since 0.01 mM might also inhibit 100%, and such a result does not provide much evidence for the importance of SH groups at the active center, although it is often so interpreted.

trations being used, or statements that both at certain concentrations inhibit 100%. Of the remaining reports, Hg^{++} is more potent in 65%, *p*-MB in 29%, and in 6% they are of equal potency. One must admit that Hg^{++} is generally more effective. In some cases it is of much greater inhibitory potency than *p*-MB or the other organic mercurials. One might expect Hg^{++} to be more potent than *p*-MB because (1) it is smaller and might be able to penetrate and react with SH groups inaccessible to the larger molecule, and (2) it could possibly in some instances induce dimerization of the enzyme (or even polymerization), since it is bifunctional. On the other hand, *p*-MB might be considered to shield off, sterically or electrostatically, a greater area on the enzyme surface once it has combined with the SH groups, due to its greater size and the charged COO^- group. The result in any case is probably a balance of these and other factors. The fact that Hg^{++} is often more inhibitory than *p*-MB does not immediately imply that it is a better or more reliable inhibitor to use for the purpose of detecting SH groups on enzymes, but it does, I think, suggest that Hg^{++} has been unnecessarily neglected by many workers. It might be proposed that both mercurials be used, since not only will the detection of SH groups be made more certain, but occasionally interesting information on the nature of the inhibition may be obtained.

The organic mercurials themselves have not often been used in the same investigation, but in 19 reports using both *p*-MB and PM, I have found PM to be more potent in 79%. The differences between them are seldom very marked, however. One would not expect much difference between *p*-MB and *p*-MPS, and examination of the eight reports using both bears this out, in two *p*-MB being the more potent, in two *p*-MPS being the more potent, and in four the potencies being the same. There is some reason for believing that the smaller uncharged alkyl mercurials, such as MM, might be better for enzyme study than any of the other mercurials, but there has been so little comparison that nothing can be said definitely about their relative effectiveness at this time.

Meaning of K_i and Methods of Expressing Inhibition by the Mercurials

The values for K_i have occasionally been reported for mercurial inhibition; e.g., for the inhibition of phosphoribosyl-ATP pyrophosphorylase by *p*-MB, $pK_i = 5.15$ (Martin, 1963), and for the inhibition of heart lactate dehydrogenase by *p*-MB, $pK_i = 4.10$ (Gruber *et al.*, 1962). In the latter case the binding of the mercurial to the noncatalytic SH groups, causing the spontaneous reversal of the inhibition, is characterized by a pK_i of 5.40. Most of the values given for pK_i are in the range 4–6. However, these values were obtained by simply taking the concentration to produce 50% inhibition, which would be valid if (1) the inhibition is classically noncompetitive (which in most cases probably it is not), and (2) the system is in zone A

(which it seldom is). If the system is in zone B or C, K_i may be a good deal smaller than pI_{50}, and we have seen that in zone C there is no way kinetically of determining K_i. The only valid calculation of a true dissociation constant for a mercurial complex with an enzyme, of which I am aware, is that of Madsen and Gurd (1956) for muscle phosphorylase and *p*-MB. They used an ultracentrifugal method to measure the concentration of free *p*-MB after equilibration and determined K_i from a plot of $1/r$ against $r/(p\text{-MB}_f)$, where r is the molar ratio of *p*-MB bound to protein and $p\text{-MB}_f$ is the free mercurial. A value of $pK_i = 6$ was found. It is likely that in most cases in which an enzyme is potently inhibited by a mercurial, a pK_i of 6 or less would be found, and in this range it is very difficult to determine the constant by the usual plotting procedures. It will be recalled that W. L. Hughes (1950) found a pK of 4.46 for the complex of mercaptalbumin and MM (page 759). We may now inquire into what values of K_i would be predicted under ordinary circumstances. Equation 7-3 gives the relation between the experimental constant and the dissociation constant for a mercurial complex, and if we alter it to correspond to inhibition of enzymes we have

$$pK_i' = pK_i - pK_a - pK_x ,$$

where pK_i' is the experimental or apparent dissociation constant. If pK_i is taken as 21, pK_a as 8.7, and pK_x (for Cl^-) as 6.5, all of these being approximations, pK_i' turns out to be around 5.5. Since pK_i certainly varies from 20 to 22, pK_a from 7.5 to 9.5, and pK_x (depending on the ligand) from 6 to 9, it is clear that pK_i' may vary over a wide range, but at least the values experimentally determined are of the correct order of magnitude. The experimental pK_i' is thus a good deal less than the true pK_i because of the competitive effects of H^+ and the ligand X^-.

The question of how best to report mercurial inhibitions is a difficult one. First, the concentration-inhibition curves are often very steep (Fig. 7-31) so that giving the results of a single concentration may be quite misleading. Therefore one can suggest that in all studies a range of mercurial concentration be used, such as to provide different degrees of inhibition, preferably from 0 to 100%. Second, values of K_i, which are so useful in other inhibitions, are difficult if not impossible to determine by the usual procedures, especially when the systems are in zones B or C, in which case pI_{50} may vary greatly depending on the enzyme concentration. It is evident that for work with pure enzymes it is best to state the amount of inhibitor present in terms of μmoles per milligram of enzyme, or if the molecular weight of the enzyme is known to express this as a molar ratio. However, when impurities are present, and especially when preparations such as homogenates are used, this method is not as useful and even a designation such as μmoles of mercurial per milligram of total protein is not very meaningful. Third, as we have discussed previously, mercurial inhibition is

strongly dependent on several factors, such as pH, temperature, and medium composition, so that these conditions should be stated accurately and completely, and it should always be realized that the results reported apply only to these particular conditions.

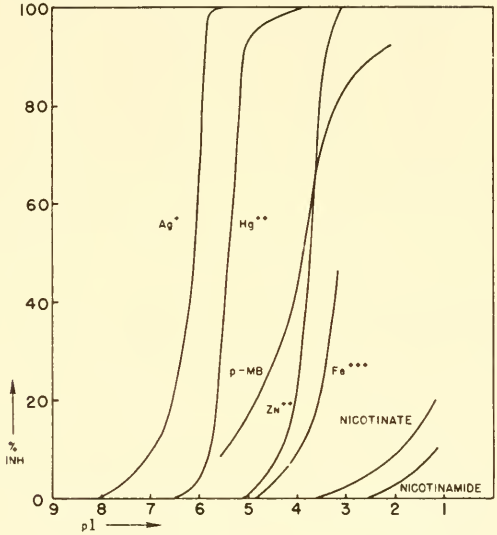


FIG. 7-31. Inhibitions of glutamate dehydrogenase, showing the relative potencies of the various inhibitors. Glutamate = 11 mM, NAD = 0.17 mM, and pH 7.6. (From Olson and Anfinsen, 1953.)

Inhibition of ATPase

The results of the actions of the mercurials on ATPase were not included in Table 7-13 because they are complex and warrant more detailed treatment, particularly as the effects of mercurials on mitochondrial and myosin ATPase are of importance in the work on oxidative phosphorylation and muscle contraction, respectively. Some of the reported inhibitions of ATPase are shown in Table 7-14; the stimulation of ATPase under certain conditions has been omitted since it was presented in Table 7-11. One immediately notes a very great variation in results. This is due partly to the different sources of the enzyme, but also to the different conditions under which the experiments were run. The response to mercurials depends on the state of activation of the enzyme, whether Ca^{++} or Mg^{++} is present, the pH, and the temperature, as well as the obvious factors of buffers and nonenzymic protein. The pH actually determines whether stimulation or inhibition will

TABLE 7-14

INHIBITION OF ATPASES BY THE MERCURIALS

Tissue	Preparation	Source	pH	Mercurial	Concentration (m*M*)	% Inhibition	Reference
—	Ghost fraction (Ca^{++}) (Mg^{++})	*Bacillus megaterium*	7.2	*p*-MB	0.1	8 36	Greenawalt *et al.* (1962)
Leaf	Chloroplasts	Spinach	7.5	*p*-MB	0.1	62	Wessels and Baltscheffsky (1960)
Spermatozoa	Tail enzymes	*Mytilus edulis*	8.6	*p*-MB	0.1	87	Nelson (1959)
Whole animal	Particulate	Mosquito	7.4	*p*-MB	0.1	0	Avi-Dor and Gonda (1959)
Electric organ	Microsomal (K^+-Na^+)	Eel	7.1	*p*-MB	0.1	96	Glynn (1963)
Kidney	Endoplasmic reticulum	Rat	6.1 6.6 7.2 7.9 8.2 6.1 7.1 7.9	Mercapto-merin Meralluride	3.4 12.1	43 43 33 20 17 0 22 38	Landon and Norris (1963)
Erythrocyte	Stroma	Human	7.4	*p*-MB	0.04	50	Herbert (1956)
			7.5	Hg^{++}	4.7	100	Caffrey *et al.* (1956)

			7.4	Hg^{++}	0.00125	50	Laris *et al.* (1962)
	Membrane	Chicken	7.4	*p*-MB	0.025	65	Engelhardt (1958)
Spleen	Purified enzyme	Beef	5.8	*p*-MB	1	40	Revel and Racker (1960)
Liver	Mitochondria	Rat	7.4	*p*-MB	0.5	73	Novikoff *et al.* (1952)
			7.4	*p*-MB	0.05	87	Sickevitz *et al.* (1958)
					0.1	99	
			7.4	PM	0.003	70	Wadkins (1960)
	Mitochondrial fragments	Mouse	7.5	*p*-MB	3	35	Löw *et al.* (1959)
Brain	Dispersion	Rat	7.4	Hg^{++}	0.0025	6	Gordon (1953)
	Membrane (K^+-Na^+)	Beef	7.6	*p*-MB	0.01	53	Skou (1963)
Heart	Mitochondrial fragments	Beef	7.4	*p*-MB	0.5	0	Penefsky *et al.* (1960)
Muscle	Mitochondria	Pigeon		PM	1.67	82	Azzone *et al.* (1960)
	Mitochondrial fragments	Rabbit	6.8	Hg^{++}	0.0005	11	Fujie (1959)
					0.001	68	
					0.002	94	
	Homogenate	Rabbit	6.8	*p*-MB	4	77	Bailey and Marsh (1952)
	Myosin	Cockroach	7.4	*p*-MB	0.005	7	Sacktor *et al.* (1953)
					0.01	40	
					0.05	82	
					0.1	89	
		Housefly	7.4	*p*-MB	0.1	0	Sacktor (1953)
					0.5	40	
					1	57	

TABLE 7-14 (*continued*)

Tissue	Preparation	Source	pH	Mercurial	Concentration (m*M*)	% Inhibition	Reference
		Mytilus	8.0	*p*-MB			
		Adductor			0.01	0	Kishimoto (1961)
					0.1	98	
		Byssus			0.0001	60	
		Rabbit	7.4	*p*-MB	0.013	100	Singer and Barron (1944)
			9.0	PM	0.6	80	Polis and Meyerhof (1947)
					1.2	100	
			7.0	PM	0.006	54	Greville and Needham (1955)
					0.01	77	
			7.6	*p*-MB	0.08 μmole/mg	66	Kielley and Bradley (1956)
			7.2	*p*-MB	0.0022	50	Gilmour and Griffiths (1957)
			7.4	*p*-MB	1	100	Gergely and Maruyama (1960)
			6.7	*p*-MB	0.07 μmole/mg	75	Tonomura and Kitagawa (1960)
			7.4	MM	0.063 μmole/mg	73	Kominz (1961)
					0.068 μmole/mg	88	
			8.5	*p*-MB	0.04 μmole/mg	8	Stracher and Chan (1961)
			9.5			65	

be exhibited over a wide range of mercurial concentration (Fig. 7-32). The fairly symmetrical curves for myosin ATPase, the maximal stimulation being observed at a pH around 7.5, are most likely the result of changes in ionizable groups at or near the active center, whereas the more complex curve for mitochondrial ATPase perhaps arises from additional factors related to mitochondrial structure or the effects of intramitochondrial components on ATPase. It may also be mentioned that Tonomura and Furuya (1960) found essentially the same behavior for myosin B ATPase, stimulation being maximal at pH 7.8 and absent at 5.7 and 10.

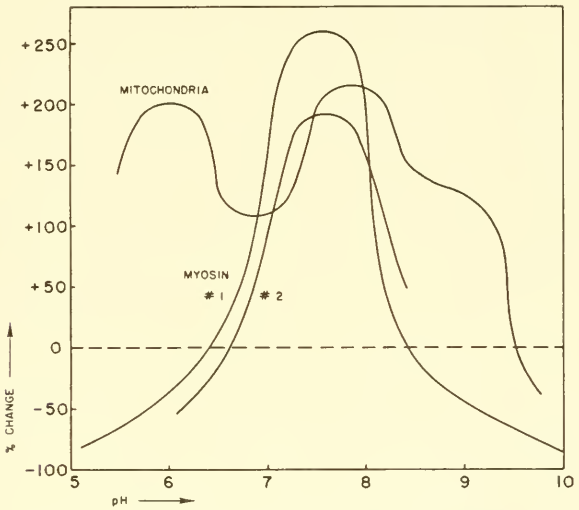


FIG. 7-32. Effects of pH on the actions of *p*-MB on ATPase. Liver mitochondrial ATPase treated with *p*-MB at 0.1 m*M* (Myers and Slater, 1957 b). Myosin ATPase curve ≷1 treated with *p*-MB at 0.04 μmole/mg (Stracher and Chan, 1961), and curve ≷2 treated with *p*-MB at 0.0872 μmole/mg (Blum, 1962 a).

When ATP is added to a preparation of myosin ATPase, there is an initial burst of phosphate release, followed by a steady level of hydrolysis. The effects of *p*-MB on these two phases of activity have been shown to be quite different by Tonomura and Kitagawa (1957, 1960). There is a progressive depression of the magnitude of the initial burst as the SH groups are titrated, but the steady rate is accelerated until around 80% of the groups have been combined (Fig. 7-33). The rate of the initial burst may be stimulated but the amount of ATP split during this period is reduced. How these observations correlate with the various theories of how mercurials activate ATPase (page 819) is not known; for example, does the initial

burst have anything to do with the postulated structural rearrangements of the enzyme following addition of ATP? In any event, there is evidence that mercurials can alter not only actomyosin stability itself (Gergely *et al.*, 1959) but the structure (Kominz, 1961) or flexibility (Levy *et al.*, 1962) of the enzyme near the active center.

The configurational changes are apparently complex inasmuch as measurements of the optical rotatory dispersion indicate that *p*-MB up to 4 moles/10^5 g myosin A increases the helical content several per cent, but addition of 8 moles/10^5 g decreases the helical content (Tonomura *et al.*, 1963 a). It was suggested that these small changes in the helical structure

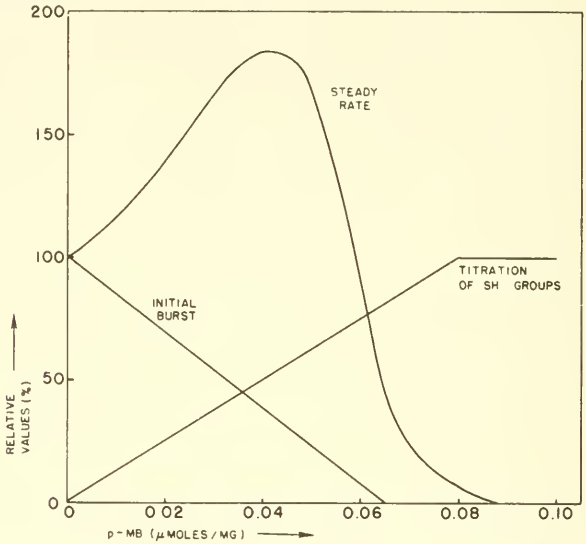


FIG. 7-33. Titration of myosin B ATPase with *p*-MB and the effects on the initial and steady-state rates at pH 6.7 and 20°. The SH reaction measured by absorption at 255 mμ. (From Tonomura and Kitagawa, 1960.)

induce modifications at the active site. If the inactivated enzyme is treated with β-mercaptoethanol the mercurial is removed, the activity is restored, and the rotatory dispersion returns to normal (Tonomura *et al.*, 1963 b). One might then assume that the effects of the *p*-MB are completely reversible; however, it was found that the substrate inhibition at high concentrations of ATP no longer occurs, and that EDTA now does not inhibit. It may be that the treatment with *p*-MB removes divalent cations, possibly the tightly bound Ca^{++}. It was indeed shown that the progressive depression of the ATPase activity is accompanied by a loss of the binding of Ca^{++} and Mg^{++} (Martonosi and Meyer, 1964).

The interesting treatment of the kinetics of myosin ATPase inhibition with *p*-MB recently reported by Walter (1963) should be consulted for the theoretical development of the equations and appropriate plotting procedures, but in connection with our present subject it is worth noting that the kinetics are complicated by two factors which should be kept in mind in such work on all enzymes. First, the reaction of the first SH groups does not lead to inactivation immediately. Second, this initial reaction reduces the mercurial concentration so that the more slowly reacting groups are exposed to a much lower concentration than was originally added. The calculated bimolecular rate constant for these catalytically important SH groups is thus quite different than that obtained from the initial rate.

The relationship between the effects of mercurials and 2,4-dinitrophenol is very interesting and the results indicate that SH groups are involved in the stimulation of ATPase activity by the latter substance. Lardy and Wellman (1953) noted that 0.04 m*M* *p*-MB almost completely abolishes the activation of mitochondrial ATP splitting by DNP, and others not only have confirmed this in general but have shown that the DNP-activated enzyme is only inhibited by mercurials (Greville and Needham, 1955; Gilmour and Griffiths, 1957; Pullman *et al.*, 1960). This is shown for myosin ATPase in Fig. 7-30. However, many factors can influence these interactions. Myers and Slater (1957 b) found that *p*-MB inhibits the DNP-activated mitochondrial ATPase from pH 6 to 8, but stimulates further at pH 9, and Cooper (1958 b) has emphasized the importance of Mg^{++}, addition of this ion decreasing the inhibition by *p*-MB of DNP-activated enzyme, as originally shown by Lardy and Wellman. Not all ATPases may behave in this fashion, and the activity of a particulate preparation from *Rhodospirillum rubrum* can still be stimulated by DNP in the presence of *p*-MB and Mg^{++} (Cooper, 1958 a). Some investigators have postulated that DNP and the mercurials activate ATPase by similar mechanisms, but it seems doubtful if the evidence is sufficient to draw this conclusion. It has recently been found that CO_2 stimulates mitochondrial ATPase markedly and this occurs in the presence of 0.005 m*M* Hg^{++},which itself has broght about activation; indeed, Hg^{++} activates about the same in the absence or presence of CO_2 (Fanestil *et al.*, 1963). These stimulations thus appear to be approximately additive. The K^+-Na^+-activated membrane ATPase contains SH groups which seem to be specially involved in this activation and react readily with mercurials (Skou, 1963).

The interesting effects of the mercurials on the P_i-ATP and ADP-ATP exchange reactions occurring in mitochondria and the relations to ATPase will be discussed later under oxidative phosphorylation (page 872).

ELECTRON TRANSPORT AND OXIDATIVE PHOSPHORYLATION

The majority of dehydrogenases are quite sensitive to the mercurials, and inspection of Table 7-13 shows that 50% inhibition is commonly produced by concentrations of 0.001–0.05 m*M*. If we define NADH dehydrogenase as the enzyme component responsible for the transfer of electrons to a variety of acceptors, it must be placed in the same category with respect to sensitivity, and, indeed, NADH-cytochrome c reductase is usually even more susceptible, often being completely inhibited by concentrations of 0.001–0.01 m*M*. One is thus tempted to attribute the inhibition of various oxidations by the mercurials to an action early in the electron transport chain, at least pre-cytochrome. Furthermore, Barron and Singer (1945) had reported that the oxidation of reduced cytochrome c by a cytochrome oxidase preparation is not affected by *p*-MB, and this has more recently been observed with *Arum* (Simon, 1957) and *Penicillium* (Sih *et al.*, 1958) cytochrome oxidases. Finally, some have found that certain oxidases and the corresponding dehydrogenases are inhibited equally by mercurials, although the different conditions of testing in such cases make accurate comparison difficult.

This simple picture of inhibition in the electron transport sequence has, however, been questioned by workers at the Institutum Divi Thomae, who from 1946 to 1957 obtained increasing evidence that the cytochrome system may not be as immune to mercurials as generally imagined. Their results may be summarized in the four following categories. (1) Cytochrome oxidase activity, as determined by the oxidation of ascorbate, is inhibited rather potently, 50% depression being observed with 0.006–0.012 m*M* PM and 0.032 m*M* *p*-MB (Cook *et al.*, 1946; Kreke *et al.*, 1950). (2) Succinate oxidase is much more sensitive to mercurials than is succinate dehydrogenase. It requires around 10 times the concentration to inhibit rat heart succinate dehydrogenase compared to the oxidase (Cook *et al.*, 1946; Kreke *et al.*, 1949; Smalt *et al.*, 1957). (3) The inhibitions of cytochrome oxidase and succinate oxidase are not reversed by thiols (Cook and Perisutti, 1947; Kreke *et al.*, 1949, 1950). This led them to suppose that the inhibition might not involve SH groups, but this conclusion, as we have seen, is not valid. (4) No evidence for reaction of the mercurials with ascorbate or cytochrome c could be obtained by spectroscopic or preincubation techniques (Cook *et al.*, 1946; Kreke *et al.*, 1950). It may also be mentioned that Boeri and Tosi (1954) found no reaction of *p*-MB with cytochrome c, and that Strittmatter and Velick (1956) likewise found no change in microsomal cytochrome spectral absorption after incubation with 1 m*M* *p*-MB. All of these data have been interpreted as indicating that the mercurials may exert a major part of their effect on cytochrome oxidase.

Slater (1949) had also observed that succinate oxidase is inhibited more strongly than the dehydrogenase by *p*-MB (and also by *o*-iodosobenzoate and oxidized glutathione), although the difference was not as great as reported by Cook, Kreke, and their co-workers, and attributed this in the particulate preparations used to an effect on some link between the dehydrogenase and the oxidase, presumably occurring before cytochrome c in the chain. This effect might be a structural disorganization of the complex to interrupt the flow of electrons. Nevertheless, Slater observed some inhibition of cytochrome oxidase. Seibert *et al.* (1950) made a solubilized deoxycholate preparation of cytochrome oxidase and found by both manometric and spectrophotometric tests that it is inhibited to the same degree as the crude preparation; they also demonstrated shifts in the spectral bands of the oxidase following treatment with the mercurials. The final conclusion of the Institutum Divi Thomae group is that the actions of the mercurials on heme enzymes may be nonspecific, may involve denaturation (which could account for the spectral shifts), and do not involve SH groups, but I doubt if there is sufficient evidence for any of these statements. However, their data, which are definite and consistent, must be explained on some basis. It is important to realize that the inhibitions reported for "cytochrome oxidase" were all obtained with ascorbate (and occasionally hydroquinone) as the substrate. Now neither ascorbate nor hydroquinone is oxidized directly by cytochrome oxidase and the electron transfer occurs through a series of components. It has usually been assumed that ascorbate reduces cytochrome c_1 or c, in which case the action of the mercurials could be on some component or link in the cytochrome sequence, rather than on cytochrome oxidase itself. It will be remembered that the work quoted at the beginning of this section showed that, when cytochrome c is used as substrate, the mercurials do not inhibit. Is it possible that there is a component which might be designated as ascorbate dehydrogenase, which is sensitive to the mercurials? Seibert *et al.* (1950) actually observed relatively little inhibition of the purified system when determined spectrophotometrically with cytochrome c as the substrate.

There are several ways of explaining the differential inhibitions of succinate dehydrogenase and oxidase. Since the activities of these two systems are determined very differently — the dehydrogenase usually by methylene blue reduction and the oxidase manometrically — one must question if this could be responsible for the different sensitivities observed. The dehydrogenase activity associated with methylene blue reduction might not be exactly the same as in the normal transfer of electrons to the cytochromes; i.e., a region of the enzyme surface, or another component in the chain, might be involved in the normal transfer but not in the dye reduction, and this part of the system could be sensitive to the mercurials. If we look into the details of the procedures (Kreke *et al.*, 1949), we find that

in the dehydrogenase test the pH was 7.2 and the succinate concentration 0.33 m*M*, whereas in the oxidase test the pH was 7.4 and the succinate 88 m*M*; the former was done in strong phosphate buffer, whereas the latter medium contained 0.7 m*M* Ca^{++} and Al^{+++}; in addition, the times for equilibration and incubation were different. When the conditions are so diverse, it is impossible to compare these two systems quantitatively. The structural interference theory of Slater must also be considered and has as much evidence as the other explanations (i.e., none). It would be important to know just how much effect mercurials can exert on the cytochrome system, inasmuch as it has obvious bearing in considerations of the actions on various oxidations, mitochondrial systems, and respiration.

A comparable situation with NADH dehydrogenase, NADH: cytochrome c reductase, and NADH oxidase has been noted by Minakami *et al.* (1963). The total oxidase and the cytochrome c reductase are very sensitive to *p*-MB whereas the dehydrogenase, as determined by ferricyanide reduction, is not as sensitive. It was postulated that two types of SH group are involved in NADH oxidation, one readily accessible to mercurials and functioning between the dehydrogenase active site and the distal respiratory chain (this SH group is not required for ferricyanide reduction), and a second concealed in the dehydrogenase complex as isolated, and exposed on degradation to the cytochrome c reductase. Such an explanation could apply to the succinate oxidase as well, as was suggested above relative to methylene blue as an acceptor for the determination of dehydrogenase activity.

Oxidative Phosphorylation

The results summarized in Table 7-15 show that mercurials are not particularly specific or effective uncouplers of oxidative phosphorylation in mitochondria, but that a fair degree of uncoupling can occur under certain circumstances. It is especially interesting that high toxic doses of the mercurial diuretics and $HgCl_2$ can often reduce the P : O ratio in the mitochondria of excised kidneys several hours after the administration, without simultaneously affecting oxidative phosphorylation in the liver, but this is undoubtedly due to the higher concentration of mercurial in the kidney. The P : O ratio is, however, not altered significantly by the ordinary diuretic doses, so that it is questionable if this action is related to diuresis. I know of no instance in which the mercurials augment O_2 uptake while simultaneously reducing the P_i incorporation, so that they are not true uncouplers in the same sense as the nitrophenols.

The P_i^{32}-ATP exchange is quite potently inhibited by mercurials in the mitochondria obtained from mosquitoes (Avi-Dor and Gonda, 1959), pig liver (Chiga and Plaut, 1959), and rat liver (Plaut, 1957; Cooper and Lehninger, 1957; Lehninger *et al.*, 1958; Löw *et al.*, 1958). For rat liver mitochondria the exchange is sometimes inhibited 50% by concentrations

TABLE 7-15

EFFECTS OF MERCURIALS ON OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIAL PREPARATIONS

Source	Substrate	Mercurial	Dose or concentration (mM)	Effect on P:O ratio	Reference
Micrococcus lysodeikticus	NADH	*p*-MB	0.01	0.33 → 0.27	Ishikawa and Lehninger (1962)
			0.1	0.33 → 0.67	
Sweet potato	Citrate	*p*-MB	0.01	2.28 → 2.23	Hackett *et al.* (1960)
Rat liver	Malate	Mersalyl	Toxic dose	No effect	Cohen (1953 b)
	Choline	*p*-MB	0.05	Decrease	Rothschild *et al.* (1954)
	β-OH-butyrate	Hg^{++}	0.1	2.7 → 0	Hunter and Ford (1955)
	Succinate	Hg^{++}	0.0033	1.64 → 1.68	Jacobs *et al.* (1956)
Rat kidney	Pyruvate	Hg^{++}	0.056	0.43 → 0.22	Hirade (1952)
	Malate	Mersalyl	0.027 millimole/kg	No effect	Cohen (1953 a)
		Mercaptomerin	10.7 mg Hg/kg	0.57 → 0.76	Cohen (1953 a)
			26.7 mg Hg/kg	0.55 → 0.092	
	Glutamate	Chlormerodrin	20 mg Hg/kg	No effect	Greif and Jacobs (1958)
	α-Ketoglutarate	Hg^{++}	3 mg Hg/kg	2.1 → 1.1	Shore and Shore (1960)
Rat brain	Pyruvate	*p*-MB	0.03	No effect	Abood and Romanchek (1955)
Beef heart	Succinate	*p*-MB	0.01	− 79%	Penefsky *et al.* (1960)

around 0.002 mM, and completely by 0.01 mM. The ADP^{32}-ATP and ADP-C^{14}-ATP exchanges are also inhibited, but perhaps not as strongly (Wadkins and Lehninger, 1958; Chiga and Plaut, 1959; Kahn and Jagendorf, 1961). These exchange reactions are related intimately with oxidative phosphorylation. Indeed, Wadkins and Lehninger (1958) postulated that the P_i-ATP exchange is a measure of the two terminal reactions in oxidative phosphorylation:

$$\text{Carrier} \sim X + P_i \leftrightarrows \text{carrier} + P \sim X$$
$$P \sim X + ADP \leftrightarrows ATP + X$$

where X is perhaps the enzyme protein, while the ADP-ATP exchange measures only the last reaction. They further suggest that the mercurials inhibit this last reaction principally, whereas 2,4-dinitrophenol acts on the penultimate step. If the mercurials act solely on the transfer of phosphate to ATP they would be good uncouplers, but actions elsewhere in the electron transport chain limit their efficiency. It is interesting that Griffiths and Chaplain (1962) have found evidence for a new phosphorylated derivative of NAD following incubation of heart mitochondria with succinate and P_i^{32}. ATP can be formed from the intermediate and this reaction is completely blocked by *p*-MB at 0.01 mM. This observation is compatible with the scheme of Wadkins and Lehninger.

FERMENTATION AND GLYCOLYSIS

The first impression from surveying the studies of mercurial action on fermentation and glycolysis is that these pathways are often surprisingly insensitive to this group of inhibitors. In many cases it requires concentrations greater than 1 mM to depress glycolysis significantly in cellular systems. Reference to Table 7-13 shows that several enzymes in the Embden-Meyerhof pathway are quite readily inhibited by mercurials, e.g., hexokinase, aldolase, 3-phosphoglyceraldehyde dehydrogenase, enolase, and lactate dehydrogenase, concentrations of 0.001-0.05 mM usually inhibiting 50% or more in muscle, although little is known about the sensitivities of the yeast enzymes. Since several enzymes in the pathway are susceptible, one might anticipate that the sequential inhibition by mercurials at concentrations above 0.05 mM would produce a very strong over-all depression of anaerobic CO_2 or lactate formation. Three explanations for the failure to do so are immediately apparent: (1) The mercurials do not penetrate into the cells readily; (2) the glycolytic enzymes are protected in the cell (e.g., by substrates or coenzymes); and (3) the large amount of nonenzymic material in cellular preparations binds much of the mercurial. Cleland (1949) found that 1 mM PM inhibits glycolysis in oyster eggs only 17% at 0–45 min and 48% at 45–90 min, whereas glycolysis in egg homogenate (with

glycogen, ATP, and NAD added) is completely blocked. Similar results were obtained with sea urchin eggs by Cleland and Rothschild (1952 a), 1 m*M* PM inhibiting lactate formation 17% in whole eggs and 97% in extracts. These results were interpreted to indicate poor penetration by the mercurial, but the other two explanations given above are probably as likely, and undoubtedly all contribute to some extent. It may be noted that Cleland found endogenous respiration to be inhibited more potently in whole eggs than in homogenates, which is more difficult to explain. There are instances of cellular glycolysis quite sensitive to the mercurials; in ascites carcinoma cells there is 50% inhibition by 0.0032 m*M* Hg^{++} (Schöm *et al.*, 1961). The glycolysis in spleen slices is also fairly sensitive, although it requires 4–5 hr to reach maximal inhibition (Fig. I-12-24) (Jowett and Brooks, 1928). The interesting questions of the penetration of mercurials and the effects exerted on cell membranes will be considered later (page 892).

Yeast Fermentation

Since the early work of Schulz (1888), who reported an initial stimulation of fermentation by low concentrations of Hg^{++} (0.005–0.008 m*M*) and inhibition by higher concentrations (>0.02 m*M*), there have been many studies of yeast fermentation with variable results. The stimulation observed by Schulz has seldom been confirmed. Joachimoglu (1922) could never demonstrate acceleration of CO_2 formation by Hg^{++}, concentrations of 0.0031–0.037 m*M* exerting no effect and 0.074 m*M* inhibiting around 70%. Meier (1926) found even more potent inhibition of aerobic fermentation, 0.009 m*M* Hg^{++} depressing 72%, while Kostytschew and Berg (1930) never observed stimulation, inhibition beginning at 0.0185 m*M* Hg^{++} and reaching 42% at 0.2 m*M*. More recently, some have found potent inhibition by Hg^{++} (e.g., Hurwitz and Chaffee, 1954), but others have not (e.g., Weitzel and Buddecke, 1959); in the latter work, 1 m*M* Hg^{++} inhibited only 70% in fermenting yeast. Organic mercurials have not been often used, but Spiegelman *et al.* (1948) reported 69% inhibition by 0.01 m*M* PM and 32% inhibition by 0.05 m*M* *p*-MB, indicating these mercurials to be fairly effective. Certainly much of the variation in the results is due to the different densities of yeast suspension used, the media employed, and the state of the yeast (by which is meant its fermentative activity and past history). One would expect mercurials to attack surface hexokinase and the initial phosphorylation of glucose, as occurs in muscle, so one can explain the examples of weak inhibition only on the basis of relatively dense yeast suspensions.

Muscle Glycolysis

The results obtained on muscle glycolysis with the mercurials have been quite inconsistent and even more difficult to explain than those with yeast.

Gemmill and Hellerman (1937) found that Hg^{++}, *p*-MB, and PM all block glycolysis in extracts of frog muscle, but the concentrations used were too high and usually unspecified. Separated fibers of cockroach muscle treated with 1 m*M* *p*-MB show no change of CO_2 formation and a rather marked increase in lactate formation if only glucose is added, but in the presence of glucose + ATP, CO_2 production is inhibited 67% and there is no effect on lactate (Barron and Tahmisian, 1948). This behavior is quite different from that of iodoacetate, which inhibits only in the absence of added ATP. The authors felt that the failure to depress lactate formation in any case is perhaps a characteristic of invertebrate muscle, since Harting (1947) had observed 1 m*M* *p*-MB to produce only stimulation of glycolysis in strips of scallop and thyone muscle. However, Krueger (1950) has shown that 2 min perfusion of frog muscle with 37 m*M* Hg^{++} essentially doubles the lactate formation. The only serious study of muscle glycolysis was done by Bailey and Marsh (1952) on rabbit psoas homogenates. Here *p*-MB produces definite inhibition (see accompanying tabulation), but the concentration is so

	Control	*p*-MB 4 m*M*
Δ pH	− 0.28	− 0.08
Δ Fructose-diP	+27	+ 6
Δ Triose-P	+ 3	− 1
Δ ATP	−14	− 5
ATP resynthesis	45	13
ATPase inhibition		77%

high that it is remarkable that the inhibition is not much greater. It was believed that aldolase inhibition is responsible for the results but from the data it is not possible to localize the site of action so closely. The authors pointed out that 3-phosphoglyceraldehyde dehydrogenase is not so readily inhibited by *p*-MB as by iodoacetate. The transfer of phosphate from creatine-P to ADP is immediately and completely blocked by *p*-MB, so that creatine-P remains at its initial level, and this must also be a factor in the inhibition, since it would prevent regeneration of ATP. It is thus impossible in this study to determine what effects *p*-MB might have directly on the Embden-Meyerhof pathway. All of the results on intact muscle tissue seem to be incompatible with the demonstration by Demis and Rothstein (1955) that glucose uptake by diaphragm is very sensitive to Hg^{++}, being almost completely inhibited by 0.2 m*M*. However, respiration and anaerobic lactate formation, being dependent on endogenous substrate, are much less sensitive and are only slowly inhibited. This will be considered in greater detail when the effects of mercurials on respiration are discussed (page 884).

Stimulation of glycolysis by the mercurials is not confined to yeast and muscle. Hg^{++} below 0.11 mM stimulates glycolysis in guinea pig blood and inhibits in higher concentration (Fuentes and Rubino, 1923), while in human blood Hg^{++} stimulates anaerobic glycolysis from 0.0185 to 1.85 mM although at 18.5 mM there is almost complete inhibition (Rubino and Varela, 1923). Glucose utilization, CO_2 release, and lactate formation in human erythrocytes are all stimulated by *p*-MB up to 5 μmoles/ml of erythrocytes (Jacob and Jandl, 1962). No explanation for these results is immediately evident.

TRICARBOXYLATE CYCLE

Despite the fact that no analysis of the effects of mercurials on the cycle or on the operation of mitochondria has been made, one would predict quite potent inhibition on the basis of the sensitivities of the individual enzymes (Table 7-13). Mercurial concentrations in the neighborhood of 0.01 mM should depress several enzymes very significantly (e.g., pyruvate oxidase, isocitrate dehydrogenase, α-ketoglutarate oxidase, succinate dehydrogenase, malate dehydrogenase, and some ancillary enzymes, such as acetate kinase), and concentrations of the order of 0.1 mM should block completely. However, since we have already noted that glycolysis is often not inhibited as much as one would expect from studies of the individual enzymes, we must be very careful in considering inhibitions of the cycle in cellular preparations. The utilization of pyruvate and acetate by a variety of cellular and subcellular preparations has been shown to be readily inhibited by mercurials (Table 7-16), but in no case was the operation of the entire cycle tested, so that the entire inhibition, as far as one knows, might be on the initial enzyme reaction (pyruvate oxidase or acetate kinase). If the cycle is operating by regenerating oxalacetate, much stronger inhibition would undoubtedly be observed. In work with mitochondria, homogenates, or cell suspensions, however, one must always remember the role of nonenzyme protein in reducing the mercurial available for inhibition, so that concentrations such as those in Table 7-16 are not of much quantitative significance, but show definite interference with cycle activity.

In order to answer some of these questions relative to the action of the mercurials on the cycle, Dr. Yang kindly consented to examine the effects of Hg^{++} on the O_2 uptake of rabbit heart mitochondria by the same techniques used in a previous study of iodoacetate (Yang, 1957). The changes over a 60 min period obtained from results on three preparations are shown in the accompanying tabulation. These data show clearly that several steps in the cycle are inhibited rather strongly as the concentration is raised from 0.003 mM to 0.01 mM, and that at 0.1 mM the cycle activity is essentially completely blocked. The stimulation observed with α-ketoglutarate as the

TABLE 7-16

INHIBITION OF THE OXIDATIONS OF CYCLE SUBSTRATES BY MERCURIALS

Preparation	Substrate	Mercurial	Concentration (mM)	% Inhibition	Reference
Acetobacter melanogenum	Pyruvate	*p*-MB	0.1	100	Bone and Hochster (1960)
Escherichia coli	Pyruvate	Hg^{++}	0.033	100	Avi-Dor *et al.* (1956)
Sweet potato mitochondria	Citrate	*p*-MB	0.1	13	Hackett *et al.* (1960)
Chicken liver suspension	Pyruvate	*p*-MB	0.5	92	Barron and Singer (1945)
Guinea pig liver mitochondria	Glutamate	*p*-MB	0.03	46	Chari-Bitron and Avi-Dor (1959 a)
Rat liver mitochondria [a]	Glutamate	*p*-MB	0.1	49	Corwin and Schwarz (1963)
Rat heart mitochondria	Pyruvate	*p*-MB	0.01	19	Reiss and Hellerman (1958)
			0.05	100	
Rat brain mitochondria	Pyruvate [b]	*p*-MB	0.03	21	Abood and Romanchek (1955)
Rat Walker carcinoma mitochondria	Pyruvate [b]	*p*-MB	0.1	87	Hellerman *et al.* (1962)
Rabbit heart mince	Acetate	*p*-MB	0.25	24	Barron *et al.* (1953)
			0.5	83	
			1	95	

[a] Mitochondria from livers of vitamin E deficient rats; if rats were fed vitamin E there was no inhibition.

[b] Malate or fumarate present to supply oxalacetate.

Substrates	% Changes from Hg^{++} at:			
	0.001 m*M*	0.003 m*M*	0.01 m*M*	0.1 m*M*
Pyruvate + malate	−10	—	−79	− 99
α-Ketoglutarate	+24	+5	−74	−100
Succinate	0	+5	−41	−100
Malate	− 6	—	−79	− 99

substrate seems to be real since it was consistently obtained. The inhibition with pyruvate + malate as substrates and Hg^{++} at 0.01 m*M* is about 50% at 10 min and then increases more slowly until it is 100% at 60 min. The figures in the tabulation are mean inhibitions over the 60 min period and even at 0.01 m*M* the activity was almost all lost in all cases by 60 min.

RESPIRATION

The effects of the mercurials on the O_2 uptake of tissues vary considerably and depend on the mercurial used, the substrate, the pH, the species, and many other factors (Table 7-17). One factor about which little is known, but which could be very important, is the thickness of the tissue when the preparation is a strip, section, or slice, inasmuch as the mercurial possibly does not penetrate equally throughout but acts primarily on the outer layers of cells. Cascarano and Zweifach (1962) examined rat diaphragm after exposure to Hg^{++} by determining the ability of the tissue to reduce a tetrazolium dye, and found that only a well-defined band of surface fibers had lost the ability, the central portions retaining activity. Measurements of respiratory inhibition in such cases do not provide true values (see page I-479); in the extreme case the inhibition may relate only to the fraction of the tissue affected, and progressively developing inhibition may exhibit time relations dependent only on the rate of penetration through the tissue. This would apply not only to respiration, of course, but to all measurements, metabolic or functional, made on all intact tissues. Failure to reach all of the cells equally must be one reason for the low degree of inhibition often observed, lower than would be predicted from the effects on glycolysis, the cycle, and the enzymes involved.

One notes several examples wherein respiration is stimulated by the mercurials, more often at low concentration but in one instance, yeast respiring endogenously (Shacter, 1953), the stimulation appears only at high concentrations of 1–2 m*M*. There are other reports of stimulation not included in the table. For example, Gremels (1929) found that when mersalyl induces diuresis in a heart-lung-kidney preparation, the kidney respiration

TABLE 7-17

INHIBITION OF RESPIRATION BY THE MERCURIALS

Organism	Preparation	Substrate	Mercurial	pH	Conc. (m*M*)	% Inhibition	Reference
Staphylococcus aureus	Suspension	Glucose	Hg^{++}	7.0	0.0037	~80	Claus (1956)
		Glucose	Hg^{++}	7.4	0.08	50	Yamada and Yanagita (1957)
			Thiomersol	7.4	0.045	50	
Bacterium lactis	Suspension	Glucose	Hg^{++}	7.0	0.0022	~65	Claus (1956)
Brevibacterium flavum	Suspension	Glucose	*p*-MB	7.0	1	100	Shiio *et al.* (1961)
Rhodospirillum rubrum	Suspension	Succinate	PM	7.0	0.013	50	Horio and Kamen (1962 b)
Aspergillus niger	Suspension (30 min)	Sucrose	Hg^{++}	7.2	0.2	65	Cook (1926)
Trichophyton rubrum	Suspension	None	Hg^{++}	4.6	1	28	Nickerson and Chadwick (1946)
					10	79	
			PM	4.6	0.45	40	
Yeast	Suspension	Glucose	Hg^{++}	—	0.009	0	Meier (1926)
					0.018	28	
					0.037	100	
		Glucose	PM	7.2	0.032	90	Cook and Kreke (1943)
		Glucose	*p*-MB	4.5	0.01	5	Shacter (1953)
					0.05	34	
					0.1	70	
					1	100	
		None	*p*-MB	4.5	0.1	20	Shacter (1953)
					0.5	40	

					1	St	15	
					2	St	110	
		Glucose	Hg^{++}	7.0	1		80	Weitzel and Buddecke (1959)
		None	Hg^{++}	7.0	1		98	
		Acetate	Hg^{++}	7.0	0.3		10	Estler *et al.* (1960)
					1.25		95	
Schizophyllum commune	Mycelial pellets	Glucose	PM	7.0	0.1		97	Niederpruem and Hackett (1961)
Chlorella vulgaris	Suspension (nitrogen starved)	Glucose	Hg^{++}	6.5	0.03		91	Bach (1961)
		None	Hg^{++}	6.5	0.03		0	
	Suspension	None	Hg^{++}	6.5	0.005	St	5	Horwitz (1957)
					0.01	St	35	
					0.023	St	150	
					0.04		70	
					0.1		100	
Scenedesmus obliquus	Suspension	None	Hg^{++}	6.5	0.005	St	80	Horwitz (1957)
					0.01	St	50	
					0.023		60	
					0.04		85	
					0.1		100	
			p-MB	6.5	0.3	St	40	
					0.65		50	
Porphyra perforata	Suspension	None	*p*-MB	8.2	0.5		40	Eppley (1960)
Fucus ceranoides	Frond fragments	None	*p*-MB	8.2	0.2		0	Klemperer (1957)
					1		80	
Hormosira banksii	Thalli	None	*p*-MB	7.5	0.1		20	Bergquist (1958)
Sunflower	Stem sections	None	*p*-MB	—	0.1	St	2	Niedergang-Kamien and Leopold (1957)
					1	St	19	
			PM	—	0.1	St	20	
					1	St	13	

TABLE 7-17 (*continued*)

Organism	Preparation	Substrate	Mercurial	pH	Conc. (m*M*)	% Inhibition	Reference
Trichomonas vaginalis	Suspension	Glucose	*p*-MB	6.0	0.05	78	Ninomiya and Suzuoki (1952)
Toxoplasma gondii	Suspension	Glucose	*p*-MB	7.4	0.1	100	Fulton and Spooner (1960)
Echinus esculentus	Egg homogenate	None	PM	7.5	1	63	Cleland and Rothschild (1952 b)
Arbacia punctulata	Spermatazoa	None	*p*-MB	8.2(?)	0.1	St 57	Barron *et al.* (1948)
					1	100	
			Hg^{++}		0.005	St 88	
					0.1	100	
Oyster	Egg suspension	None	PM	7.5	0.01	0	Cleland (1949)
					0.1	10	
					1	80	
	Egg homogenate	None	PM	7.5	1	50	
	Spermatazoa	None	PM	7.5	0.01	20	Humphrey (1950)
					0.1	75	
Australorbis glabratus	Mince	None	*p*-MB	7.4	1	34	Weinbach (1953)
Echinococcus granulosus	Scolices	None	*p*-MB	7.4	10	76	Agosin *et al.* (1957)
Marinogammarus marinus	Whole amphipod	None	Hg^{++}	8.2	0.18	4	Hunter (1949)
Gasterosteus aculeatus	Whole fish (15 min)	None	Hg^{++}	—	0.02	St 25	Jones (1946)
Frog	Skiu	None	Hg^{++}	7.4	0.2	15	Linderholm (1952)
					1	68	
	Gastrula dorsal lip	None	*p*-MB	—	0.1	14	Ornstein and Gregg (1952)
					1	64	
	Gastrula ventral lip	None	*p*-MB	—	0.1	0	Ornstein and Gregg (1952)
					1	65	

Guinea pig	Kidney cortex slices	Glucose	Hg^{++}	—	0.0037	10	Meier (1933)
					0.037	63	
					0.37	100	
Mouse	Gastric mucosa	Glucose	*p*-MB	7.4	0.178	18	Davenport *et al.* (1954)
					0.71	26	
					1.41	54	
	Ehrlich ascites cells	Glucose	*p*-MB	7.4	0.1	5	Shacter (1957)
					0.46	50	
Rat	Liver mitochondria	β-OH-butyrate	Hg^{++}	7.4	0.1	99	Hunter and Ford (1955)
	Kidney slices	None	Mercapto-merin	7.4	0.09	11	Robinson (1956)
					0.18	22	
					0.37	29	
					1	31	
	Kidney slices	None	Mercapto-merin	7.4	0.02	6	Koishi (1959 b)
					0.1	9	
					1	28	
					5	42	
	Diaphragm	Glucose	*p*-MB	6.8	1	15	Haft and Mirsky (1952)
	Diaphragm	Glucose	Hg^{++}	7.4	0.5	0	Demis and Rothstein (1955)
					2	90	
	Heart slices	Glucose	Meralluride	7.4	5.8	57	Ruskin and Ruskin (1953)
Rabbit	Heart mince	Acetate	*p*-MB	7.4	0.25	24	Barron *et al.* (1953)
					0.5	83	
					1	95	
	Kidney slices	Glucose	Hg^{++}	7.4	0.33	0	Mudge (1951)
					1	22	
Sheep	Thyroid slices	Glucose	Hg^{++}	7.4	1	77	Slingerland (1955)
			p-MB	7.4	1	88	
			PM	7.4	1	81	

is increased. There has been no study of the mechanism whereby such stimulation is produced. One might postulate that the mercurials can increase membrane permeability so that substrates can enter cells more rapidly, but although this may be a factor it is clear that endogenous respiration can be stimulated, as in the work of Shacter. It is also known that subcellular preparations, and indeed certain enzymes themselves, are stimulated (page 815), so that one cannot expect to provide a comprehensive theory based only on cell and tissue responses. Shacter felt that the mercurials might react with certain thiols which regulate metabolism, but despite all the discussion of such regulators in the past, there seems to be little evidence at present for their importance. Of the several mechanisms suggested previously (page I-453), one is at a loss to select any that would apply particularly to the mercurials. Since mercurials have been shown to reduce the P:O ratio in various isolated systems, it is possible that in the cell an uncoupling action might increase O_2 uptake in a certain limited range of concentration, and it is also possible that the mercurials by a membrane effect might alter ion movements and concentrations, thus secondarily bringing about metabolic stimulation, but there is no direct evidence for either of these mechanisms. Intracellular changes are undoubtedly so complex that metabolic effects usually defy analysis. Consider the situation described by Estler *et al.* (1960) in yeast treated with Hg^{++} (Fig. 7-34), the levels of all the adenine nucleotides increasing at 0.2 m*M*, although O_2 uptake is scarcely affected, while at higher concentrations the nucleotides change in a complicated fashion and respiration is severely depressed. Unfortunately stimulation was not recorded here, but it is only by thorough studies of this type that one can hope to penetrate into the mysteries of inhibitor stimulation.

Although Meier (1926) reported that aerobic fermentation in yeast is more potently inhibited than respiration by Hg^{++} — at 0.009 m*M* the former was inhibited 72% and the latter not at all — subsequent work on a variety of cells has indicated no general relationship between the sensitivities of glycolysis and respiration, and even in yeast Weitzel and Buddecke (1959) found both to be inhibited similarly, at least at high concentration (1 m*M*) of Hg^{++}. The respiration of oyster eggs is inhibited more than glycolysis by PM (Cleland, 1949), whereas in oyster spermatozoa the respiration is inhibited by PM when glycolysis as measured by lactate formation is increased (Humphrey, 1950). The utilization of pyruvate in these cells may be more sensitive to mercurials than the glycolytic pathway. Certainly the inhibition of respiration does not imply a reduction in glucose uptake: In diaphragm, 1 m*M* *p*-MB depresses O_2 uptake 15%, lowers the glycogen content, and yet increases glucose utilization somewhat (Haft and Mirsky, 1952). In most cases (e.g., yeast and *Chlorella*), glucose respiration is more sensitive than endogenous respiration to mercurials, but this

has not been investigated sufficiently to draw valid conclusions. Of course, in tissues such as most muscle and heart, in which endogenous substrates are responsible for the bulk or all of the respiration for some after time excision, glucose would not be expected to have much effect on the inhibition by mercurials. Little is known about the effects of mercurials on the pentose-P pathway or other routes of glucose degradation. In crude extracts of *Pseudomonas* converting gluconate-6-P to 3-phosphoglyceraldehyde and

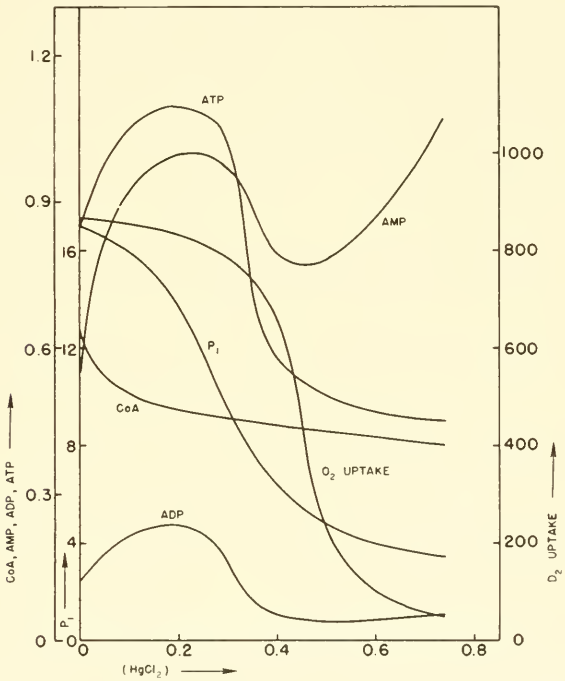


FIG. 7-34. Effects of Hg^{++} on the respiration and the levels of substances in yeast. Concentrations given as μmoles/g dry weight. Run at pH 6.8 for 60 min. (From Estler *et al.*, 1960.)

pyruvate, *p*-MB at 1 m*M* inhibits completely (Kovachevich and Wood, 1955). The site of the inhibition is not clear, but it is presumably not on the gluconate-6-P dehydrase, which was purified and found to be only moderately sensitive to *p*-MB. The pentose-P pathway is operative in extracts of tobacco leaves, the oxidations being NADP specific, and *p*-MB at 0.1 m*M* almost completely blocks the reduction of NADP by both glucose-6-P and fructose-1,6-diP (Clayton, 1959). The utilization of pentose-P by extracts of *Lactobacillus brevis* is inhibited only 20% by 0.1 m*M* *p*-MB

(Eltz and Vandemark, 1960). There are no data for comparing the relative sensitivities of the Embden-Meyerhof and pentose-P pathways.

As appears to have been the case with most of the workers who have examined the effects of the mercurials on respiration, I find little to say that seems worthwhile. The most interesting aspects of respiratory inhibition probably pertain to the metabolic basis of certain cellular functions, e.g., gastric secretion (page 914) or renal transport (page 917). The site of action to inhibit respiration is unknown and multiple sites are likely. We know little of the penetration of mercurials into cells and the intracellular concentrations attained, and the information is lacking to evaluate the importance of nonenzymic effects. These gaps in our knowledge apply not only to respiration but essentially to all cellular activities. One can at least state with fair certainty that the mercurials do not act like other SH reagents, such as iodoacetate or the arsenicals, i.e., their pattern of inhibition is quite different.

VARIOUS METABOLIC PATHWAYS

In this section we shall consider briefly some of the important types of metabolism which are readily inhibited by the mercurials. Only the more interesting aspects and interpretable investigations will be mentioned. The effects of the mercurials on metabolism are complex and vague in all cases, so it is essential to emphasize those studies in which clear-cut results have been obtained, even though the work is limited to only a certain phase of the over-all pathway and the exact site or mechanism of action is unknown. The few systems discussed will at least point out clearly the manifold potent inhibitions which can be exerted by the mercurials and will serve to establish the fact that specific effects on metabolism can seldom, if ever, be achieved in cellular systems. Perhaps with the increasing knowledge of the detailed actions of the mercurials, there will arise situations in which selective blocks can be produced under controlled conditions, but at the present time there is not much reason for optimism.

Lipid Synthesis

The long sequence of reactions in the biosynthesis of sterols seems to be strongly inhibited by mercurials at different sites. The total incorporation of mevalonate-C^{14} by *Lactobacillus casei* over 4 hr is inhibited 59% by 0.1 m*M* *p*-MB and 96% by 1 m*M* (Thorne and Kodicek, 1962). The conversion of farnesyl-PP and mevalonate to squalene by various fractions of rat liver is depressed 50% by *p*-MB, *p*-MPS, and Hg^{++} at concentrations near 0.05 m*M*, and essentially completely by concentrations much above 0.1 m*M* (Popják *et al.*, 1958; Anderson *et al.*, 1960; Goodman and Popjak, 1969). The further conversion of squalene to sterols is 97% blocked by 0.33 m*M*

p-MB (Goodman, 1961). The sensitive enzymes are probably all located in the microsomes. One of the enzymes on the pathway from mevalonate to farnesyl-PP, the isopentenyl-PP isomerase, is inhibited completely by 0.1 m*M* *p*-MB (Agranoff *et al.*, 1960), so this could account for part of the block in squalene formation, but there are undoubtedly other sensitive steps.

Fatty acid biosynthesis from acetate in mammary gland homogenates is inhibited 95% by 0.1 m*M* Hg^{++} (Popják and Tietz, 1955), and from acetyl-CoA and malonyl-CoA in purified fractions from pigeon liver 95% by 0.075 m*M* *p*-MB (Bressler and Wakil, 1962). The inhibition is probably early in the sequence, since various acyl-CoA's protect, but it is not on the NADPH: acetoacetyl-CoA oxidoreductase. The incorporation of acetate-1-C^{14} into lipid by chloroplast suspensions is also strongly depressed: 22% by 0.001 m*M* *p*-MB, 50% by 0.01 m*M*, and 88% by 0.1 m*M* (Mudd and McManus, 1964). Fatty acid oxidation is potently inhibited by the mercurials, and one likely site is the initial activation by ATP (with or without CoA), catalyzed by fatty acid thiokinase, since this is completely inhibited by 0.1 m*M* *p*-MB (Jencks and Lipmann, 1957). The incorporation of P_i^{32} into mycobacterial phospholipids is not depressed so readily, 1 m*M* *p*-MB inhibiting only 24% (Tanaka, 1960), although the synthesis of phospholipid in rat liver mitochondria from α-glycerophosphate is completely blocked at this concentration (Wojtczak *et al.*, 1963).

The direct actions of the mercurials on lipid biosynthesis combined with other actions which would secondarily inhibit these pathways, e.g., the reactions with coenzyme A or the depletion of available ATP, must lead to serious interference in the formation of fatty acids and sterols in proliferating microorganisms and contribute to the suppression of growth, and it is interesting to speculate whether they play a role in chronic mercurial poisoning in animals.

Protein Synthesis

In the preparations which have been examined it appears that protein synthesis is not particularly sensitive to the mercurials. The incorporation of leucine-C^{14} into chloroplast protein is inhibited only 30% by 5 m*M* mersalyl (Stephenson *et al.*, 1956) and into reticulocyte protein only 9% by 0.1 m*M* *p*-MB, although 1 m*M* inhibits almost completely (Borsook *et al.*, 1957), while the incorporation of phenylalanine-C^{14} into rat liver soluble proteins is inhibited 85% by 1 m*M* *p*-MB (Haining *et al.*, 1960), of amines into guinea pig liver soluble proteins 100% by 1 m*M* *p*-MB (Clarke *et al.*, 1959), and of amino acids into the acid-soluble proteins of frog egg supernatant fractions 100% by 0.77 m*M* *p*-MB (Burr and Finamore, 1963). Although these results do not conclusively indicate the exact sensitivity of protein synthesis to the mercurials, one is somewhat surprised to find that such high concentrations apparently must be used to inhibit effectively. The only instance of potent inhibition of which I am aware is that found in

Pseudomonas aeruginosa by DeTurk and Bernheim (1960), the induction of enzymes for the oxidation of putrescine, benzoate, and γ-aminobutyrate being reduced 50% by 0.0028 m*M* *p*-MB. The enzymes themselves are not inhibited at this concentration. Partial protection by Fe^{++} when it is added with the mercurial or shortly after was observed. It is now known that enzyme induction is not a valid system for estimating the effects of inhibitors on protein synthesis in general, because there are many other factors involved. In the inhibition cited, it was in fact postulated that some transport process in the membrane requires Fe^{++} and that this is the site of attack by the mercurial.

Porphyrin Synthesis

The formation of porphyrins from glycine and α-ketoglutarate by *Rhodopseudomonas spheroides* is completely blocked by 0.04 m*M* *p*-MB, and from aminolevulinate by 0.1 m*M* (possibly by lower concentrations since they were not tested) (Lascelles, 1956). The formation of aminolevulinate from glycine, phosphoenolpyruvate, and succinyl-CoA is completely prevented by 0.44 m*M* *p*-MB (Gibson *et al.*, 1958). It would thus appear that steps both pre- and post-aminolevulinate are vulnerable. The report of Granick (1958) that 1 m*M* *p*-MB does not interfere with protoporphyrin synthesis from glycine and α-ketoglutarate in chicken erythrocytes is surprising, but may be attributed to the high density of the cell suspension (around 45% by volume) and the consequent binding of the mercurial to nonenzyme proteins. The condensation of porphobilinogen to uroporphyrinogen is almost totally blocked by 0.02 m*M* Hg^{++} and 0.1 m*M* *p*-MB (Lockwood and Benson, 1960), and the subsequent conversion of uroporphyrinogen to coproporphyrinogen is again essentially blocked by 0.012 m*M* Hg^{++} and 0.7 m*M* *p*-MB (Mauzerall and Granick, 1958), if the results on the isolated enzymes catalyzing these reactions can be applied to cellular preparations. The incorporation of Fe^{++} into protoporphyrin to form heme is not so sensitive, in chicken erythrocyte hemolyzate being inhibited 64% and 58% by 1 m*M* Hg^{++} and *p*-MB, respectively (Kagawa *et al.*, 1959). The purified chelating enzyme from rat liver is inhibited 75% by 0.1 m*M* Hg^{++} (Labbe and Hubbard, 1961), the greater effect probably being due to the relative purity of the preparation. Again one can speculate that a depression of porphyrin synthesis may be of some significance in growth studies or chronic poisoning.

Bioluminescence

One of the very few thorough, quantitative, and interesting investigations on mercurial inhibition was made by Houck (1942), who studied the effects of Hg^{++} on the luminescence of *Achromobacter fischeri*. The standard conditions were as follows: pH 7.3, temperature 25°, 25 m*M* glucose as

substrate, and a suspension density of 4×10^8 cells/ml. Both respiration and luminescence of these cells are inhibited potently by Hg^{++}, the latter being somewhat more sensitive (Fig. 7-35). The rate of inhibition is much more rapid than with most cellular activities, half maximal inhibition being reached in about 30–40 sec (Fig. 7-36). It is not immediately evident why the inhibition is more potent in the rate experiments than in the studies on the effect of concentration. The effects of cell density on the inhibitions are very striking (Fig. 7-37). The initial suspension here contained 6×10^9 cells/ml and this was diluted as indicated in the graph. At 0.001 m*M* no

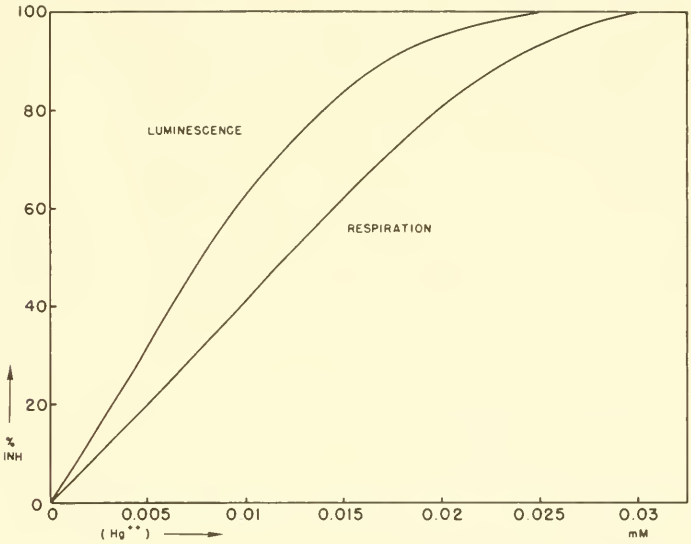


Fig. 7-35. Effects of Hg^{++} on the respiration and luminescence of *Achromobacter*. (From Houck, 1942.)

inhibition is observed until sufficient dilution is made and at low cell densities the inhibition is complete. These curves illustrate very well what essentially must occur in all cell or tissue preparations, whatever activity is measured. The meaninglessness of statements that such and such a concentration of mercurial produces a certain degree of inhibition of some cellular process is all too clear; in this case with 0.001 m*M* Hg^{++}, one might observe any inhibition from 0 to 100% depending on the cell density chosen. Inhibition was determined with 0.001 m*M* Hg^{++} at three values of the pH, and was greatest at 5.3 and 8.4, and least at 7.3 (one can estimate the mean per cent inhibitions at 1 min to be 91%, 60%, and 86% at pH 5.3, 7.3, and 8.4, respectively). The light intensity is much greater at pH 7.3 and this may possibly be related to the rate of glucose uptake. The effects of temperature have already been illustrated (Fig. I-15-9) and discussed (page

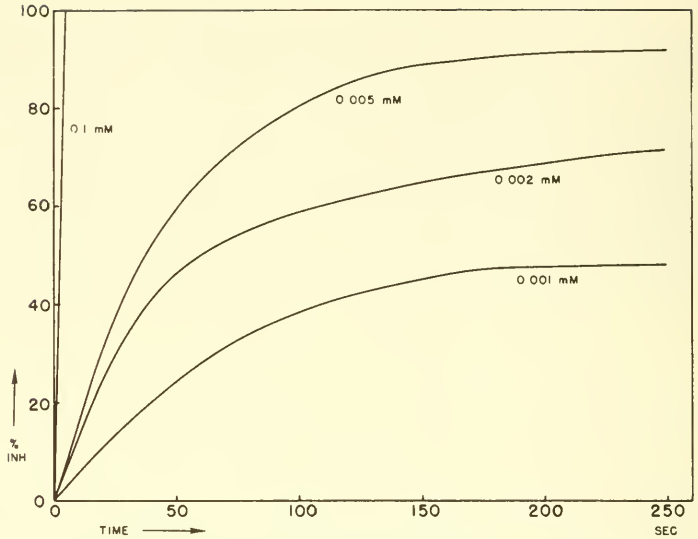


FIG. 7-36. Effects of Hg^{++} at different concentrations on luminescence of *Achromobacter*. (From Houck, 1942.)

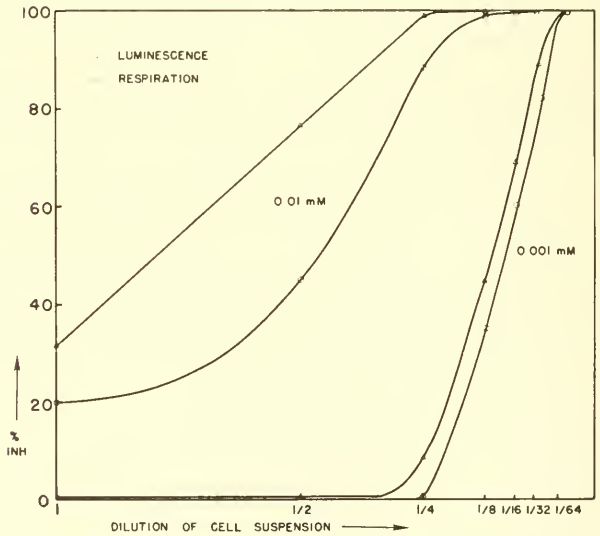


FIG. 7-37. Effects of dilution of the *Achromobacter* suspension on the inhibitions of respiration and luminescence by Hg^{++}. (From Houck, 1942.)

I-786). The increase of the inhibition with rise of temperature was interpreted by Houck in terms of an equilibrium between active and denatured forms of the attacked enzyme, especially luciferase. Actually from this work one cannot locate the site of the inhibition, or even be certain it is on the bioluminescent reactions themselves, since interference with glucose uptake or oxidation, or the supply of ATP, could be responsible. However, it has been found that *Achromobacter* luciferase is markedly inhibited in the range of Hg^{++} concentrations found to inhibit luminescence (Table 7-13), so it may well be that luciferase is the major site of action. This is somewhat substantiated by the fact that luminescence in extracts of *Renilla reniformis*, the sea pansy, is strongly inhibited by *p*-MB (Cormier, 1960).

Photosynthesis and Photophosphorylation

The marked inhibition of certain phases of photosynthesis by iodoacetate and iodoacetamide (III-1-156) indicates the necessity of SH groups, so that one would expect the mercurials to be effective inhibitors, and this is borne out. The photoreduction of various dyes in isolated chloroplasts or grana (Hill reaction) is very sensitive. In spinach chloroplasts it is inhibited 90% by 0.005 m*M* Hg^{++} (Macdowall, 1949). The dye reduction may be mediated through NADPH, which is the initial acceptor. The photosynthetic NADP reductase from spinach is inhibited 50% by 0.012 m*M* and 90%by 0.016 m*M* *p*-MB (San Pietro and Lang, 1958) and the photoreduction of NADP in chloroplasts is similarly inhibited, although slightly less potently (J. S. C. Wessels, 1959). The photoreduction of cytochrome c and NADP by the chloroplast enzyme is 50% reduced by 0.004 m*M* *p*-MPS and the enzyme is bleached by the mercurial (Keister and San Pietro, 1963). In *Chromatium*, illumination causes a blue fluorescence presumably due to bound NADH, indicating that here there is a photoreduction of NAD. This fluorescence change is completely abolished by 0.02 m*M* PM (Olson *et al.*, 1959). Finally, a NADPH diaphorase from chloroplasts, possibly involved in the reduction of the Hill dyes by NADPH, is inhibited 53% by 0.023 m*M* Hg^{++} and 50% by 0.13 m*M* *p*-MB (Avron and Jagendorf, 1956). The initial photoreductive changes upon illumination are thus quite potently inhibited by the mercurials, and this must certainly be one site of action on over-all photosynthesis. Other evidence for a primary interference with the photolysis of water was obtained by Damaschke and Lübke (1958), who showed that *Chlorella* under anaerobic conditions produces a sudden burst of H_2 upon illumination and that this is completely inhibited by 0.2 m*M* *p*-MB (lower concentrations not tested), and by Whittingham (1956), who found that although 0.12 m*M* *p*-MB does not inhibit the initial evolution of O_2 by illuminated *Chlorella*, the steady-state formation of O_2 is strongly depressed. It may be mentioned that even high concentrations of Hg^{++} do not react with chlorophyll (Macdowall, 1949).

Photophosphorylation to form ATP is not necessarily coupled with NADP reduction (J. S. C. Wessels, 1959), but nevertheless one might predict that it would be reduced by mercurials. It has been found that the incorporation of P_i into ATP in illuminated chlorophasts is inhibited around 50% by *p*-MB at 0.05–0.1 m*M* (Arnon *et al.*, 1956; J. S. C. Wessels, 1958, 1959; Jagendorf and Avron, 1959), and similar effects were reported for *Rhodospirillum rubrum* (Smith and Baltscheffsky, 1959). Photophosphorylation is not inhibited as potently as photoreduction.

The photochemical fixation of $C^{14}O_2$ by chloroplasts is inhibited 14% and 88% by 0.01 and 0.05 m*M* *p*-MB, respectively (Gibbs and Calo, 1959 b), but a reconstructed system (extract + chloroplast fragments) is more sensitive, 61% and 94% inhibition being exerted by these concentrations of *p*-MB (Gibbs and Calo, 1960 b). It is not known if this implies some barrier to penetration in the intact chloroplast. Both the initial and steady-state rates of fixation of CO_2 in illuminated dahlia leaves are only slightly reduced (15–25%) by 0.5 m*M* *p*-MB, even though plenty of time is provided for penetration (Massini, 1957), and in *Scenedesmus obliquus* photosynthesis is inhibited only 50% by 1 m*M* *p*-MB after 260 min exposure (Horwitz, 1957). The failure to inhibit more potently in these cases can at present be explained only on the basis of inadequate penetration into the cells or a certain structural integrity of the photosynthetic apparatus which makes it difficult for a mercurial to exert such inhibition as is observed with isolated chloroplasts. No detailed study of the effects of mercurials on the rapidly labeled C compounds has been made, but Miyachi (1960) has found that *p*-MB decreases the level of what he calls the primary photogenic agent (measured by 3-sec $C^{14}O_2$ fixation) in *Chlorella*, although it does not interfere with the participation of this substance in the subsequent photosynthetic pathway. Nonphotosynthetic $C^{14}O_2$ fixation is usually inhibited strongly by mercurials, e.g., the autotrophic fixation by *Hydrogenomonas facilis* (McFadden and Atkinson, 1957) or the fixation associated with sulfide oxidation in *Thiobacillus thiooxidans* (Iwatsuka *et al.*, 1962), both being inhibited around 50% by 0.01 m*M* *p*-MB — which is not surprising considering the sensitivity of the various enzymes usually involved in CO_2 fixation. This dark fixation is possibly related in some manner to photosynthesis, and it has occasionally been pointed out that the same inhibitors are effective in both.

THE CELL MEMBRANE AS A SITE FOR MERCURIAL ACTION

In the following section we shall discuss the effects of the mercurials on permeability and membrane transport systems, as a background for understanding the responses of tissues to these inhibitors, but it may serve to clarify the problem if we take up the theory of the role of the cell membrane

in heavy metal ion inhibitions as an introduction. Although many workers have considered the effects of heavy metal ions on membranes, the concepts presented here will be mainly those of Rothstein and his group at Rochester, since they have been actively engaged for over 10 years in studying this problem. Although much of the evidence is based on work with copper, molybdate, and uranyl ions, and the theory is meant to apply to heavy metal ion action in general, Hg^{++} has been used frequently and it is impossible to discuss the effects of the mercurials on cells and tissue without considering this aspect of their actions. The basic concepts of Rothstein (1959) may be summarized as follows. (1) The cell membrane is exposed directly to the heavy metal ions in the medium and is that part of the cell which reacts initially when the heavy metal ions are added. Ligand groups at the surface or within the membrane will combine with the heavy metal ions as they diffuse, and hence the membrane will experience the first effects, and certain changes in cellular metabolism or function may at this time relate to a selective membrane action. (2) The cell membrane usually presents a barrier to the penetration of the heavy metal ion into the cell and thus protects the cytoplasmic enzymes. (3) Nonenzymic or nonfuctional ligand groups in the membrane or within the cell combine with the heavy metal ions and thereby protect the active sites by reducing the amount of heavy metal ion which is free to react. (4) As a result of the second and third postulates, enzymes within cells are less readily attacked by metal ions than when they are isolated from the cells. (5) As a result of the first statement and the fourth deduction, it would be likely that the major site of heavy metal ion action on cells and tissues is often the cell membrane, rather than the enzyme and metabolic systems within the cell. (6) The most important active sites in the membrane are enzymes or other components involved in the transport of substances across the membrane. Much of the toxicity would therefore be due to interference with the movements of substrates or ions into or out of the cell.

Glucose Uptake and Respiration of Diaphragm Muscle

The uptake of glucose by rat diaphragm is almost completely abolished within 20–30 min by 0.2 mM Hg^{++}, but the respiration is not affected before 30 min and is inhibited only 30% maximally after 2 hr (Fig. I-12-31) (Demis and Rothstein, 1955). It requires 2 mM Hg^{++} to inhibit the respiration 90% and this occurs after 1.5 hr. Thus glucose uptake is depressed much more rapidly and is more sensitive than respiration by at least a factor of 10. These results might imply that Hg^{++} acts initially on the membrane to block glucose transport, and later on intracellular respiratory systems; depression of glucose uptake does not in itself inhibit respiration since the latter is dependent on endogenous substrate. Of some confirmatory evidence is the fact that cysteine will reverse the inhibition of glucose uptake

but will not restore the respiration once it is inhibited; i.e., the surface-bound Hg^{++} is available to the cysteine, but penetration of the amino acid into the cells is inadequate to remove the Hg^{++} responsible for reducing the respiration.

Uptake of Hg^{++} by Diaphragm Muscle

Logarithmic plots of Hg^{++} uptake with different initial concentrations in the medium are shown in Fig. 7-38. There appears to be two components, a fast phase with a half-time of 12 min and a slow phase with a half-time of around 60 min. The uptake essentially ceases after 20–30 min at low

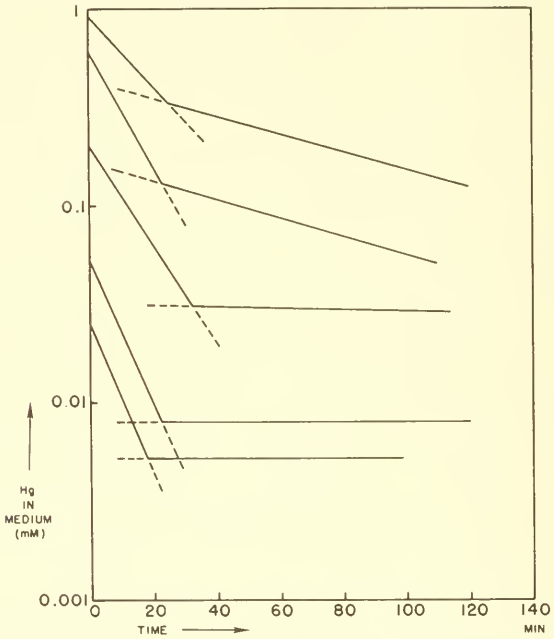


Fig. 7-38. The uptake of Hg^{++} by rat diaphragm, at pH 7.4 and 38°, with time, as determined by the Hg^{++} remaining in the medium. (From Demis and Rothstein, 1955.)

initial concentrations. It was assumed that the fast phase corresponds to the diffusion of Hg^{++} into the extracellular space and binding to the plasma membranes, the slow phase to the penetration into the cells. The time relations point to a correlation between the membrane binding and the inhibition of glucose uptake, and between penetration and respiratory inhibition.

There are certain aspects of these results which are puzzling and seem to me to be difficult to reconcile with the simple theory presented. Why does the Hg^{++} uptake cease after the fast phase for the lower initial concentrations (0.2 m*M* or below) (Fig. 7-38)? Let us estimate how much Hg^{++} is taken up by the diaphragm in the fast phase (see accompanying tabulation). If this represents Hg^{++} bound to membranes and as much as

Initial Hg^{++} concentration (m*M*)	% Uptake	Quantity of Hg^{++} taken up (μmoles/g tissue)
0.9	63	9.4
0.6	77	7.7
0.2	85	2.8
0.05	84	0.70
0.025	78	0.33

9.4 μmoles/g of tissue can be taken up, why does uptake stop when so little is bound at the lower concentrations? One would expect all the Hg^{++} to disappear from the medium when the initial amount of Hg^{++} is less than that required to saturate the membranes. The maximal Hg^{++} bound finally at the highest concentration was stated to be about 15 μmoles/g of tissue. Inasmuch as the plasma membranes cannot contribute more than 1% of the tissue mass, how is it that they can bind over half this amount?

The amount of Hg^{++} diffusing into the extracellular space and existing there unbound must be negligible, since the diaphragms weighed 0.6 g and the total medium volume was 10 ml, so that the extracellular space would be approximately 1% of the total volume. We have mentioned that Cascarano and Zweifach (1962) found diaphragm exposed to Hg^{++} to show evidence of dehydrogenase inhibition only in the outer few layers of cells (page 879). Thus Hg^{++} does not appear to penetrate readily throughout the tissue. One must ask if the fast phase of uptake may be correlated with binding only to the membranes of the outermost layer of cells. A rough estimate of the protein contained in the outermost membranes on both sides of diaphragms, assuming a generous membrane thickness of 200 Å, gives 1.5×10^{-4} μmole/g of tissue. If all the Hg^{++} taken up were bound by these membranes, there would be 62,000 Hg^{++} ions bound per protein molecule (of assumed molecular weight 100,000), and since this value is impossibly large, one must conclude that most of the Hg^{++} must be bound deeper in the tissue. Demis and Rothstein (1955) assumed that the Hg^{++} is not bound entirely to the outermost cells, but to the plasma membranes throughout the diaphragm. It is difficult to estimate the amount of protein

in the total membrane, but it seems very unlikely that it could accommodate all the Hg^{++} taken up at the higher concentrations, especially if penetration deep into the tissue does not occur.

The uptake data by themselves could be explained in a variety of ways. Binding to proteins often shows different phases due to the different reactivities of the various types of SH group, and in cellular systems one must perhaps also consider ligands other than SH groups. But how can one interpret the results on glucose utilization and respiration, especially as they seem to be correlated in time with the Hg^{++} uptake phases? Particularly, why is there such a long lag period before respiration is depressed? It may be noted that a lag period is not always observed in other tissues or cell suspensions. One possibility which cannot be ignored is that the Hg^{++} enters the cells early but is initially and preferentially bound to SH groups not involved with respiration. In muscle cells this might be more evident than in other tissues because of the large amounts of actin and myosin, each of which possesses numerous SH groups; only when these groups become saturated with Hg^{++} would effects on the oxidation enzymes be observed. It is unfortunate that the effects on muscle contraction were not determined, since if this explanation is valid, contractile activity should be reduced during the fast phase of uptake. In this case the fast phase would refer to the binding to membrane and actomyosin (and any other reactive ligands), the membrane contributing only slightly. The kinetics of the effects of mercurials on diaphragm contraction have apparently not been studied, but one notes that the diaphragms exposed to 1 m*M* *p*-MB for 30 min by Kono and Colowick (1961) were stated to be in contracture. On the other hand, the results obtained with rat atria exposed to 0.05 m*M* *p*-MB indicate that no effect on the contractile amplitude occurs during the initial 22 min, although effects on the membrane are evident (decrease in magnitude and duration of the action potential), and that depression of the contraction proceeds subsequently (Webb and Hollander, 1959). These results on atria thus would fit into the theory of Rothstein. However, it must be remembered that in obtaining transmembrane potentials one examines only the outermost cells, and that contractile amplitude involves the entire tissue; for this reason one would expect a delay in contractile response. A decision cannot be made until direct experiments on respiratory and contractile response are made in diaphragms. It must be emphasized that any modification of the concepts of Rothstein suggested here are not necessarily applicable to other heavy metal ions or other cells (especially yeast), but relate to mercurials only.

Another factor which must be considered in tissue uptake studies with the mercurials is the possibility of damage to the external layers, manifested by increased permeability and exposure of reactive SH groups, especially with the higher concentrations often used. The high degree of bind-

ing observed by Demis and Rothstein (1955) with 0.6–0.9 mM Hg^{++} might be due in part to this, the outer layers of cells picking up the Hg^{++} not only in the membranes but within the cells. One might try to estimate roughly the amount of Hg^{++} required for saturation of membrane sites by determining the initial concentration so that all the Hg^{++} is removed from the medium. If one plots as accurately as possible the amount of Hg^{++} remaining in the medium after the fast phase against the initial concentration, one finds that the nearly linear curve passes almost exactly through the origin. All this shows is that it must require very little Hg^{++} to saturate the ligands involved in the fast phase uptake.

It is of some interest to attempt an estimate of the concentration of membrane SH groups in certain cellular suspensions in order to obtain some idea of the order of magnitude. In the experiments of Houck (1942) the suspensions contained 4×10^8 cells/ml of *Achromobacter fischeri* under standard conditions. *Achromobacter* is a rod with dimensions 0.9×1.8 μ and thus the surface area of a single cell is around 3.8×10^{-8} cm^2. If one assumes that the membrane is 200 Å thick (which is probably too high), that the membrane is 50% water (since it is perhaps more condensed than the cytoplasm), that the membrane solids include 65% protein (values of this magnitude have been obtained for the membranes of other bacteria), that protein specific gravity is 1.4., that the mean molecular weight of the membrane proteins is 100,000, and that there are approximately 10 reactive SH groups on a protein of this molecular weight, one can calculate that the concentration of membrane SH groups is close to 10^{-5} mM. The lowest Hg^{++} concentration to produce reduction of luminescence was 10^{-3} mM, so that even at this lowest concentration the Hg^{++} was around 100 fold in excess of the membrane SH groups. Of course, ligands other than SH groups may occur in the membrane. This suspension of *Achromobacter* is fairly dilute relative to most suspensions used, since calculation of total cell volume indicates that the cells occupy 0.046% of the total volume. In more concentrated suspensions, such as are often used, the situation can be quite different. A 10% suspension of human erythrocytes (1.16×10^9 cells/ml, cell surface area $= 1.4 \times 10^{-6}$ cm^2, membrane thickness = 82 Å, and 10 SH groups/protein molecule of molecular weight 100,000) would be 0.059 mM with respect to membrane SH groups, so that an appreciable amount of Hg^{++} might be bound by the membranes in this case. In any study relating to a theory of membrane binding of heavy metal ions, it would be well to make some reasonable estimates of the concentration of membrane ligand groups. Although such calculations cannot be very accurate, the experimental results may be of an entirely different order of magnitude, which should impel the investigator to question the validity of the theory.

Comparison of Effects of Hg^{++} on Intact Diaphragm and Homogenates

The endogenous respiration of diaphragm homogenates fortified with ATP was claimed by Demis and Rothstein (1955) to be inhibited faster and less potently than the respiration of intact diaphragm by Hg^{++}. Actually, from the data given, it is not evident that the rate of inhibition in homogenates is much faster; at 10 min after adding Hg^{++}, for example, there is no significant difference in the rates judged from the points presented, although from then on the rate in intact diaphragm falls off, so that the inhibitions are not equivalent again until 50 min. It was stated that it requires 10 times the concentration of Hg^{++} to inhibit homogenate respiration compared to intact tissue [in a later review Rothstein (1959) stated 200 times], but no data on this point are given (the only experiment reported is with the extremely high concentration of 9 mM), and in any case it depends on what time is chosen to compare the inhibitions (e.g., up to 50 min, homogenate respiration is inhibited more strongly by 9 mM Hg^{++}). It is, furthermore, very difficult to interpret differences in inhibitions of intact cells and extracts, since the substrates utilized, the pathways taken, and the states of the enzymes are probably very different. Mercurial inhibition has usually been found to be more potent in cell extracts than intact cells, e.g., Nakayama (1959) reported that while 0.077 mM *p*-MB inhibits ethanol oxidation 9% in *Acetobacter*, it requires only 0.0077 mM to inhibit 14% in extracts. How much role the membrane plays in any of these observations is impossible to determine.

Binding of Hg^{++} to Yeast Cells and Loss of K^+

The efflux of K^+ from yeast is accelerated by Hg^{++} as it is from most cells. Although the effects of the mercurials on permeability and active transport will be taken up later, the work done by Rothstein and his coworkers will be treated here since it has bearing on the concept of differential membrane binding. Rothstein and Bruce (1958) studied the efflux of K^+ into a K^+-free medium flowing through a yeast cell column; since the pH of the medium was 3.5, and lowering the pH enhances the efflux rate, it was assumed that the process is mainly a K^+-H^+ exchange. The loss of K^+ from the cells is very sensitive to Hg^{++}, 0.001 mM producing a slight effect after a long lag period, and 0.003 mM producing at least a tripling of the rate; at the highest concentration used, 0.1 mM, 80% of the cell K^+ is lost in 1 hr.* Passow and Rothstein (1960) used a different technique in that the rate of K^+ loss into a medium (distilled water adjusted to pH 3 with HCl) from a suspension of yeast cells was measured. The minimal effective concentration of Hg^{++} to accelerate the efflux was found to be 0.2

* Dr. Rothstein informed me that Fig. 6 of the paper by Rothstein and Bruce (1958) presents the cumulative K^+ loss rather than the rate of K^+ loss as stated.

mM, and 1.6 mM produces essentially a complete loss of the cell K^+ in 2 hr.* It is possible from the results with the yeast columns that Hg^{++} at low concentrations has a specific effect on K^+ permeability without depressing active transport, and this is borne out in the work with erythrocytes. It was stated that the curve obtained by plotting log(Hg^{++}) against maximal K^+ loss is sigmoid, which fits a "normal distribution" (presumably of susceptibility of different yeast cells to Hg^{++}), and that the loss of K^+ is probably an all-or-none phenomenon, this being confirmed by determinations of staining by certain dyes in Hg^{++}-treated cells. Although yeast cells undoubtedly show a variation in the sensitivity to Hg^{++}, it is doubtful if the evidence is sufficient to categorize the K^+ loss as all-or-none, especially since sigmoid curves of this type (they are not given so one cannot directly evaluate them) are also compatible with graded effects and, in fact, are the commonest relations observed in the actions of most inhibitors on cell metabolism or function. There is an increase in general membrane permeability produced by Hg^{++}, as proved by the loss of a variety of substances from the cells and a greater penetration of dyes, and this could be a graded phenomenon occurring simultaneously with the alterations in K^+ efflux, without the need for assuming cytolysis as the necessary concomitant of K^+ loss.

Hg^{++} is bound relatively rapidly to yeast cells, the half-time being 2–4 min and maximal binding occurring in 15–20 min. Passow and Rothstein (1960) determined the uptake of both Hg^{++} and Cl^-, and found that initially only Hg^{++} is bound, the Cl^- entering when the concentration of Hg^{++} is sufficiently high. The binding at low concentrations was thus claimed to represent "binding of Hg^{++} rather than $HgCl_2$." Since the concentration of the Hg^{++} ion is actually extremely small, it seems more likely that $HgCl_2$ or other chloride complexes react with the yeast cell wall and membrane, releasing the Cl^- which diffuses into the medium. When the concentration of the mercurial becomes great enough to lead to a significant increase in permeability, Cl^- then enters, either alone or with Hg^{++}. The general conclusion is that the membrane effect of Hg^{++} is not specific for K^+ but is a more or less nonspecific breakdown of the membrane, caused by the "mol-

* The approximately 1000-fold difference in sensitivity observed in these two types of experiment deserves some comment and Dr. Rothstein has kindly provided me with the reasons. In the suspension experiments the yeast density was 60 mg/ml and at 0.4 mM Hg^{++} the maximum binding of the metal would be about 7 millimoles/kg of cells. In the column experiments with 600 mg of cells and a flow rate of 5 ml/min, the maximum binding in 30 min at 0.05 mM Hg^{++} would be only 0.015 millimole/kg. Thus the yeast in the column would be much more readily affected since less of the Hg^{++} is removed. Second, the suspension experiments measure the steady-state flux and the net loss of K^+, whereas the column experiments measure the rate of efflux into K^+-free medium. It is therefore difficult to compare the results by the two techniques on a quantitative basis.

ecular stress" brought about by the formation of S—Hg—S bridges in the membrane; when this stress reaches a critical level, the membrane disintegrates and cellular components are released (Rothstein, 1959). Little consideration is given to the possible effects of Hg^{++} on the active transport mechanisms by which K^+ is accumulated and emphasis is placed on the structural changes occurring in the membrane. Most of the studies on K^+ loss from tissues have been interpreted in terms of an inhibition of active transport (page 907), and it seems that this would be the more direct and logical explanation. It should also be pointed out that, as in all studies of the effects of substances on transmembrane fluxes, it is very difficult to distinguish between actions on the membrane and within the cells, and that therefore these results in themselves cannot be taken as evidence for a direct or specific membrane effect.

Erythrocyte Permeability and Hemolysis

Organic mercurials increase erythrocyte fragility and promote hemolysis, often at quite low concentrations, but the effects of Hg^{++} are more complex, hemolysis being either favored or inhibited depending on the conditions, of which the concentration of Hg^{++} and the type of hemolysis are the most important. If hemolysis in isotonic glycerol is studied, Hg^{++} can markedly delay the hemolysis. Human erythrocytes hemolyze rapidly in isotonic glycerol at pH 7.2; as the concentration of Hg^{++} is increased, inhibition is first observed at 0.025 m*M* and very strong inhibition at 0.05 m*M* (Wilbrandt, 1941). This was interpreted as an inhibition of glycerol entry into the cells by Hg^{++}. On the other hand, if hypotonic hemolysis of human erythrocytes is examined (i.e., hemolysis in Tyrode solution diluted to varying degrees), Hg^{++} can either accelerate or slow hemolysis (Fig. 7-39) (Jung, 1947). In normal or weakly diluted medium, Hg^{++} favors hemolysis, but at low concentration it suppresses hemolysis in markedly hypotonic media. Jung believed that the resistance to osmotic effects is mediated through a denaturation of the membrane. Arbuthnott (1962) has recently confirmed the dual action of Hg^{++}, hemolysis of rabbit erythrocytes being promoted by low concentrations and inhibited by concentrations around 1 m*M*. Organic mercurials (*p*-MB, ethyl-Hg^+, and thimerosal), however, are only lytic, even at high concentrations. Arbuthnott related this to the number of charges on the mercurials, although it is more likely a matter of the ability of Hg^{++} to form S—Hg—S bridges which increase the stability of the membrane. These effects of the mercurials on erythrocytes may or may not depend on metabolic inhibition, but they are important nervertheless in understanding the actions of the mercurials on cell membranes in general, since the mammalian erythrocyte presents an especially simple system for investigation and has been well studied.

Hg^{++} appears to have greater lytic potency than the organic mercurials.

Minatoya *et al.* (1960) reported the ED_{50} for the lytic action on rabbit erythrocytes to be 0.0034 m*M* for Hg^{++} and 0.0174 m*M* for mersalyl, and Arbuthnott (1962) found that lysis can occur in 1 hr with 0.017 m*M* Hg^{++} whereas it requires 1 m*M* *p*-MB or ethyl-Hg^{+}. The effectiveness depends on the temperature and must also depend on the medium used, since a much less potent action of Hg^{++} on rabbit erythrocytes was observed by Joyce *et al.* (1954), lysis occurring in 2 hr with 0.13 m*M*. *p*-MB is much more lytic to rat erythrocytes than is PM, 0.1 m*M* of the former lysing almost completely in 40–60 min, whereas at this time 0.5 m*M* PM produces only about 50% hemolysis (Moore, 1959), and Hg^{++} is about 3 times as potent as *p*-MB, 50% hemolysis being given by 0.4 m*M* Hg^{++} and 1.2 m*M* *p*-MB (these values estimated from data given) in 90 min (Tsen and Collier, 1960). It is obviously difficult to compare results obtained by different investigators, even when the same species is used, but the definite difference in potency between the various mercurials is clear. Although the role of SH groups in erythrocytic membrane structure and function is important, exactly how they operate in this capacity is unknown, so it is difficult to speculate on either the mechanisms of hemolysis by the mercurials or the reasons for the differences between the mercurials. Other cells do not lyse so easily in the presence of mercurials, but this does not necessarily prove that SH groups are of more importance for the erythrocytic membrane, since the inherent stability may be less.

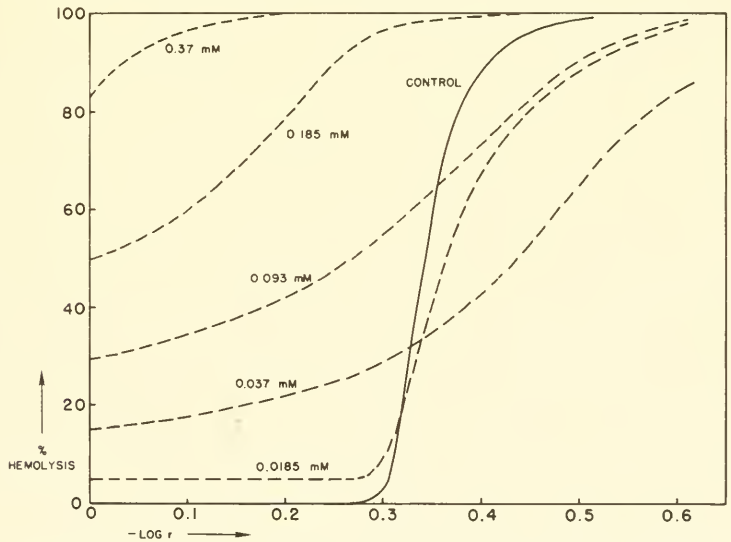


FIG. 7-39. Hemolysis of human erythrocytes by Hg^{++} at different fractional dilution of Tyrode solution (*r*). The control curve shows the hemolysis in the absence of Hg^{++}. (From Jung, 1947.)

It is interesting to inquire into how much Hg^{++} must be bound to the erythrocytic membrane to cause hemolysis. The data of Meneghetti (1922) indicate about 1.5×10^7 atoms/cell, but Jung (1947) believed this to be too low and revised the figure on the basis of his results to 1.4×10^8 atoms/cell. The data of Vincent and Blackburn (1958) allow a rough calculation that K^+ loss is induced by Hg^{++} at binding levels around 2×10^7 atoms/cell, although no hemolysis occurs, while maximal K^+ loss and inhibition of glucose uptake in human erythrocytes were found by Weed *et al.* (1962) to be produced by $3.6–4.5 \times 10^8$ atoms/cell. If there are 10 reactive SH groups for each membrane protein of molecular weight 100,000, one can estimate there to be around 3×10^7 SH groups per erythrocyte membrane. However, although stromal SH groups have a greater affinity for Hg^{++}, hemoglobin SH groups account for around 85% of the total binding, so the figures given above should be reduced if only membrane binding is desired. All one can say is that the amount of Hg^{++} to alter membrane properties is of the same order of magnitude as the estimated SH content of the membrane. On the other hand, the number of molecules/cell of the organic mercurials required for hemolysis is greater than necessary to cover the surface of the sheep erythrocyte (Benesch and Benesch, 1954). For PM there is a 4-fold excess and for mersalyl a 24-fold excess. Of course, the organic mercurials probably do not lie flat on the membrane, but, more important, it is not known how much of the mercurial is bound to hemoglobin or other nonmembrane components.

The kinetics of mercurial hemolysis are generally characterized by a lag period, the duration of which is dependent on the mercurial concentration, followed by a rather sudden hemolysis (Fig. 7-40). For sheep erythrocytes there is a lag period of around 80 min when treated with 0.45 m*M* PM (Benesch and Benesch, 1954), and for human erythrocytes the lag period is 90 min at 37° when exposed to 0.5 m*M* *p*-MB (Sheets *et al.*, 1956 a). The temperature is an important factor, since in the latter case the lag period is around 200 min at 25°. The lag period is partly due to the slow binding of these organic mercurials. Washing the erythrocytes 1 min after exposure to *p*-MB protects completely, after 30 min protects partially, and after 60 min there is no protection (Sheets *et al.*, 1956 a). On the other hand, there is maximal uptake of Hg^{203} by erythrocytes within 5 min (Weed *et al.*, 1962). The osmotic fragility is altered after 3-min exposure to Hg^{++}: At $5.2 \times 10^7–5.5 \times 10^8$ atoms/cell there is a decrease in the fragility, at 4.5×10^9 atoms/cell there is an increased fragility and hemolysis. The kinetics for Hg^{++} and the organic mercurials appear to be quite different. The very marked effects of PM concentration on the kinetics of hemolysis may be seen in Fig. 7-40 for sheep erythrocytes, and a similar dependence has been noted in rat erythrocytes (Moore, 1959). This might indicate a rather critical level of membrane binding to produce hemolysis. The uptake of

Hg^{++} by erythrocytes or ghosts is very rapid but the situation with chlormerodrin is different, in that binding to ghosts is rapid but uptake into erythrocytes continues for 2 hr or more; the binding of chlormerodrin is also perhaps more specific for certain SH groups (Rothstein, 1964). Chlor-

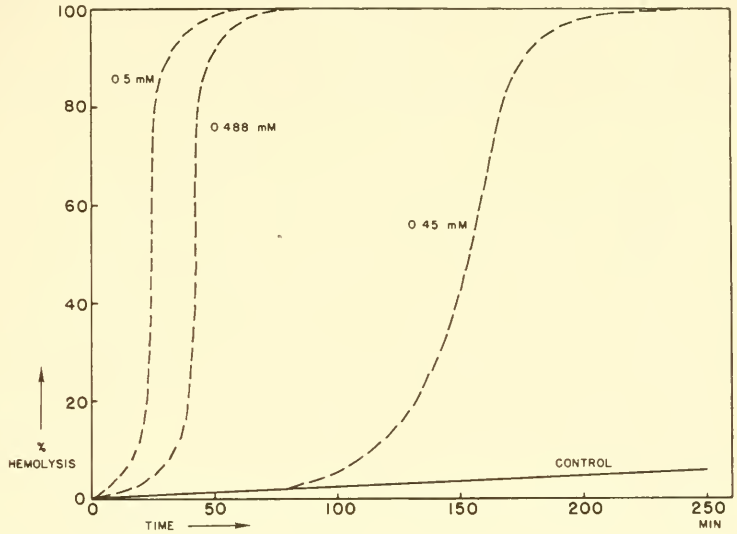


FIG. 7-40. Hemolysis of sheep erythrocytes in a 2% suspension by PM at pH 7.4 and 37°, showing the marked differences over a narrow concentration range. (From Benesch and Benesch, 1954.)

merodrin thus might be useful in separating the effects of membrane and internal binding.

Is hemolysis by the mercurials in any way related to effects on glucose uptake or metabolism? This question cannot be satisfactorily answered since there has been little work where metabolic and hemolytic actions can be compared, and the results available are divergent. The utilization of glucose by human erythrocytes is inhibited moderately within a range of Hg^{++} concentration, the inhibition disappearing as the amount of Hg^{++} bound is increased (Fig. 7-41) (Weed *et al.*, 1962). In the reversal range, a change in the hemoglobin was observed and some agglutination of the cells occurred. The question arises as to whether the effects on K^+ loss and glucose utilization result from some action on all the cells or are due to hemolysis of a few cells. Weed *et al.* (1962) assumed that high Hg^{++} concentrations denature the membrane, causing a decrease in permeability, which could explain the reversal of the effect on K^+ loss, but is difficult to reconcile with the disappearance of the effect on glucose utilization. It is interesting to compare their results on osmotic fragility with these actions. At the Hg^{++}

level producing the maximal K^+ loss and inhibition of glucose utilization (6.0–7.5 $\times$ 10^{-16} mole Hg/cell), it is claimed that the fragility is decreased, which it is in very hypotonic media, but examination of the curves shows that some hemolysis (probably around 2-5%) has occurred in normal medium. At a higher level of bound Hg^{++}, where the effects on K^+ and glucose have been partly reversed (7.6 $\times$ 10^{-15} mole Hg/cell), fragility is definitely increased, and some 20% hemolysis has occurred in the normal medium within 3 min. It seems clear, therefore, that the K^+ loss and suppression of glucose utilization at lower levels of bound Hg^{++} (left of the

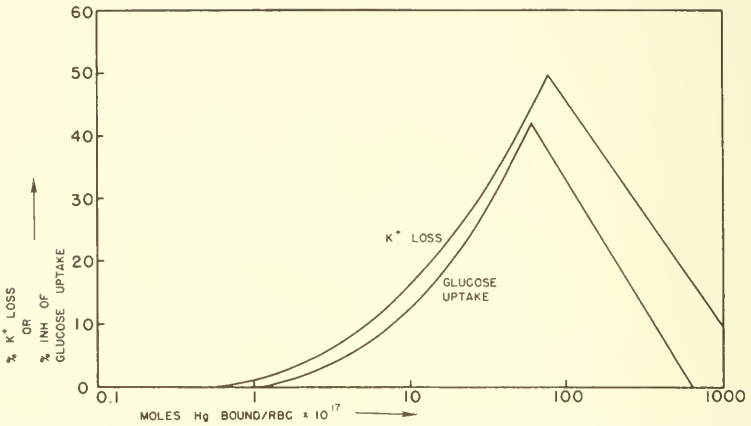


Fig. 7-41. Effect of Hg^{++} on the loss of K^+ from human erythrocytes and the uptake of glucose. (From Weed *et al.*, 1962.)

maximum in Fig. 7-41) are due almost entirely to effects on all the cells and not on lysis of a fraction of the cells. However. at higher levels of bound Hg^{++}, lysis must contribute to both K^+ loss and interference with glucose utilization; e.g., at 7.6 $\times$ 10^{-15} mole Hg/cell bound, there is 20% lysis and around 20% loss of K^+. If this is so, the disappearance of the effect on K^+ loss from intact cells must occur even more precipitously than appears in the figure (the reversal is, of course, really not precipitous, since it is a logarithmic scale; to reverse these effects appreciably it requires the binding of about 10 times that amount of Hg^{++} necessary for maximal K^+ loss). The results of Jacob and Jandl (1962), also on human erythrocytes, are quite different, since they observed that *p*-MB does not inhibit glucose uptake or lactate formation — indeed, stimulates these somewhat — up to 5 μmoles *p*-MB/ml of cells, which is around 2 $\times$ 10^{-16} mole *p*-MB/cell. There is also no reaction of *p*-MB with the intracellular glutathione. These results point to a failure of *p*-MB to penetrate through the membrane. Hemolysis occurs and is presumably due to an action on the membrane. However, it is rather strange that sufficient mercurial can be bound to the

membrane to cause lysis and yet have no inhibitory effect on glucose uptake. One might conclude that Hg^{++} penetrates into the erythrocytes more readily than *p*-MB, which is undoubtedly the case, but it is also possible to speculate that the bifunctional Hg^{++} can distort the membrane pores by forming S—Hg—S bridges in such a way that glucose penetration is slowed while K^+ permeability is increased, as in the concept of critical pore sizes formulated by Mullins (1960). Another problem is how these results can be reconciled with those of LeFevre (1948), who showed that glucose utilization by human erythrocytes is inhibited by 0.002 m*M* *p*-MB and abolished by 0.01 m*M*.

The very rapid loss of K^+ from human erythrocytes observed by Weed *et al.* (1962) — maximally 50% of the total cell K^+ in 3 min at 7.5×10^{-16} mole Hg/cell — is not seen in rabbit erythrocytes, from which there is a slow loss of K^+ in the presence of Hg^{++}, a rapid loss occurring only upon hemolysis, a result which is quite reasonable (Joyce *et al.*, 1954). It is difficult to compare the results of these two groups of investigators because the Hg^{++} concentrations are expressed differently. However, it is possible to estimate that when 7.5×10^{-16} mole Hg/cell is bound, the free Hg^{++} concentration is roughly 0.01 m*M* (see Fig. 2 of Weed *et al.*). In the work of Joyce *et al.*, 0.032 m*M* Hg^{++} caused a 30% loss of total K in 4 hr, so that apparently there is a very marked difference in the response of rabbit and human erythrocytes to Hg^{++}.

The question as to the relation of glucose metabolism to hemolysis is still unanswered. There is one observation which suggests a relation, the finding by Moore (1959) that 10–100 m*M* glucose inhibits the hemolysis induced by *p*-MB, this being manifest mainly in a lengthening of the lag period. No reaction between *p*-MB and glucose can be detected spectroscopically and it is not an osmotic effect. Certain other sugars, e.g. fructose and sorbose, are also effective. It was postulated that glucose may combine with some component of the rat erythrocyte and protect it from the mercurial; if so, this would probably be the transport system for glucose, which is inhibited readily by *p*-MB, the situation being similar to the protection of enzymes by substrates. Sheets *et al.* (1956 a) had found that glucose exerts no protection against hemolysis of human erythrocytes by *p*-MB, but the glucose was added at various times after the *p*-MB and was only 3.3 m*M*.

Membrane or transport ATPase is inhibited by the mercurials but the Hg^{++} or chlormerodrin which is initially bound is without effect (Rothstein, 1964). Chlormerodrin can bind to about 3% of the total SH groups without inhibition of ATPase, but by the time of maximal binding with 25% of the SH groups the ATPase is inactivated, possibly leading to the loss of K^+, although some increase in permeability may also play a role.

The possibility that mercurial hemolysis is related to the reaction of erythrocytic glutathione with SH reagents was considered by Tsen and

Collier (1960). However, Hg^{++} and *p*-MB can produce hemolysis without significant loss of glutathione, whereas iodoacetate and *N*-ethylmaleimide reduce the glutathione completely without lysis. Jacob and Jandl (1962) also showed that *p*-MB reacts readily with glutathione in solution, but does not attack erythrocyte glutathione, and concluded that *p*-MB does not penetrate into the cells. However, Weed *et al.* (1962) found that of the three major sources of SH groups in the erythrocyte — stroma, hemoglobin, and glutathione — the last has the lowest affinity for the mercurial and constitutes only 5% of the total SH groups. It is thus possible that glutathione would be reacted only when all the other SH groups are saturated. In any event, it is evident that glutathione does not play a significant role in hemolysis.

Hg^{++} is able to produce structural changes in the erythrocytic membrane which are detectable by electron microscopy (Jung, 1947). Isolated hemoglobin-free membranes treated with high concentrations of Hg^{++} (37 m*M*) show gross changes in structure — a crumpling with increased density and apparent thickness — but with lower concentrations (0.37 m*M*) the picture is different, a network of holes appearing in the otherwise unaltered membrane. Intact erythrocytes treated with 1.85 m*M* Hg^{++} for several hours no longer lyse in distilled water, and the membrane is seen to have been replaced by a thick mass of coagulated protein. Certain changes in the over-all erythrocyte configuration were also observed by Vincent (1958) in preparations allowed to bind Hg^{++} for 5 min, especially sphering and crenation. Possibly a more detailed study of structural changes induced by low prohemolytic concentrations of the mercurials would be useful in clarifying the mechanism of hemolysis.

We have assumed with others in this discussion of hemolysis and permeability changes brought about by the mercurials that SH groups only are attacked. Certain nonelectrolytes, such as glucose and glycerol, enter the erythrocyte by facilitated diffusion and, since the transport is usually effectively blocked by SH reagents, it has been thought that SH groups are involved in some manner. We have seen that Wilbrandt (1941) claimed a marked reduction in glycerol permeability with 0.05 m*M* Hg^{++}. Furthermore, it was believed that inhibition of glycerol penetration occurs only while the Hg^{++} is entering the cells, i.e., when the Hg^{++} is bound to the membrane. When the Hg^{++} has been picked up by the hemoglobin, there may be little left in the membrane and the permeability to glycerol is restored. LeFevre (1948) established that *p*-MB likewise blocks glycerol entry into the human erythrocyte. However, Barnard and Stein (1958) have suggested that an imidazole group is involved in this transport. The fact that histidine as well as cysteine can reverse the inhibition (it requires a 5- to 10-fold excess of histidine) is not valid evidence; it simply shows that mercurials are bound to histidine. It was also claimed that mercurial action

was characterized by a lag period, this being due to the preferential binding to SH groups before the imidazole groups are attacked; however, there are other possible reasons for such a lag period, and indeed Wilbrandt (1941) claimed the inhibition occurs before the mercurial is bound intracellularly. They also point out that Cu^{++} is much more potent than *p*-MB in depressing glycerol entry and that this favors an imidazole group; *p*-MB is, however, rather ineffective relative to Hg^{++}, which exerts an effect at 0.025 m*M*, due possibly to steric factors. It does not appear that the evidence is sufficient to establish an imidazole group as involved in the glycerol transport, but one cannot argue against this theory, and it is quite possible that in the complex mechanisms of penetration there are both SH and imidazole groups. In either case, one cannot attribute an active role to these groups in the transport on the basis of the evidence available.

EFFECTS ON PERMEABILITY AND ACTIVE TRANSPORT

The general discussion of the mechanisms by which transport systems in the membrane may be affected by SH reagents (see III-1-171, 180) is applicable to the mercurials. We shall confine our attention to certain important problems and interesting results, as far as possible, and only summarize most of the studies in Table 7-18. The effects of the mercurials on renal transport will be taken up in the following section. It is clear from the results in the table that the mercurials often cause a loss of intracellular substances, e.g., K^+, carbohydrate, and amino acids. It is likely that a good many substances leak out of cells treated with the mercurials as a result of not only interference with active transport but direct distorting effects on the membrane structure leading to increases in permeability. Possibly coenzymes, enzymes, and other large molecules may be lost. Ohr (1960) observed the release of some ultraviolet-absorbing material from diaphragm exposed to Hg^{++}, and Weed *et al.* (1962) detected the early release of some Hg^{++}-complexing material from human erythrocytes, this altering the binding kinetics at low concentrations of Hg^{++}. It is not easy to determine if the action is primarily on active uptake or on outward diffusion, even with labeled substances. For example, if one is studying K^{42} efflux, an inhibition of a pump involved in maintaining a high intracellular K^+ level might alter this efflux, either by changing the membrane potential or directly if part of the K^+ efflux is mediated by the pump, since a Na^+ pump might not be completely specific for Na^+ and might carry some K^+ out of the cell. If K^{42} influx is measured, an alteration of the permeability could easily change the rate at which active transport occurs, particularly if K^+ loss accelerates the pump. In most cases there seems to be a decrease in the intracellular K^+/Na^+ ratio, but the mechanism is not clear. Furthermore, a decrease in transport is occasionally not accompanied by a signifi-

TABLE 7-18

EFFECTS OF MERCURIALS ON ACTIVE TRANSPORT PROCESSES[a]

Transported substance	Organism and tissue	Mercurial	Concentration (mM)	% Inh.	$\Delta(X)_t$(%)	General effects	Reference
Inorganic cations							
K^+	Yeast	Hg^{++}	0.2	—	− 15	—	Passow and Rothstein (1960)
			0.6	—	−100	—	
	Yeast	Hg^{++}	0.67	—	—	T/M 8.86 to 3.54	Kleinzeller (1961)
	Hormosira banksii	*p*-MB	0.1	—	0	—	Bergquist (1958)
	Porphyra perforata	*p*-MB	0.1	—	− 24	K/Na 3.7 to 2.4	Eppley (1958)
			0.5	—	− 55	K/Na 3.7 to 1.2	
	Porphyra perforata	*p*-MB	0.5	61	—	—	Eppley (1960)
	Rabbit erythrocytes	*p*-MB	0.1	—	—	K^+ loss $>100\times$	Green and Parpart (1953)
	Human erythrocytes	Hg^{++}	0.4 μmole/ml cells	—	− 50	—	Vincent and Blackburn (1958)
	Human erythrocytes	*p*-MB	2.5 μmole/ml cells	—	− 30	—	Jacob and Jandl (1962)
	Mouse ascites carcinoma	Hg^{++}	0.1	—	− 80	—	Riggs *et al.* (1958)

	Rat liver mitochondria	Hg^{++}	0.01	—	− 92	K/Na 2.3 to 0.25	Spector (1953)
	Rabbit liver mitochondria	Hg^{++}	0.0072	—	− 50	—	Scott and Gamble (1961)
		p-MB	0.054	—	− 50	—	
		PM	0.081	—	− 50	—	
	Rabbit heart mitochondria	*p*-MB	0.01	0	—	—	Ulrich (1960)
			1	100	—	—	
Na^+	*Porphyra perforata*	*p*-MB	0.1	—	+ 17	—	Eppley (1958)
			0.5	—	+ 38	—	
	Porphyra perforata	*p*-MB	0.5	55	—	—	Eppley (1960)
			1	85	—	—	
	Goldfish gills	Hg^{++}	0.001	25	+ 43	—	Meyer (1952)
			0.01	100	—	—	
	Rat liver mitochondria	Hg^{++}	0.01	—	− 29	—	Spector (1953)
Rb^+	Mung bean roots	PM	0.0006	60	—	—	Tanada (1956)
Mg^{++}	Rat liver mitochondria	Hg^{++}	0.01	—	− 4	—	Spector (1953)
			0.1	—	− 23	—	
Ca^{++}	Rat liver mitochondria	Hg^{++}	0.01	—	+ 7	—	Spector (1953)
			0.1	—	− 17	—	
	Rat intestine	Hg^{++}	1	—	—	T/M 4.6 to 1.1	Schachter and Rosen (1959)
	Rat intestine slices	Hg^{++}	1	—	—	T/M 5.8 to 1.5	Schachter *et al.* (1960)
	Rat kidney mitochondria	Hg^{++}	0.1	80	—	—	Vasington and Murphy (1962)

TABLE 7-18 (*continued*)

Transported substance	Organism and tissue	Mercurial	Concentration (m*M*)	% Inh.	$\Delta(X)_i$(%)	General effects	Reference
	Rat liver mitochondria	Hg^{++}	0.1	100	—	—	Vasington (1963)
Cu^{++}	Rat liver slices	*p*-MB	0.1	Stim 5	—	—	Saltman *et al.* (1959)
Inorganic anions							
Cl^-	Dog ileum	Hg^{++}	0.1	Slight	—	—	Ingraham and Visscher (1936)
			0.7	100	—	—	
I^-	*Fucus ceranoides*	*p*-MB	0.05	50	—	—	Klemperer (1957)
	Sheep thyroid slices	Hg^{++}	1	77	—	—	Slingerland (1955)
		p-MB	1	88	—	—	
	Rat intestine	*p*-MPS	0.1	30	—	—	Acland (1962)
			0.67	75	—	—	
$HPO_4^=$	*Micrococcus aureus*	Hg^{++}	0.1	48	—	—	P. Mitchell (1953)
		PM	0.1	72	—	—	
	Micrococcus aureus	Hg^{++}	0.14	—	− 10	—	Hotchkiss (1956)
	Human erythrocytes	*p*-MB	0.25	87	—	—	Schauer and Hillmann (1961)
Carbohydrates							
Glucose	Sugar cane sections	Hg^{++}	0.01	5–10	—	—	Bieleski (1960)
			0.1	100	− 37	—	

		p-MB	0.01	0	—	—	
			0.1	0–10	—	—	
			1	100	− 39	—	
	Human erythrocytes	Hg^{++}	0.0012	50	—	—	LeFevre (1954)
	Human erythrocytes	*p*-MB	0.02	100	—	—	LeFevre and Davies (1951)
Xylose	Rat diaphragm	*p*-MB	0.01	20	—	—	Battaglia and Randle (1960)
Sucrose	*Rhizoctonia solani*	Hg^{++}	0.185	47	—	—	Tolba and Salama (1962)
Miscellaneous							
Aminopterin	*Bacillus subtilis*	*p*-MB	1	—	—	T/M 1.64 to 0.61	Pine (1960)
Indoleacetate	*Avena* colcoptile	*p*-MB	0.01	0	—	—	Johnson and Bonner (1956)
			0.1	40	—	—	
Triiodothyro-acetate	Rat intestine	Hg^{++}	1	93	—	—	Herz *et al.* (1961)
Uracil	Rat intestine	*p*-MB	1	100	—	—	Schanker and Tocco (1962)
Water	Rabbit cornea	Chlorme-rodrin	10	100	—	—	Harris (1957)

[a] $\Delta J(X)_i$ is the change in the tissue concentration of the substance, K/Na is the ratio of the intracellular concentrations of K^+ and Na^+, T/M is the ratio of concentrations in the tissue and the medium; the per cent inhibition refers to changes in the rates of transport.

cant depression of respiration, as in the uptake of I^- by *Fucus*, where 0.05 m*M* *p*-MB inhibits transport 50% but 0.2 m*M* does not affect respiration (Klemperer, 1957), or the accumulation of K^+ by *Porphyra*, where *p*-MB decreases the number of ions pumped per O_2 consumed (Eppley, 1960), or the active transport of Na^+ through frog skin, which is blocked by Hg^{++} at a concentration not altering respiration (Linderholm, 1952). In such cases it has generally been assumed that the action is on the transport system itself, but this is not necessarily true. It is rather surprising that so few have reported instances of decreased permeability brought about by the mercurials, particularly the organic ones, inasmuch as they might be expected to react with SH groups in or around the membrane pores to impede the passage of substances across the membrane; perhaps this would be observed more often if lower concentrations were examined. The permeability of frog skin to Cl^- is decreased by *p*-MB, and Janacek (1962) postulated that the mercurial sterically hinders the movement of anions through the pores.

Certain results occasionally point to an effect of mercurials on the endergonic phase of transport rather than a simple depression of ATP formation. The fact that Hg^{++} at 0.5 m*M* inhibits the 20-sec uptake of acetate by diaphragm without a lag period (Foulkes and Paine, 1961), taken with the rather slow depression of metabolism, is indicative of an action directly on the membrane. We have also seen that *p*-MB lyses erythrocytes without reacting with intracellular glutathione or inhibiting glycolysis, in contrast to *N*-ethylmaleimide, and that this has been attributed to a failure to penetrate into the cells, so that the effects observed must involve an attack on the membrane (Jacob and Jandl, 1962). Hg^{++} very potently inhibits stromal ATPase — 50% inhibition at around 0.00125 m*M*, and plots of log $(v/v_i - 1)$ against log (Hg^{++}) suggest that 3 Hg^{++} ions are required for each ATPase molecule (Laris *et al.*, 1962). The same type of behavior was observed for the inhibition of glucose uptake, and the concentrations of Hg^{++} required to inhibit are comparable (LeFevre, 1954). Laris *et al.* replotted LeFevre's data and found that roughly 6 ions of Hg^{++} are necessary for the inhibition of the transport of each glucose molecule. The similar sensitivities and kinetics allowed them to postulate that the two inhibitions may be closely related. The stimulation of the uptake of certain sugars (e.g., D-xylose and L-arabinose) into diaphragm by *p*-MB, and the inhibition of the stimulation produced by insulin, may well be on the muscle membranes (Kono and Colowick, 1961). There is certainly no correlation with the level of ATP, and *p*-MB actually seems to increase ATP somewhat. A block between ATP and the transport system was considered a possibility.

The reaction of mercurials with a membrane carrier was adduced to explain the inhibition of phosphate transport in *Micrococcus pyogenes* by Hg^{++} and PM (P. Mitchell, 1953). An inhibition of 50% is given by 2.2 μmoles

PM/g cells and by 4.7 μmoles Hg^{++}/g cells. The inhibition-concentration curves are said to conform to the equation $K = (I)\ (1 - i)/i$ and hence to suggest reaction of the mercurial with a phosphate carrier X, according to $I + X \leftrightarrows IX$.* This equation is simply that for noncompetitive inhibition and it is difficult to understand how it would serve to indicate any particular mechanism by which transport is depressed. Mitchell then proceeds to calculate the number of carrier molecules for 100 molecules of intracellular phosphate; since the cells contained 147 μmoles P_i/g cells, 1.5 and 3.2 molecules of PM and Hg^{++}, respectively, are required for 50% inhibition per 100 molecules of internal P_i. It was apparently assumed that if 50% inhibition is given by these numbers of molecules, 100% inhibition would be given by twice these, namely, 3.0 and 6.4 molecules of PM and Hg^{++}, respectively. If this were true titration or zone C inhibition, this would be correct, but inspection of the curves shows that it is not; indeed, it is evident that approximately 20 and 40 μmoles/g cells of PM and Hg^{++} are needed for 90% inhibition (curves do not reach complete inhibition, which would require appreciably more of the mercurials). Therefore, his conclusion that the number of carriers is not more than 3% of the internal P_i is not valid. In addition, the mercurials must be bound to cell components other than a hypothetical carrier, so that under any circumstances it would be difficult to estimate the relative amount of carrier present, just as it is impossible to calculate the amount of an enzyme present in a complex mixture by the quantity of mercurial required for 50% inhibition.

Transmembrane and Transcellular Transports

The uptake of a substance into a cell is often a process different from the transport across a layer of the cells. If a substance is moved against a concentration gradient from one medium into a similar medium, it is an active transport, whereas accumulation of a substance within a cell can be the result of binding. A good example of this is the transport of triiodothyroacetate by rat intestine (Herz *et al.*, 1961). The mucosal → serosal transport is inhibited 93% by 1 m*M* Hg^{++}, but the accumulation in the tissue is actually accelerated 16%. The cellular uptake was postulated to be due mainly to binding. The accumulation of Fe^{+++} (Saltman *et al.*, 1955) and Cu^{++} (Saltman *et al.*, 1959) by rat liver slices is slightly stimulated by *p*-MB, and it is very likely that these are instances of binding to intracellular ligands. There is sometimes not so clear a separation of transmembrane and transcellular transports. Rat intestinal slices accumulate Ca^{++} to a tis-

* Mitchell gives the equation as $K = (I)i/(1 - i)$, changing his symbols to those used in the present work, which is obviously incorrect, since it would mean that the inhibition would vary inversely with the inhibitor concentration, so I have taken the liberty of rewriting it.

sue/medium ratio of 5.8, and 1 mM Hg^{++} reduces this to 1.5 (Schachter *et al.*, 1960). The transport across the intestinal wall leads to an inside-outside ratio of 4.6, and 1 mM Hg^{++} drops this to 1.1 (Schachter and Rosen, 1959). Thus in this instance there is no significant difference in the mercurial inhibition, but certain other inhibitors affect the transintestinal process more strongly. The results will often depend on the location of the active transport mechanism. We shall see that this is an important point in considering the effects of the mercurials on the kidney.

Mitochondrial K^+

Mitochondria isolated from rat liver in 0.25 M sucrose contain 620–640 μmoles K^+/g N and this can be lost if the mitochondria are placed in hypotonic media or treated with saponin, 2,4-dinitrophenol, or Hg^{++} (Spector, 1953). Most of the K^+ appears to be free, but a fraction may be bound; the retention of the free K^+ is dependent on oxidative phosphorylation. However, Gamble (1957) found that mitochondrial fragments catalyzing oxidative phosphorylation can bind K^+. This binding is not dependent on ATP but is abolished by 2.4-dinitrophenol, and a relation between the K^+ binding and the sites for oxidative phosphorylation was postulated. Hg^{++} at 0.01 mM and p-MB at 0.03 mM produce a 5-fold increase in the K^+ exchange rate. This was later investigated in detail (Scott and Gamble, 1961), and Hg^{++} in concentrations around 0.01 mM was found to increase the exchange rate, reduce the bound K^+ by 50%, and inhibit phosphorylation 50%. The organic mercurials are less effective. These three actions are presumably not mediated through the same mechanism, since EDTA prevents the effects of Hg^{++} and p-MB on K^+ binding, has no effect on the stimulation of the exchange rate, and protects oxidative phosphorylation from Hg^{++} but not from p-MB. These complex relationships are not completely understood at the present time, but obviously are of importance in certain cases of K^+ accumulation and transport.

Gastric Acid Secretion

Reduction of gastric acidity by 0.25 mM Hg^{++} introduced into the stomach was shown by Mann and Mann (1939), and the mechanisms involved were studied by Davenport and his group at Utah. Gastric secretion of HCl is depressed to a basic level by 1 mM p-MB; if the secretion is stimulated by carbachol or histamine, the inhibition appears to be greater but the rate is reduced to the same level (Fig. 7-42), i.e., p-MB effectively abolishes the secretion brought about by these drugs (Davenport, 1954, Davenport *et al.*, 1954). There is thus a basal level of secretion (around 30% of maximal) resistant to the mercurials. Graphical analysis indicated that 2 SH groups are involved in the inhibition. Lactate formation when glu-

cose is the substrate is inhibited by *p*-MB, one SH group being involved here, but it is unlikely that glycolytic inhibition is the mechanism by which acid secretion is depressed, inasmuch as inhibition occurs when pyruvate or acetoacetate is the substrate. Respiration associated with secretion is also inhibited by *p*-MB; it was felt that this is not a generalized effect on oxi-

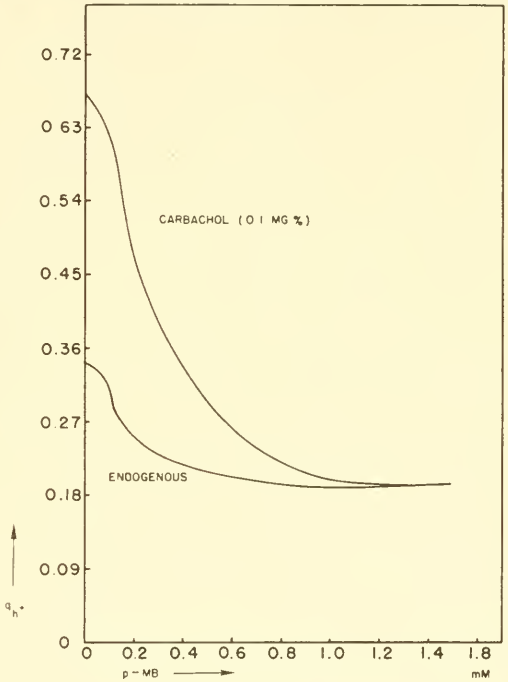


FIG. 7-42. Inhibition of gastric acid secretion by *p*-MB, in the presence of 20 m*M* glucose and in the absence and presence of carbachol. (From Davenport *et al.*, 1954.)

dative reactions, but that the site of attack is some unknown system intimately concerned with the secretory process. The relation between the inhibitions of respiration and secretion by *p*-MB is reasonably linear (Fig. 7-43), in contrast to the results with antimycin and 2,4-dinitrophenol (Davenport and Chavré, 1956). Possibly the primary inhibition by *p*-MB is on the transport system itself, the respiration being reduced secondarily. Other SH reagents inhibit secretion but apparently act at somewhat different sites than *p*-MB (Davenport *et al.*, 1955), so that it is difficult to correlate the blockade of SH groups with the secretory suppression or to locate exactly these SH groups.

Intestinal Transport

The transports of Na^+, water, and glucose across the rat intestine are inhibited by Hg^{++} (Clarkson and Cross, 1961). The transintestinal electric potential is dependent on the Na^+ transport and the ionic permeabilities of the lumenal membranes. Hg^{++} 0.01–1 m*M* causes a rapid brief elevation of the potential which is followed by a fall, the rapidity of which is determined by the Hg^{++} concentration. There are two phases in the response: (1) an immediate loss of K^+ and phosphate from the intestinal wall and a marked inhibition of glucose uptake, and (2) a delayed (occurring after 20 min or longer) inhibition of transintestinal transport of Na^+, water, and

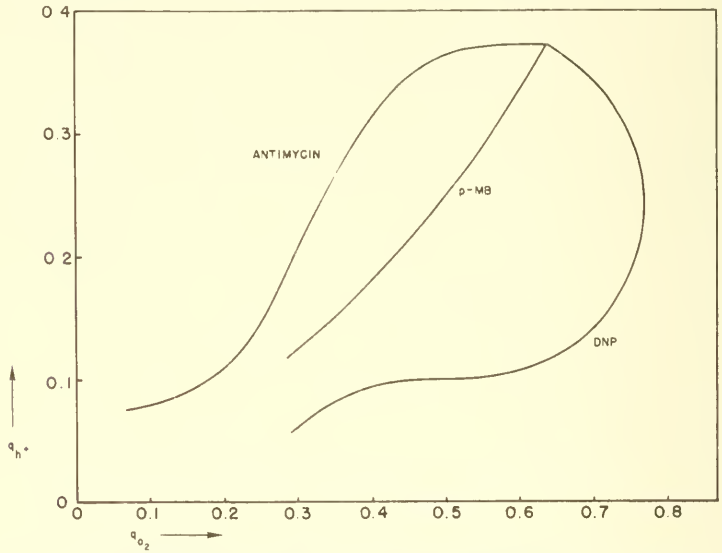


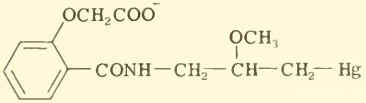
Fig. 7-43. Effects of inhibitors on the respiration and acid secretion of mouse gastric mucosa stimulated by carbachol. (From Davenport and Chavré, 1956.)

glucose, with a suppression of lactate formation. The uptake kinetics of Hg^{++} show two phases, a fast component dominant during the initial 20–30 min of exposure ($k_1 = 0.0032$ min^{-1}) and a slower component ($k_2 = 0.0017$ min^{-1}). It was pointed out that the system is so complex that it is difficult to interpret the uptake data, but possibly there is some correlation with the initial and delayed responses discussed above. Analysis showed that the potential changes are produced when certain quantities of Hg^{++} are bound to the intestine; i.e., when different concentrations of Hg^{++} are applied to the intestine, the potential changes occur at different rates which are related to the uptake rates. Since the transports and potential are de-

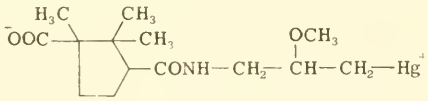
pendent on glucose, it was suggested that the inhibitions of transintestinal transport are perhaps all the result of interference with glucose uptake into the cells. This does not explain the immediate responses, which may be due to the direct effect of Hg^{++} on the cell membranes. Indeed, all the changes observed may arise from modifications in the permeability properties of the lumenal membranes.

EFFECTS ON THE KIDNEY

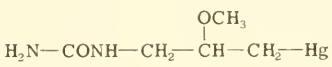
The clinical effectiveness of the mercurials in certain edemas has stimulated much investigation directed at discovering the mechanism by which diuresis is produced. It is now clear that the action is on tubular transport processes. Since these transports are mainly active and depend on tubular cell metabolism, as well as on certain specific carrier and enzyme systems, we shall be primarily concerned with the possible effects of the mercurials on renal metabolism as a basis for their diuretic activity. The pharmacology textbooks and recent reviews (Beyer and Baer, 1960; Farah and Miller, 1962; Kessler, 1960; Mudge and Weiner, 1958; Orloff and Berliner, 1961; Pitts, 1958, 1959) cover the general renal actions of the mercurials and also discuss many of the controversial points. We shall confine ourselves here to a summary of these actions and then proceed to the mechanisms which may be involved in the alteration of the transport systems. The structures of the four most common mercurial diuretics used experimentally are shown in their ionic forms. The preparations provided for clinical use are complexed with different ligands (OH^-, Cl^-, thioacetate, or theophylline), but once introduced into the body or experimental media, the mercurials usually establish new equilibria with the available ligands, as discussed previously



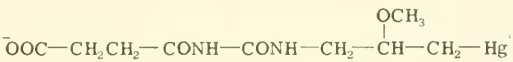
Mersalyl (Salyrgan)



Mercurin (mercaptomerin, Thiomerin)



Chlormerodrin (Neohydrin)



Meralluride (Mercuhydrin)

(page 742). Thus mercurin (Mercuzan) is the un-ionized acid, the Na^+ salt is Novurit, the complex with thioacetate is mercaptomerin (Thiomerin), and the complex with theophylline is Mercurophylline (Mercuzanthin), but the fundamental active structure is the same in all cases. The complexers alter the solubility, local actions on tissues, and rates of absorption, but probably do not significantly affect the basic effects on the tubular transport systems.

Summary of the General Renal Actions of the Mercurials

(A) *The diuretic action is entirely renal.* Since the classic transplantation and unilateral injection studies of Govaerts in 1928 and Bartram in 1932 it has been clear that any extrarenal actions of the mercurials are unimportant for diuresis. Effects on tissues other than the kidneys may of course occur, especially at high doses, but do not contribute significantly nor are they necessary for diuresis.

(B) *The primary action is on tubular transport rather than glomerular filtration.* Clearance studies have demonstrated that glomerular filtration rates are not altered much or at all during marked clinical diuresis. Analyses of the changes in composition of the filtrate as it passes down the nephron show that the increased flow of urine can be accounted for entirely by the depression of certain tubular transport processes. Mercurials given intramuscularly or orally to human subjects do not affect renal blood flow or glomerular filtration (Brun *et al.*, 1947), but injected intravenously in animals they produce definite effects which may possibly modify the primary diuretic action. Vasoconstriction and a reduction in blood flow are usually observed. Jackson (1926 b) reported that intravenous mersalyl causes a rise in blood pressure and a profound constriction of the kidney (measured oncometrically), while Farah (1952) observed the renal blood flow to fall 50% from mersalyl at 10 mg/kg intravenously and 70% from 4 mg/kg intraarterially, these changes occurring before the onset of diuresis. Kessler *et al.* (1957 b) invariably found a significant decrease in glomerular filtration rate following various mercurial diuretics given intravenously, and such often persists long after the maximal diuretic effect occurs. Occasionally a transient antidiuresis is noted after intravenous diuretics and this could be the result of renal vasoconstriction and a reduced glomerular filtration rate (Vargas and Cafruny, 1959; Cafruny and Palmer, 1961). It is interesting to note that the nondiuretic *p*-MB likewise produces these vascular changes. Thus the effects on glomerular filtration when observed experimentally would be antidiuretic and might reduce the over-all diuretic response instead of favoring it.

(C) *The diuretic action is mainly due to an inhibition of active Na^+ resorption in the proximal tubules.* It is now generally agreed that the mercurials

are primarily natriuretics, and that the resorption of Cl^- and water by the proximal tubules follows the movement of Na^+ for electrostatic and osmotic reasons. The evidence for the proximal site of action of the mercurials will be presented later (page 920). The fundamental mechanism of action must therefore be sought in the modifications of active Na^+ transport by the mercurials.

(D) *The mercurials can also act elsewhere on the nephron to modify urine composition and flow rate.* Sufficient evidence has been accumulated to show that the mercurials can exert minor effects throughout the nephron — on the loop of Henle, the distal tubule, and the collecting duct — to further alter the urine volume, and that transport processes and exchange reactions of various types can be inhibited. That is, the primary site of action may be on active Na^+ transport in the proximal tubules, but this is by no means the sole site of action. The transports of a variety of substances, including K^+, Ca^{++}, urate, *p*-aminohippurate, amino acids, and various dyes, are depressed by the mercurials.

(E) *Only a relatively small effect on Na^+ resorption need be exerted to produce marked diuresis.* Inasmuch as 98–99% of the filtered Na^+, Cl^-, and water is resorbed, it is evident that a reduction of this to 90–95% would cause up to a 10 fold increase in excretion rate. Consequently one might predict that only a small metabolic disturbance by the mercurials would be necessary for diuretic action, and that so small an effect might be difficult to detect under the usual conditions. Furthermore, only 15–30% of the total NaCl resorption can be inhibited by the mercurials, the remainder presumably being mediated through mercurial-resistant systems (Pitts, 1958).

(F) *The selective action of the mercurials on the kidney is mainly a consequence of the accumulation of mercurial.* Mercurials exert demonstrable effects only on the kidneys over a dosage range, and this is primarily due to the relatively high concentrations of mercurial reached in the renal tissue, whether this is achieved by tubular secretion or filtrate resorption (page 928). The transport systems are probably no more sensitive than in other tissues to the mercurials (at least there seems to be no clear evidence for this). However, the point mentioned in the previous paragraph that only small effects on renal transport need be exerted may be a factor in increasing the apparent sensitivity of the kidney.

(G) *The reported renal responses to the mercurials are quite variable.* One cannot fail to be impressed by the general lack of agreement on certain basic actions of the mercurials despite the great amount of work done over many years, and it is disturbing that almost every hypothesis can be refuted by evidence of apparent validity. It may be helpful to list some of the reasons for these disagreements. (1) Work done with different species can

frequently not be compared. For example, mercurials in diuretic dosage inhibit glucose resorption and *p*-aminohippurate secretion in man, but have no effect on these transports in the dog; also *p*-MB is not diuretic in the dog, but increases urine flow in the rat (Cafruny and Palmer, 1961). (2) Animals in different states of water load, ion load, or pH will respond differently to the mercurials. (3) The use of theophylline-containing mercurials has often confused interpretation, since theophylline itself is a diuretic acting by a mechanism quite different than the mercurials. Thus Goldstein *et al.* (1961) found Mercuhydrin (meralluride complexed with theophylline) to produce two phases of diuresis, the first due to the theophylline. Certainly some of the results attributed to the mercurials have had their origin in the theophylline present, and for this reason it is always advisable to use mercurials complexed with inactive substances if a pure mercurial action is to be investigated. (4) Much of the work on distribution of the mercurials and their actions on enzymes in the kidney has been done with toxic or lethal doses or concentrations. If a mechanism for the normal diuretic effect is to be found, one must use mercurial concentrations which do not deviate appreciably from those producing maximal diuresis. (5) Different routes of administration often lead to different results. We have seen that intravenous injection causes changes in blood flow and glomerular filtration not seen with the usual routes of administration.

Sites of Action in the Nephron

Several types of evidence have been used to locate the major sites of mercurial action on renal transport processes; these will be discussed briefly, since they also provide interesting information on the mechanisms involved.

(A) *Inhibition of transport processes located in different regions of the nephron.* The mercurials interfere with the transport of a variety of substances by the proximal segment of the nephron. This includes the resorption of glucose, amino acids, urate, phosphate, bicarbonate, Na^+, K^+, and Ca^{++}, and the active secretion of *p*-aminohippurate, iodopyracet (Diodrast), tetraethylammonium ion, phenol red, and various dyes. Izar (1909) noted an increase in urinary urate in dogs given $HgCl_2$ intravenously, and Dale and Sanderson (1954) demonstrated that urate excretion in man rises rapidly following administration of mersalyl. However, if oliguria is produced by lethal doses of $HgCl_2$, urate excretion is impaired and the tissue concentration will rise (Wells, 1916). Mild poisoning by mercurials leads to an aminoaciduria in man (Clarkson and Kench, 1956). There has been disagreement with respect to the effects on glucose resorption, and perhaps in man there is little reduction at diuretic doses, but Vander (1963) has shown a very definite inhibition in the dog, Δ Tm being around -100 during maximal diuresis. Mercaptomerin in dogs lowers the bicarbonate threshold of the proximal tubules by 35% without significant effect on the distal tubules

(Gardier and Woodbury, 1955). Since the resorption of bicarbonate is about equal in the proximal and distal segments, this indicates an exclusively proximal action. Increased excretion of Ca^{++} and Mg^{++} in both man and dog treated with mercurials has been reported, and it is likely that the site is proximal (Wesson, 1962). The inhibition of the secretion of *p*-aminohippurate, tetraethylammonium ion, and phenol red has been shown not only in intact animals but with low concentrations of the mercurials in isolated tubules or slices (Forster and Taggart, 1950; Farah and Rennick, 1956; Koishi, 1959 b). Thus 0.01 m*M* Hg^{++} completely blocks phenol red transport in the flounder tubule. These and other observations all point definitely to a major site of action in the proximal segment of the nephron. However, there is also evidence that more distal transports can be affected. For example, the secretion of K^+ and the H^+ and NH_4^+ exchanges in the distal segment (Dale and Sanderson, 1954), and the resorption of solute-free water by the loop of Henle (Lambie and Robson, 1960) and the distal segment (Goldstein *et al.*, 1961), are depressed by the mercurials. It is difficult to compare the actions on proximal and distal portions of the nephron because of the different magnitudes of the transport processes; i.e., effects on proximal transport would be much more marked because of the major role of this segment in resorption.

(B) *Disappearance of renal SH groups.* Histochemical determination of the free SH groups in different regions of the kidney in normal and mercurial-treated animals might provide some information on the site of action if clear-cut differences are observed. Cafruny *et al.* (1955 b) determined the free protein SH groups in rat kidney sections by treatment with the SH reagent DDD (2,2′-dihydroxy-6,6′-dinaphthyldisulfide), coupling of the naphthol moiety with the azo dye Fast Blue RR, and photometric analysis. Following injection of a large dose of mersalyl (20 mg/kg), reduction of SH groups was observed in all portions of the nephron except the proximal and distal convoluted portions (see accompanying tabulation). Even at the markedly nephrotoxic dose of 40 mg/kg there is no decrease in SH groups

	Extinction values		% Change
	Control	Mersalyl	
Proximal tubules (convoluted)	0.619	0.613	− 1
Proximal tubules (terminal)	0.415	0.227	−45
Brush borders (terminal)	0.701	0.545	−22
Loop of Henle (descending)	0.355	0.238	−33
Loop of Henle (ascending)	0.373	0.229	−39
Distal tubules (convoluted)	0.592	0.596	+ 1
Collecting duct (medullary)	0.279	0.136	−51

in the convoluted segments. With low doses (2.5 mg/kg), disappearance of SH groups was observed only in the terminal portion of the proximal tubules and the ascending loops, the latter being the most sensitive region of the nephron. Time studies showed that the terminal proximal tubules are affected first and up to 1 hr show more reduction than the loops of Henle. Incubation of kidney sections with 20 m*M* mersalyl (Cafruny *et al.*, 1955 b) or saturated $HgCl_2$ (Cafruny *et al.*, 1955 a) produces marked nonspecific reduction in free SH groups, indicating that the pattern seen in the whole animal is due in part to the factors involved in the resorption and secretion of the mercurials. Cafruny and Farah (1956) later used dogs so that correlation between diuresis and SH group disappearance might be made. Kidneys were removed at the peak of diuresis (around 90 min) from 10 mg/kg of mersalyl, urine flow and Na^+ being increased 5- to 6-fold, and the changes given in the accompanying tabulation were observed, indicat-

	Extinction values		% Change
	Control	Mersalyl	
Proximal tubules (convoluted)	0.537	0.541	+ 1
Proximal tubules (terminal)	0.410	0.267	−35
Loop of Henle (ascending)	0.360	0.261	−28
Distal tubules	0.495	0.510	+ 3
Collecting ducts	0.242	0.192	−21

ing that selective reaction with SH groups in certain regions of the kidney does occur. It should be pointed out that the nature of these SH groups is not known; they may be on enzymes, carriers, or nonfunctional proteins.

Farah and Kruse (1960) used seven mercurials at 4 mg Hg/kg in rats and found moderate reduction of the protein SH groups (around 20–30%) in the terminal proximal tubules, the loops of Henle, and the collecting ducts, and it was concluded that maximal diuresis occurs when 20% of the protein SH groups of the proximal tubule are reacted, and thus that no more than this can be related to the diuresis. However, there is no correlation between diuresis and decrease in the SH groups, since *p*-MB and MM, both nondiuretic, produce similar changes in these groups. $HgCl_2$ and mersalyl at equimolar doses cause comparable decreases in renal SH groups in rats, and this was noted particularly at the bases of the proximal tubular cells (Gayer and Partowi, 1962). Renal S—S groups do not change for several hours after injections of $HgCl_2$ or chlormerodrin, but from 6 to 24 hr there is an increase in S—S groups at the expense of SH groups (Shore and Shore, 1962). This may be related to the potent inhibition of protein disulfide reductase, but is probably not correlated with diuresis since it occurs

some time after maximal urine flow. Renal damage and conversion of SH to S—S groups could be related in some as yet unexplained way.

These results all demonstrate that mercurials react with renal SH groups, and that some selectivity on certain regions may be exerted, but do not necessarily have any bearing on the site of transport inhibition, since the SH groups involved in the transport (assuming they are) may be only a very small fraction of the total in the tissue; indeed, it is quite possible that only 1–2% of the total SH groups need be reacted to produce maximal diuresis.

(C) *Reduction of electrical potentials of tubular cells.* There are two electrical potentials of the proximal tubular cells of the isolated *Necturus* nephron, a transmembrane potential of −72 mv and a transtubular potential of −20 mv (lumen negative) (Giebisch, 1958, 1960, 1961). Chlormerodrin in a concentration around 220 μg Hg/g tissue reduces both potentials; in the perfused nephrons the transmembrane potential is decreased 62% and the transtubular potential 63%. Since these potentials are dependent on active ion transports, quite possibly they relate to renal function. These results show that mercurials can affect the proximal tubules, but whether this is related to the diuretic effect is impossible to say.

(D) *Pattern of accumulation of mercurials in the kidney.* The kidneys of rats poisoned with $HgCl_2$ (3 mg/kg intraperitoneally) were examined from 5 min to 48 hr afterward by the silver sulfide method, and mercury was found to be deposited first in the endothelial cells of the interstitial capillaries, then in the glomerular tufts, and eventually in the epithelium of the proximal tubules, beginning apically and progressing toward the bases of the cells (Wöckel *et al.*, 1961). The mercury in the proximal cells is particularly associated with the basally situated mitochondria. It was concluded that Hg^{++} is filtered through the glomerulus and picked up by the tubular cells during resorption, which is the most obvious route for Hg^{++} and one which explains the early and marked effects on the proximal tubule. However, it has recently been claimed that another route is more important. Brun *et al.* (1947) suggested that mersalyl is secreted by the tubular cells, and that this accounts for the high concentration of mercury in the tubules and the selective effects on proximal transport. It was claimed by Borghgraef *et al.* (1956) that the excretory rate of chlormerodrin is too fast for glomerular filtration, especially considering that a large fraction of the plasma mercurial is bound and not filtered, and that tubular secretion is responsible for essentially all the mercury in the tubules. This theory has also been proposed by Weiner *et al.* (1956), Kessler *et al.* (1957 a, b), and Campbell (1959). Greif (1960) held that the uptake of Hg^{203} by *Phascolosoma* nephridia is an active transport, presumably because it is inhibited by cyanide; however, no inhibition by 2,4-dinitrophenol, azide, or iodoacetate was noted. Despite the evidence for the tubular secretion of

mercurials, I am not convinced that it is more important than resorption from the glomerular filtrate. If 1–5% of the total plasma mercurial is in a freely diffusible form, this fraction will certainly be filtered readily and concentrated in the lumen; renal accumulation occurs with many drugs which are bound to the plasma proteins. Second, only the free mercurial is available to the tubular cells from the peritubular fluid, and it is difficult to envision a mechanism by which the cells can pick up or actively secrete protein-bound mercurial. The tubular cells undoubtedly accumulate mercurials from the plasma as do other tissues, and may secrete them to some extent, but the rates of excretion are not such as to imply secretion as the major pathway. It is also strange that mecurials of so many different structures would be actively secreted; those with carboxylate groups might be carried by the transport system for acids, as Campbell (1959) postulated for mercaptomerin, but neither probenecid nor *p*-aminohippurate interferes with the excretion of mercurials in the dog (Kessler *et al.*, 1957 b). In any event, there is little likelihood that the pattern of mercurial distribution in the kidney can be directly correlated with the site of action. Weiner *et al.* (1959) emphasized that there is no obvious relationship between diuresis and the total amount of mercurial in the kidney or its parts, and stated, "Diuresis is a consequence of the action on a specific renal receptor by a very small amount of mercury."

(E) *Stop-flow technique.* Serial sampling of urine following ureteral clamping allows an analysis of the composition changes throughout the nephron, and such studies have uniformly pointed to a proximal rather than a distal site of mercurial action (Kessler *et al.*, 1958; Vander *et al.*, 1958). This applies exclusively to the site of inhibition of Na^+ resorption and diuresis.

(F) *Differential damage to renal cells.* It has been thought that those portions of the nephron most readily damaged by toxic doses of the mercurials might be the same portions primarily affected to produce diuresis. Suzuoki (1912) was the first to show by adequate methods that mercurials can damage rather selectively the more terminal portions of the proximal tubules. Edwards (1942), on the other hand, claimed that Hg^{++} exerts selective damage on the central region of the proximal convoluted tubule, injury to the distal convolution being rarely seen. The loops of Henle are too thin and squamous to permit satisfactory examination. Simonds and Hepler (1945) confirmed the observations of Suzuoki in finding selective damage to terminal proximal tubules. More recent work has not greatly extended our knowledge. Relatively little damage to the glomeruli has been confirmed (Staemmler, 1956) even when a severe nephrosis is produced, although Schörcher and Löblich (1960) found some changes in the glomerular filtration membrane by electron microscopy. Tubular cells show apical edema and vacuolization, these occurring primarily in the proximal segment in rats injected with meralluride (Sanabria, 1963). The brush border may show

a separation of the villi, and mitochondrial disintegration occurs. With large doses, damage may be observed with the electron microscope within 10 min. The maximal diuretic dose of mersalyl (6 mg/kg) in the rabbit produces nuclear pycnosis, mitochondrial changes, and vacuolization in the convoluted tubule (Dejung, 1963). Again, these results show selective effects, but may be the result of differential distribution and may not relate to the diuretic site of action.

The evidence taken together suggests that various portions of the nephron are affected in one way or another by the mercurials, and what portion may be involved will depend on the particular transport considered. The diuretic action, i.e., the inhibition of Na^+ resorption, appears to be limited mainly to the proximal tubule; whether this is primarily in the convoluted or terminal segments is not known. Analyses of the filtrate composition throughout the nephron in the presence of mercurials are very difficult to interpret, but most of the data are compatible with a proximal action (Welt *et al.*, 1953). More indirect evidence will be provided by studies on enzyme inhibition in the following section, but it is clear that the basic mechanism of mercurial action must be sought in the Na^+ transport system of the proximal tubules.

Effects on Enzyme Activity in the Kidneys

Much of the work has unfortunately been on succinate dehydrogenase, presumably because the activity is easily measured, but this enzyme is probably not directly involved in renal transport and, in fact, is much less sensitive to the mercurials than are many other enzymes. The results, however, may be taken as a rough indication that the mercurials can in diuretic doses inhibit various renal enzymes, and to some extent provide evidence for the primary site of action. Handley and Lavik (1950) found that meralluride injected intravenously at a dosage of 8 mg Hg/kg in dogs and rats reduces the succinate dehydrogenase activity around 45% in the kidney at the peak of diuresis, whereas no inhibitions were observed in the liver or heart. Fawaz and Fawaz (1951), on the other hand, could detect no changes in succinate oxidation by renal homogenates from rats given the diuretic dose (4 mg Hg/kg) of mersalyl, and concluded that if the mercurial is acting on an SH enzyme, succinate dehydrogenase is not involved. Mustakallio and Telkkä (1953) reported that high doses (10–60 mg Hg/kg) of mercurophylline lead to loss of succinate dehydrogenase activity in the distal tubules, using a tetrazolium staining technique, and later (Telkkä and Mustakallio, 1954) found some inhibition in the proximal tubules and loops of Henle, their conclusion being that there is little correlation between such inhibition and transport inhibition. Somewhat different results were obtained by Rennels and Ruskin (1954), who found marked inhibition of succinate dehydrogenase in the proximal tubules of the rat following 40 mg Hg/kg of meralluride, little or no effect being exerted on the distal tubules or loops

of Henle. However, this is a very high toxic dose and the maximal inhibition occurred at 24–48 hr, although some effect could be observed at 3 hr. In doses under 10 mg Hg/kg, no inhibition could be demonstrated, so that again there is no reason to relate the inhibition to diuresis. Wachstein and Meisel (1954) also observed succinate dehydrogenase inhibition in the proximal tubules of the rat following large nephrotoxic doses of meralluride. Bahn and Longley (1956) confirmed these results in that diuretic doses of meralluride (4 mg Hg/kg) produce insignificant inhibition of succinate dehydrogenase while toxic doses (12 mg Hg/kg) inhibit moderately, especially in the terminal proximal tubules. Finally, Bickers *et al.* (1960) found that meralluride at 3–4 mg Hg/kg does not inhibit succinate dehydrogenase, whereas 5 mg Hg/kg does to some extent after 10 hr. Later changes in enzyme activity, at a period of tubular damage, may be the result of secondary changes and necrosis, so have little bearing on the mechanism of the diuretic action.

α-Ketoglutarate oxidase is more sensitive to mercurials than is succinate dehydrogenase, and is inhibited 64% at 3–4 hr and 91% at 6 hr in rats given $HgCl_2$ at 3 mg Hg/kg (Shore and Shore, 1960). In animals fed sucrose, the inhibitions are less, and sucrose also protects somewhat against the nephrotoxic effects of Hg^{++}, the mechanism being unknown. But again it is impossible to correlate this action with the diuretic effect, since mersalyl in diuretic dose (4 mg Hg/kg) does not alter the renal concentration of α-ketoglutarate, although toxic doses (10 mg Hg/kg) produce an early and prolonged rise in α-ketoglutarate (Dzúrik and Krajči-Lazáry, 1962). No changes in sorbitol dehydrogenase activity in the kidneys of rats given nephrotoxic doses of mercurin are detectable, and it would probably not be very significant if they were (Pietschmann *et al.*, 1962). Moderate inhibitions of NAD and NADP diaphorases are found after toxic doses of the mercurials (Bickers *et al.*, 1960; Wachstein and Meisel, 1957), but in all cases where enzyme inhibition is seen, there is histologically demonstrable damage to the tubules. Protein disulfide reductase is apparently rather potently inhibited in the kidney during mercurial action, but this is undoubtedly unrelated to the diuretic effect (Shore and Shore, 1962).

Various phosphatases have been the subject of investigation although there is little reason to expect a relation to ion transport. Nephrotoxic doses of $HgCl_2$ inhibit renal phosphatase slightly (10–20%) without affecting serum phosphatase, but subtoxic doses somewhat increase the phosphatase activity (Hepler *et al.*, 1945). There is no correlation between glucosuria and phosphatase inhibition (Hepler and Simonds, 1946). Very high doses (175–260 mg/kg) of $HgCl_2$ decrease tubular phosphatase, although not in capillaries or glomeruli, but low doses (6–10 mg/kg), which are lethal in 3 weeks, do not alter the phosphatase (Sachs and Dulskas, 1956). Diuretic doses of various mercurials do not inhibit alkaline phosphatase, β-glycero-

phosphatase, or glucose-6-phosphatase (Shore *et al.*, 1959; Bickers *et al.*, 1960), but toxic doses lead to a diffuse distribution of various phosphatass in the tubular cells, and some inactivation (Wachstein and Meisel, 1957). Very little work has been done on ATPase, but there is slight evidence that it can be inhibited in the kidney by both mersalyl (DeGroot *et al.*, 1955) and *p*-MB (Padykula and Herman, 1955). It is probably fair to say that none of these investigations of phosphatases has established a relation to their role in transport or mercurial diuresis.

Effects on Renal Metabolism

Mercurials can certainly depress the respiration of kidney at sufficiently high doses, and this may possibly be a factor in the renal damage produced, but there is little evidence that nontoxic diuretic doses of the mercurials reduce O_2 consumption. The early work of Gremels (1929) indeed showed that, in the heart-lung-kidney preparation, mersalyl actually increases renal respiration during diuresis. Cohen (1953 a,b) showed particularly well the difference between diuretic and toxic doses. Mercaptomerin at a dosage of 10.7 mg Hg/kg in rats produces a marked diuresis. After 1 hr the animals were sacrificed and a mitochondrial suspension prepared from the kidneys; no change in the O_2 uptake was noted, and the P : O ratio may actually have been increased. However, with a toxic dose of 26.7 mg Hg/kg, the O_2 uptake was depressed 35% and the P:O ratio dropped from 0.55 to 0.092. Very similar effects were observed on kidney slices, only toxic doses reducing the respiration. Even toxic doses do not affect the O_2 uptake or P:O ratio of liver mitochondria obtained from poisoned animals. That mercurials exert very little effect on the cycle when given in diuretic doses was demonstrated by Fawaz and Fawaz (1954). Rats were poisoned with fluoroacetate, and citrate accumulation was determined in the heart and kidney of control animals and those given mersalyl at 4–5 mg Hg/kg; no significant change in citrate formed was noted, so that the operation of the cycle would seem to be unaltered. Although these results indicate no appreciable interference with coenzyme A, Leuschner *et al.* (1957) claimed that the coenzyme A induced acetylation of sulfanilamide is reduced by $HgCl_2$, mersalyl, and mercaptomerin in the same order of potency as for diuresis. Toxic doses reduce the coenzyme A reaction, but it is not certain if diuretic doses are able to do this. The mercurial diuresis is less in coenzyme A-deficient rats, but the significance of this is unknown. Results on oxidative phosphorylation are contradictory. Greif and Jacobs (1958) found that even large doses of chlormerodrin (up to 20 mg Hg/kg, which is 20 times the diuretic dose) do not alter the P:O ratio of kidney mitochondria with glutamate as the substrate, but Shore and Shore (1960) reported a marked fall in P:O ratio with α-ketoglutarate as the substrate following toxic doses (3 mg Hg/kg) of $HgCl_2$.

The results obtained on kidney slices are generally in agreement that, although respiration may be reduced by reasonably high concentrations of mercurials (0.25–1 m*M*), the inhibition of various transports is much greater. Thus Cross and Taggart (1950) found that 1 m*M* Hg^{++} depresses O_2 uptake of rabbit kidney slices 35% while inhibiting *p*-aminohippurate accumulation 89%, and Mudge (1951) showed that respiration is scarcely affected by Hg^{++} at concentrations markedly altering Na^+ and K^+ transport. Mendelsohn (1955) confirmed that Hg^{++} can reduce *p*-aminohippurate accumulation as much as 70% without affecting respiration in kidney slices. Robinson (1956) believed that the inhibition of respiration by mercaptomerin might be the basis for the swelling of rat kidney slices and the interference with water transport, but there was no direct evidence for a relationship. Maizels and Remington (1958) also observed moderate respiratory inhibition with mercaptomerin and meralluride, but did not feel that this was the chief factor in the increase in water and Na^+ of the slices. Furthermore, the lowest concentration of the mercurials which exerts an effect *in vitro* is much greater than the maximal tolerated plasma concentration in rats *in vivo*, so it is doubtful if these inhibitions of respiration are relevant to the diuretic action.

Summarizing all the data obtained on enzyme and metabolic inhibition in the kidney, it is disappointing that no system particularly sensitive to the mercurials has been found, and that no correlation between inhition and transport processes has been demonstrated. If the basis of mercurial diuresis is metabolic, no clear evidence for this has yet been provided.

Accumulation and Excretion of Mercurials by the Kidneys

All mercurials are rather slowly accumulated by the kidneys and the high levels are sustained for periods of several days. The kidney/plasma ratio is maximally around 1000 in the rat and dog when chlormerodrin is given (Borghgraef and Pitts, 1956; Giebisch and Dorman, 1958), but these ratios are reached only after many hours, and are in part due to the retention by the kidney with falling plasma levels. The correlation between distribution and excretion of a mercurial and the diuresis is well shown in the results of Borghgraef *et al.* (1956) (Fig. 7-44). The loss of mercurial from the plasma is divided into three components: that excreted in the urine, that entering the various tissues, and that accumulated by the kidneys. Some mercurials are excreted fairly rapidly and others slowly; meralluride administered intramuscularly in man is 50% excreted in 3 hr (Burch *et al.*, 1950) but chlormerodrin given by the same route to rats is only 21% excreted after 24 hr, 67% remaining in the kidneys (Borghgraef and Pitts, 1956). We have discussed the theories of the excretory mechanisms (page 923) and the possible role of tubular secretion. The mercurials are not excreted entirely in the form administered. Some may be split into inorganic Hg^{++} but most

is excreted as a complex with cysteine, or other thiols (Weiner and Müller, 1955). The origin of this cysteine complex is not known, and it may be in the kidney or the blood. The various mercurials are distributed differently throughout the tissues, as one might expect from the different properties of their molecules, and this must play some role in the effects they produce,

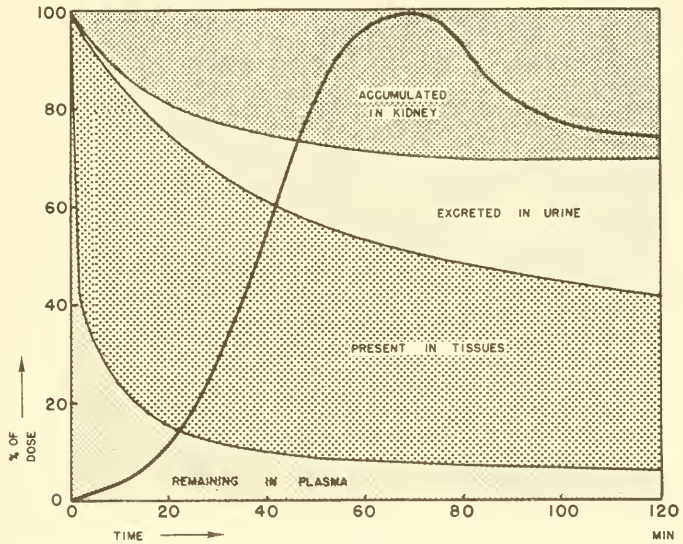


FIG. 7-44. Distribution of Hg^{203} after intravenous injection of chlormerodrin in dogs at 1 mg Hg/kg. The curve shows the change in urine flow (diuresis) estimated from the figures given. (From Borghgraef *et al.*, 1956.)

not only on the kidney but on other tissues in higher doses. The most thorough study has been made by Kessler *et al.* (1957 a) and some of their results are summarized in Table 7-19. Hg^{++} behaves quite differently than the organic mercurials; it does not enter the kidney rapidly but eventually reaches very high levels after several hours. It may be noted that the distribution of *p*-MB is not markedly different from the diuretic mercurials. Little is known about the cellular fractions of the kidney which accumulate the mercurials, but it is somewhat surprising that Greif *et al.* (1956) found that by far the most mercury after injection of chlormerodrin to rats, followed by fractionation of kidney homogenates in sucrose solutions, is in the soluble fraction, about one third the amount in granules, and much less in the nuclei. Although all of these distribution studies are important in understanding many facets of mercurial action, they do not appreciably contribute to our knowledge of where or how the mercurials produce disturbances in the renal function.

Table 7-19

Concentration of Mercury in Tissues of the Dog After Intravenous Administration of Mercurials [a]

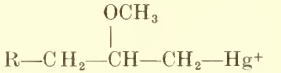
Tissue	Tissue concentration (μg/g wet weight)				
	Chlormerodrin	Meralluride	Mersalyl	$HgCl_2$	*p*-MB
Renal cortex	155	19	7.7	113	36
Renal medulla [b]	125	13	4.0	79	—
Renal papilla	4.5	1.4	0.4	2.3	—
Liver	3.9	0.8	0.8	6.6	2.8
Heart	0.3	0.2	0.4	0.5	0.7
Spleen	1.4	0.6	1.1	74	2.1
Lung	1.2	0.9	0.9	2.0	2.8
Diaphragm	0.2	0.2	0.1	0.3	0.8
Intestine	0.7	0.5	0.7	1.1	2.5
Skin	0.4	1.0	0.7	0.8	1.0
Plasma	1.1	1.3	1.2	2.9	—

[a] At 2 mg Hg/kg and sacrifice of the animals at 160 min. (From Kessler *et al.*, 1957 a; *p*-MB data from Kessler *et al.*, 1957 b.)

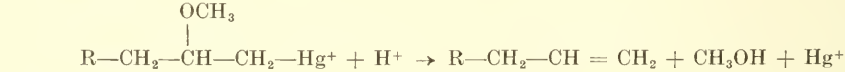
[b] Only outermost sections of medulla considered here.

Active Form of the Mercurials and Relation of Action to Structure

The concept that the organic mercurials in order to be active diuretics must dissociate into inorganic mercury is an old one and has been revived recently to explain some of the differences between mercurials and the effects of pH on the activity. Most diuretic mercurials have the structure:

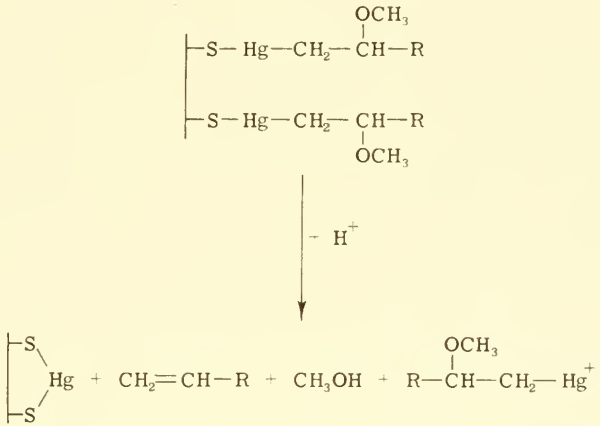


The methoxy group arises because these mercurials are synthesized by the oxymercuration of alkenes in methanol; the nature of this group is not particularly important for the activity. It is possible that the reverse of this reaction

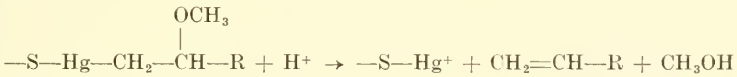


might occur under physiological conditions, as suggested by Hughes (1957).

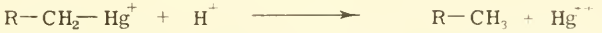
Such a splitting would occur more rapidly the lower the pH. Certainly this release of Hg^{++} is not generally responsible for the actions and toxicity of the organic mercurials, and most of the mercurials, such as *p*-MB, PM, and MM, are quite stable. Hepp (1887) emphasized long ago that alkyl mercurials do not release Hg^{++} in the body and exert a toxic action much different than Hg^{++}. However, the diuretic mercurials present a different situation and the theory of Hg^{++} release must be given serious consideration. The possibility that the above reaction might be catalyzed or accelerated by thiols through the formation of mercaptides was presented by Benesch and Benesch (1952) as a result of their polarographic investigations of the reaction between mersalyl and dimercaprol. In this scheme, free Hg^{++} may not be produced directly; instead, a cyclic mercaptide is formed, which could



conceivably be the inhibiting complex in renal transport, although it is also possible that monothiols can act similarly:



since Mudge and Weiner (1958) showed that cysteine increases the splitting of mersalyl in acid medium. Other acid-stimulated types of splitting would be the simple reactions:



but these usually occur fairly slowly, especially for the alkyl mercurials.

The hypothesis that organic mercurial diuretics to be active must release inorganic Hg^{++} in the kidney was proposed by Mudge and Weiner (1958) and the evidence was presented by Weiner *et al.* (1962). This had been suggested occasionally ever since the first use of the organic mercurials but very little evidence either for or against was reported, and the idea generally was not taken seriously because all the other metal compounds used clinically had been shown to act directly without splitting off the metal ion. The evidence now accumulated impels one to consider this possibility. If such splitting occurs, it is important not only for the diuretic action but for many other effects of the mercurials, even *in vitro*. The evidence is mainly of two sorts: (1) a correlation between acid lability of organic mercurials and their diuretic activity, and (2) the potentiation of diuretic activity by the acidifying NH_4Cl.

Mudge and Weiner (1958) pointed out that mersalyl does not split* in acid medium over 3 hr, but in the presence of cysteine the half-time for splitting is 105 min and with dimercaprol 5 min. The acid lability of 32 mercurials was tested by Weiner *et al.* (1962) by incubating the mercurial at 1 mM with cysteine at 2 mM in an O_2-free medium at pH 4 and 37° for 3 hr. The diuretic activity was expressed as ΔCl (μmoles/min/kg). All of the 22 mercurials which are diuretic are acid-labile, while of the 9 nondiuretic mercurials 6 are stable and 3 labile (1 mercurial is indeterminate in diuretic activity). There is thus a reasonably good correlation between lability and diuretic activity. The 3 labile nondiuretic mercurials are all of the ether series with structures of the type $R—CH_2—O—CH_2CH_2—Hg^+$, and possibly their distribution is such that Hg^{++} is not released in the proper region. The pH dependence of the splitting, according to the reactions of Benesch and Benesch (1952), indicate the rate of splitting to be approximately one thousandth as fast at pH 7 as at pH 4. If one assumes that $X = X_0e^{-kt}$, where X is the amount of organic mercurial, it may be calculated that at pH 7 around 0.14% splitting would occur in 3 hr, since the mean splitting of the labile mercurials is about 75%. Since maximal diuresis occurs in 1–2 hr, approximately 0.1% would be split in this time. Now, this calculation is not very valid because one does not know the conditions for splitting in the kidney; e.g., dimercaptides may be formed there and split more rapidly than the cysteine complexes. Diuretic activity was examined by injecting the mercurials with a 10-fold excess of cysteine, but presumably in the kidney there would be a transfer of the mercurial from cysteine to other thiols. Thus it is difficult to obtain an idea of the amount of inorganic mercury which is released in the kidney. If much splitting occurs, one might expect to find Hg^{++} excreted in the urine in some form.

* The term "split" will be used to designate the dissociation of the mercurial into inorganic mercury so that there will be no confusion with the term "dissociation" which is used to indicate the reaction $R—Hg—X \rightleftharpoons R—Hg^+ + X^-$.

Moyer *et al.* (1957) and Handley and Seibert (1956) could detect no inorganic mercury after administration of meralluride, but Weiner *et al.* (1962) pointed out that Hg^{++} would be excreted mainly as the cysteine complex and this would be included with the organic mercurial in their chromatographic fractionations. It is also possible that very tight binding of the active Hg^{++} in the kidney would occur, so that the excretion would be slow. Weiner *et al.* (1962) could detect Hg-cysteine in the urine following injection of 3 particularly labile mercurials, but of course this is not valid evidence that the Hg^{++} is the active form.

Clinical diuretic refractoriness to the mercurials has been known for years and it is often possible to restore the diuretic response by giving NH_4Cl. The potentiating action of NH_4Cl has been the subject of much work and speculation, but the mechanism is still unknown. One hypothesis is that the urinary acidification is the major factor. Weiner *et al.* (1962) assume that this acidification increases the splitting of the labile mercurials. Administration of NH_4Cl drops the pH of the urine below 5 and an optimal effect is usually seen around 4.5; thus the splitting of the mercurial in the urine will be significantly accelerated. However, the Hg^{++} in the urine will presumably be complexed with cysteine or other simple thiols and the rate of splitting will not be very great. It has also generally been assumed that the splitting occurs in the tubular cells. Although the intracellular pH undoubtedly falls after NH_4Cl, the decrease is certainly not as great as in the urine. Pending determinations of intracellular changes, one cannot estimate the effect this would have on mercurial splitting. The mercury contents of the proximal tubules in the dog were determined histochemically by Cafruny (1962), using di-β-naphthylthiocarbazone, and acidosis was shown to increase the levels for chlormerodrin and Hg^{++}, although a decrease occurs with *p*-MB. He felt that acidosis either increases the available receptors for mercurials or somehow alters the affinity of the mercurial for the receptors. Change in the acid-base balance does not alter the excretion of the mercurials, so it is presumably not a matter of the tubular concentration of mercurial. If the fall in pH is responsible for increased splitting of the mercurials and thus a greater diuretic effect, NH_4Cl administration or other acid-base changes should not affect the diuresis produced by Hg^{++} complexes. Mudge and Weiner (1958) and Levy *et al.* (1958) reported that the action of meralluride is altered more than Hg-cysteine by variations in the urinary pH. However, Hg-cysteine diuresis is increased 2.4-fold in going from alkalosis to acidosis, so that the results are not as clear-cut as one might wish. If acidosis is responsible for greater mercurial action, one would also expect that any type of acidosis would be effective. However, Kessler (1960) points out that inhalation of 12% CO_2 actually decreases mercurial diuresis, although not as much as alkalosis produced by HCO_3^- infusion. But inhalation of 12% CO_2, although it produces an acidification

of the plasma (pH 7.4 to 7.14), does not alter urinary pH significantly, as does administration of NH_4Cl; no one knows what happens to intracellular pH in the tubules. It may also be pointed out that alkalinization of the urine with acetazolamide or K^+ does not alter the diuretic response to mercurials (Pitts, 1958).

It is not immediately apparent why Hg^{++} must be formed from organic mercurials to inhibit renal transport, since in most cases the organic mercurials react readily with SH groups which may be involved. If mercaptides or dimercaptides participate in the splitting of the mercurials, the Hg^{++} formed must dissociate from these SH groups and attach to others, because the cell component originally binding the mercurial must be blocked and there would be no necessity for splitting. If Hg^{++} is necessary for diuresis, it must be that either (1) a cyclic mercaptide is required, or (2) the important SH groups are not sterically accessible to the larger organic mercurials. Weiner *et al.* (1962) assume that the specific receptor for the diuretic action contains two groups, one being an SH group and the other either an SH group or some other ligand complexing with Hg^{++} (e.g., an amino group). The complete scheme as outlined by Weiner *et al.* (1962) is shown

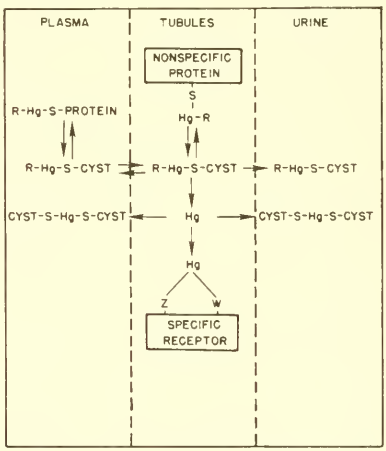


FIG. 7-45. Scheme of mercurial reactions in the kidney. (From Weiner *et al.*, 1962.)

in Fig. 7-45. It is strange that this transport component would not be inhibited by organic mercurials bound to one SH group, or that certain potent SH reagents, such as MM or *p*-MB, would not inactivate it. The nondiuretic *p*-MB prevents and reverses the diuretic effects of the mercurials, while MM does not do this (Miller and Farah, 1962 a). A competition between *p*-MB and Hg^{++} for the receptor SH groups was suggested. Miller and Farah

(1962 b) also postulate a two-group receptor, one group being SH; mercurials which are diuretics attach to the SH group, split, and the resulting Hg^{++} makes a two-point attachment. The block by *p*-MB is due to its binding to the SH group; being stable it does not split. If this is so, *p*-MB might be expected to displace mercurials in the kidney, and this was demonstrated using Hg^{203}-labeled chlormerodrin. The decrease in radioactivity of the kidneys parallels the antagonism of the diuresis by *p*-MB.

Another hypothesis for diuretic mechanism was outlined by Kessler *et al.* (1957 b), who assumed that the organic mercurials act as intact molecules by a two-point attachment to a receptor. The basic structure for diuretic activity was given as R—C—C—C—Hg^+, i.e., a hydrophilic group separated from the Hg by three carbon atoms, the R group interacting in some manner with a group spaced appropriately in relation to an SH group. This hypothesis in its simple form has had to be abandoned in the light of further work showing that various mercurials not conforming to this structure are diuretic, e.g., some of the aryl mercurials (Weiner *et al.*, 1962). However, the idea that there is some relationship between structure and diuretic action should not be given up, inasmuch as the situation may be more complex than originally assumed. If one considers the three simple alkyl mercurials (see accompanying tabulation), one sees that diuretic activity is

Alkyl mercurial	Diuretic activity	Lability
$CH_3—CH_2—Hg^+$	−	−
$HO—CH_2—CH_2—Hg^+$	+	+
$HO—CH_2—CH_2—CH_2—Hg^+$	−	−

correlated with acid lability, and that $HO—CH_2CH_2CH_2—Hg^+$, which should be diuretic in the scheme of Kessler *et al.*, is not. It is intriguing that $HO—CH_2CH_2—Hg^+$ is 95% split under the conditions of the lability test, whereas the other two compounds are completely stable; it is also surprising that HO—*o*—Hg^+ and H_2N—*o*—Hg^+ are 73% and 88% split, respectively, while ^-OOC—*o*—Hg^+ and *o*—Hg^+ are not split at all. The structural requirements of lability seem to be very rigid. Could it be that the structural requirements for splitting are the same as for combination with a receptor to produce diuresis, splitting not being a necessary prelude to an effect? It is clear that a final decision as to these important matters cannot be reached at this time and that more data must be accumulated; one would like to have some information on the rates of splitting of mercurials in homogenates or extracts of kidney, and the effects of pH on this.

Mechanism of Transport Inhibition

If one knows essentially nothing of the cellular site of action of the mercurials, and is completely ignorant of the molecular nature of ion transport, it is difficult to discuss possible mechanisms of inhibition without becoming ethereal. All the evidence points to a lack of significant depression of the exergonic phases of renal metabolism at concentrations markedly affecting transport, so it is likely that the action is on some component of the functional system. If the postulated specific receptor for mercurials is a carrier, then there is the problem of accounting for the inhibition of many types of transport; furthermore, it is not at all certain that a carrier is involved in ion transport. On the basis of what was said in the previous section on the effects of mercurials on membranes and permeabilities, it is most likely that the site of action is the tubular cell membrane. The Na^+ pump is probably located in the peritubular membrane and the diffusion of Na^+ across the lumenal membrane is passive along concentration and electrical gradients. The fact that the transmembrane potential is around -43 mv at the lumenal surface and -64 mv at the peritubular surface was interpreted by Giebisch (1960) as indicating the greater Na^+ permeability of the lumenal membrane. Mercurials could thus depress Na^+ resorption by acting in three ways: (1) inhibition of the Na^+ pump, (2) decrease of the permeability to Na^+ of the lumenal membrane, or (3) increase of the permeability to Na^+ of the peritubular membrane. It has frequently been assumed that the mercurials inhibit active transport directly but, as has been discussed for other systems, it is possible that the primary effect is on the membrane structure controlling permeability. The evidence from the changes in electrical potentials brought about by chlormerodrin shows that both membranes are affected (Giebisch, 1961). The potential across the peritubular membrane is decreased to -25 mv and, since the transtubular potential is simultaneously decreased to -7 mv, it would seem that the lumenal membrane potential is decreased to -18 mv. These changes can be interpreted as due to increases in the Na^+ permeability of both membranes, but it is also possible to conclude that there is a general decrease in the ionic permeability. Until information on the effects of mercurials on ion fluxes is available, one cannot distinguish between these possibilities. The results of Mudge (1951) on rabbit kidney slices point either to an increase in permeability to Na^+ and K^+, or to an inhibition of active transport. However, the relationship of these *in vitro* results to mercurial diuresis is obscure, especially as Auditore and Holland (1956) found that diuresis can be produced without appreciable loss of cell K^+, although the latter can occur with minimal toxic doses. That mercurials can alter ion permeabilities without appreciably depressing active transport is demonstrated by studies on other tissues, such as atria (page 945). Mercurials could conceivably alter pore sizes by distorting membrane structure, or actually clog ion-

transporting pores by reacting with SH groups on the walls, or interfere with the open-closed transition postulated to occur in the membrane by Kavanau (1963). White *et al.* (1961) have provided the only direct evidence that mercurials increase the permeability of the proximal tubule cells to Na^+. They infused Na^{22} into the renal artery during mannitol diuresis and found the Na^+ flux to be increased by meralluride at diuretic doses. It was thus concluded that the net resorption of Na^+ is decreased because of the augmented backward leak.

EFFECTS ON TISSUE FUNCTIONS

Surprisingly few thorough or quantitative investigations of the effects of mercurials on tissue function have been made, especially considering the long-known toxic and therapeutic actions of these substances, and most of them are on the heart. Since in essentially no case have functional and metabolic disturbances been correlated, most of the results will be treated cursorily and presented principally to point out some fields in which interesting work may be done. There is great need for the study of the metabolic changes produced in tissues isolated from animals treated with mercurials, since only in this way can one be certain that the effects observed are related to the *in vivo* interference with function. This has been done with the kidney but is notably absent with other tissues. Much of the *in vitro* work with mercurials has been done with relatively high concentrations (around 1 m*M* or higher) so that it is impossible to determine if the results are applicable to the effects seen in the whole animal. Indeed, studies of the simultaneous changes in fuction and metabolism under any conditions are very rare.

Skeletal Muscle

Resting rat diaphragm treated with 1–2 m*M* mersalyl soon exhibits fibrillatory twitches accompanied by rapid small (1 mv) variations of the membrane potential, which persist for several minutes, followed by a rise in the resting tension during the next 10 min, and finally by a further slowly developing irreversible contracture and loss of excitability (Kuschinsky *et al.*, 1953). Stimulation during the early action of mersalyl produces a normal contractile tension but there is a marked retardation of relaxation, the duration of contraction increasing 5- to 10-fold. Hg^{++}, on the other hand, causes only the slow contracture and loss of excitability. Decamethonium and curare abolish the fibrillation due to mersalyl, indicating that the action of the mercurial is on the end-plate. Furthermore, chronically denervated muscle does not show mersalyl fibrillation. Inasmuch as physostigmine and neostigmine cause a similar fibrillation, and since mersalyl

inhibits muscle cholinesterase, it was concluded that the fibrillation results from inhibition of cholinesterase, allowing acetylcholine to accumulate (Kuschinsky and Lüllmann, 1954). None of the other actions of mersalyl appears to be related to this inhibition. The delayed relaxation was claimed to be similar to that produced by veratrine but, if so, it does not provide much information on the mechanism, since one is ignorant of how veratrine acts.

Contracture of frog muscle by Hg^{++} had been noted by Bacq (1942), Beck and Bein (1948), and Krueger (1950). Bacq assumed this to be an effect such as that given by iodoacetate, i.e., a Lundsgaard effect due to glycolytic inhibition, but Krueger showed that Hg^{++}, in contrast to iodoacetate, does not bring about a reduction in lactate concentration during rigor. Kuschinsky and Lüllmann (1954) found that mersalyl causes a rapid loss of muscle K^+ and attributed the initial rapid contracture to a depolarization of the fibers, a conclusion shared by Muscholl (1958), who demonstrated a fall in the membrane potential coincident with contracture. However, the delayed contracture must have another origin and, although Kuschinsky and Lüllmann postulated a Lundsgaard mechanism, there is no evidence one way or the other. The action potential traces presented by Muscholl (1958) show that mersalyl reduces the magnitude so that the overshoot is lost, slows both depolarization and repolarization, and hence prolongs the duration of the action potential, effects quite different than those seen in heart (page 945). Frog muscle after-potentials seem to be unaffected by mercurials between 0.1 and 2 m*M* (Macfarlane and Meares, 1958). Contracture by mersalyl is dependent on Ca^{++} in the medium, but this may be true for most types of contracture (Kutscha, 1961).

The contraction of glycerinated muscle fibers by ATP is inhibited by Hg^{++} at concentrations around 0.01–0.1 m*M* (Godeaux, 1944, Korey, 1950; Hasselbach, 1953; Edman, 1958) and by mersalyl at similar concentrations (Portzehl, 1952; Edman, 1959). A preparation contracted by ATP is relaxed upon addition of the mercurial. These effects are irreversible by washing or treatment with cysteine. Weber and Portzehl (1954) suggested that the inhibition of the ATP effect is due to a block of ATPase so that ATP can act only as a plasticizer, but there is also, according to Edman (1958), a direct effect since there is some relaxation in the absence of ATP.

A great deal of work has been done on the behavior of muscle contractile proteins exposed to mercurials, and the importance of SH groups has been conclusively demonstrated. The effects of the mercurials are summarized in Table 7-20. Both actin and myosin possess SH groups of differing degrees of reactivity and function. In the complexing of actin and myosin to form actomyosin, it is the SH groups of myosin which are important (Bailey and Perry, 1947; Kuschinsky and Turba, 1951). Bailey and Perry felt that

TABLE 7-20

EFFECTS OF THE MERCURIALS ON THE CONTRACTILE PROTEINS OF MUSCLE

Effect produced	Mercurial	Concentration (mM)	Reference
Inhibition of actomyosin formation	*p*-MB	1/3 SH's reacted	Bailey and Perry (1947)
	Hg^{++}	—	Turba and Kuschinsky (1952)
	Mersalyl	1–7 moles/10^5 g	Bárány (1959)
	MM	—	Bárány and Bárány (1959 a)
Dissociation of actomyosin	*p*-MB	—	Gergely *et al.* (1959)
	Mersalyl	—	
	Mersalyl	—	Bárány (1959)
Inhibition of actomyosin gel contraction by ATP	Mersalyl	0.3	Kuschinsky and Turba (1950)
Prevention of plasticizing effect of ATP on myosin and actomyosin films	*p*-MB	0.01	Cheesman *et al.* (1959)
Inhibition of G-actin → F-actin polymerization	*p*-MB	2.5	Tsao and Bailey (1953)
	p-MB	2–5 SH's reacted	Tonomura and Yoshimura (1962)
	p-MB	4–6 DH's reacted	Katz and Mommaerts (1962)
	p-MB	—	Drabikowski and Gergely (1963)
	Hg^{++}	—	
	Mersalyl	—	Drabikowski and Gergely (1963)
	Mersalyl	0.3	Kuschinsky and Turba (1950)
Depolymerization of F-actin	*p*-MB	1	Bárány *et al.* (1962)
Prevention of binding of ATP to G-actin	Hg^{++}	0.11	Martonosi and Gouvea (1961)
	Mersalyl	0.12	
Dissociation of G-actin-bound ATP	*p*-MB	0.1	Strohman and Samorodin (1962)
Release of G-actin-bound Ca^{++}	*p*-MB	1	Bárány *et al.* (1962)
	MM	1	
	Mersalyl	1	
Increase of the viscosity of myosin	Mersalyl	0.3	Kuschinsky and Turba (1950)
Decrease of the flow birefringence of myosin B	Mersalyl	—	Noda and Maruyama (1960)
Release of subunit from myosin	MM	—	Kominz (1961)

the binding might be through SH groups but, as Gergely *et al.* (1959) pointed out, it only indicates that SH groups are in the vicinity of the binding groups. The myosin SH groups concerned with the binding of actin react with mercurials more readily than the SH groups upon which ATPase activity depends (Fig. 7-22) (Bárány, 1959). However, MM presents an exception, in that it inhibits ATPase and actomyosin formation in a parallel fashion (Bárány and Bárány, 1959 a), possibly indicating that the size of the group on the mercurial is important. G-actin is more reactive than F-actin, due perhaps to shielding of the SH groups in the polymerized form. G-actin cannot polymerize unless ATP is present and, since mercurials release ATP from actin, the possibility of the effect on the G-actin $\leftrightarrows$ F-actin transformation being due to an interference with ATP binding was examined, but most mercurials were found to cause a rapid loss of polymerizability without appreciable loss of ATP (Drabikowski and Gergely, 1963). When the ATP is finally lost, the actin has been changed irreversibly, and there is further evidence from optical rotation that structural changes are produced (Tonomura and Yoshimura, 1962). Katz and Mommaerts (1962) consider the six SH groups of G-actin to fall into three categories: two rapidly reacting, two intermediately reacting, and two slowly reacting, only the last two being necessary for polymerization. It is interesting that the SH groups of G-actin are made more reactive to *p*-MB by Mg^{++} and less reactive by Ca^{++} (Katz, 1963). It was postulated that Mg^{++} brings about an open configuration whereas Ca^{++} tends to produce a closed configuration, the SH group being in a crevice.

The effects of the mercurials on extracted muscle proteins are certainly interesting and often obtained at low concentrations, but there is at present essentially no way of determining if they are at all responsible for any of the changes observed in intact muscle. It would be particularly important to know if rigor is related to any of the actions on actomyosin, but actually most of the actions described above could not very well explain why a muscle goes into contracture. The mechanisms by which mercurials alter muscle function are thus obscure, but it is not unlikely that the earliest effects are on the permeability and transport systems in the membrane. More information will be provided in the following section in which the effects of the mercurials on cardiac muscle will be discussed.

Heart

The detrimental effects of the mercurials on the heart have long been recognized and many cases of clinical deaths from intravenous injections of mercurial diuretics have been reported. It is generally agreed that death is attributable to the direct action on the heart during temporary high plasma concentrations of the mercurials, whereas at the usual low concentrations required for diuresis there are no detectable cardiac effects. The

actions of the mercurials on the heart have been well studied but the mechanisms involved, and whether these actions involve metabolic disturbances, are not known. Hepp (1887) observed that the toxic effects of the organic mercurials differ from those produced by Hg^{++}, and that ethyl-Hg^{++} stops the frog heart in diastole. Dreser (1893) studied the cardiac effects of several complexes of Hg^{++}, both *in vivo* and on perfused isolated frog hearts. Cardiac depression was noted after injection of around 2.5 mg of the rhodanate, succinimide, and cyanide complexes of Hg^{++}, and standstill of the isolated heart was brought about by 0.45 m*M* of the thiocyanate complex. These and other early investigations showed only that the heart can be depressed by mercurials and that the relative potencies depend on the substances with which the Hg^{++} is complexed. For example, Müller *et al.* (1911) showed that compounds of the type R—Hg—OH or R—Hg—CN are around 10 times as cardiotoxic in cats as compounds of the type R—C—Hg—C—R in which the Hg is bonded to two C atoms, and that the toxicity is related to the rate at which these compounds react with sulfide. The first serious study of the effects of the mercurials on the heart was undertaken by Salant and his co-workers in Georgia from 1921 to 1931, the results of which will be discussed throughout the following sections.

(A) *Isolated heart preparations.* The effects of the mercurials depend on the species, the type of preparation, the mercurial used and its concentration (Table 7-21). As would be expected, there is generally depression of contractile amplitude, rate of beating, and rate of conduction, the last leading to varying degrees of a-v block and dissociation of the atria and ventricles. Ventricular standstill often occurs before the atria cease to beat. Tachycardia and fibrillation are frequently seen in animals poisoned acutely with the mercurials, but are not noted in isolated preparations, although occasionally with low concentrations, or initially, some increase in rate and contractile amplitude may be observed. The ventricular dysrhythmias *in vivo* may be in part the result of altered a-v and ventricular conduction, but in isolated preparations there is little evidence for the appearance of rapidly discharging ectopic foci. Most of these effects are irreversible, or only partially reversible at the lowest concentrations and with short exposures, but dimercaprol or cysteine is occasionally able to reverse rather advanced degrees of depression (Ruskin and Johnson, 1949). The selective depression of the rat atrial rate is marked; at 0.0013 m*M* Hg^{++} the rate is reduced 35% in 15–20 min while the amplitude is unaffected, and at 0.0025 m*M* the rate may be inhibited 90% and the amplitude some 15% greater than normal (Berman, 1951). Glutathione and dimercaprol effectively protect both the atria and ventricles.

The development of contracture is not nearly as common with the mercurials as with iodoacetate, and in fact has been noted only in frog and turtle hearts. Rat atria treated with *p*-MB are slowly and markedly depressed

TABLE 7-21

SUMMARY OF SOME EFFECTS OF THE MERCURIALS ON THE ISOLATED HEART

Preparation	Mercurial	Concentration (m*M*)	Effect on:		Other effects	Reference
			Contraction	Rate		
Frog heart	Hg^{++}	0.037	0	0	—	Salant and Kleitman (1922)
		0.08	Slow <	0	—	
		0.37	Fast <	Variable <	a-v Block	
	p-MB	0.6	Some >	<	—	Haley (1945)
	p-MB	0.056	<	Standstill	a-v Block and some contracture	Méndez and Peralta (1947)
		0.56	Fast <	Standstill	Full contracture	
Turtle heart	Hg^{++}	0.00037	—	—	Delirium cordis	Salant and Kleitman (1922)
		0.0037	—	—	Delirium cordis	
		0.037	Slow <	Slow <	Dysrhythmias and some contracture	
		0.37	Fast <	Standstill	Contracture	
	Hg^{++}	0.09	Some >	Some >	—	Johnston (1941)
		0.18	Moderate <	Moderate <	—	
		0.37	Marked <	Marked <	—	
	p-MB	0.6	<	—	a-v Block	Haley (1945)
Guinea pig atria	Mersalyl	0.04–0.2	<	<	—	Stein *et al.* (1960)

Rat atrium	Hg^{++}	0.0005	0	Slight <	—	Berman (1951)
		0.0013	0	Moderate <	—	
		0.0025	Slight >	Marked <	—	
	p-MB	0.05	Slow <	—	No contracture	Webb and Hollander (1959)
Rat ventricle	Hg^{++}	0.01	Slow <	—	—	Berman (1951)
Rabbit heart	Mercaptomerin	60 mg Hg/g	<	<	< a-v and ventricular conduction	Ruskin and Johnson (1949)
	Meralluride	36 mg Hg/g	—	—	< a-v and ventricular conduction	Ruskin and Johnson (1949)
		75 mg Hg/g	—	Standstill		
Cat heart	Hg^{++}	0.00013	Some <	—	—	Lehman *et al.* (1950)
		0.00025	Marked <	Standstill	—	
		0.0005	Marked <	Standstill	Dysrhythmias	
	Meralluride	0.0003	Some <	—	—	Lehman *et al.* (1950)
		0.0009	Marked <	—	—	
		0.0025	Marked <	Standstill	—	
	Mercaptomerin	0.044	Some <	—	—	Lehman *et al.* (1950)
		0.11	Marked <	—	—	
		0.15	Marked <	—	Dysrhythmias	
		0.4	Marked <	Standstill	—	
Dog atrium	Mersalyl	0.02–0.08	—	—	> Refractory period and < excitability	Farah and Mook (1961)
Dog heart-lung	Mersalyl	0.27	Some <	—	—	Long and Farah (1946)
		0.33	Moderate <	—	—	
		0.39	Marked <	—	—	

but no elevation of resting tension is seen even after 2 hr (Webb and Hollander, 1959). It may well be that the mercurials so readily depress membrane functions — pacemaker discharge, conduction, etc. — that the hearts cease beating before they are depleted of ATP. Méndez (1946) noted that frog heart treated with 0.56 m*M* *p*-MB stops beating quite soon and before full contracture has developed; however, if the heart is electrically stimulated it can be put into complete rigor. It has been observed many times that the heart stops in diastole *in vivo* following intravenous injections of the mercurials.

(B) *Cardiac effects in whole animals.* Sudden death during or following the intravenous injection of diuretic mercurials clinically has usually been attributed to ventricular fibrillation. In animals (usually cats and dogs) the mercurials produce the following cardiac effects: initial cardiac depression, disturbances in a-v conduction leading occasionally to a temporary ventricular bradycardia, atrial flutter or fibrillation (rarely), slowing of ventricular conduction, and often ventricular tachycardia before the terminal fibrillation (Jackson, 1926 a, b; Salant and Nadler, 1927; Macht, 1931 b; DeGraff and Lehman, 1942). The effects may be quite complex and are undoubtedly due to a variety of actions. McCrea and Meek (1929) felt that one of the major actions is a descending stimulation followed by a depression of the cardiac conducting tissue. The innervation of the heart probably plays a role in the initiation of the dysrhythmias, since atropinization or cutting the vagi in dogs prevents ventricular fibrillation due to mersalyl (Jackson, 1926 a). It is also known that epinephrine potentiates the fibrillatory action of the mercurials. The fall in blood pressure invariably observed during intravenous infusion of the mercurials must induce sympathetic activity and a rise in plasma catecholamines. Various salts of Hg^{++} apparently are not so likely to induce dysrhythmias as the organic mercurials and are more directly depressant (Salant and Kleitman, 1922). These effects are not dependent on the vagi since they occur after atropinization. However, Hg^{++} can produce conduction disturbances and dysrhythmias in dogs, and occasionally ventricular fibrillation (McCrea and Meek, 1929). The intravenous lethal dose depends on the mercurial, the species used, and the rate of injection; in most cases it falls between 10 and 50 mg Hg/kg for the common diuretic mercurials (DeGraff and Lehman, 1942; Chapman and Shaffer, 1947; Lehman *et al.*, 1950; Farah *et al.*, 1951). Inorganic Hg^{++} is somewhat more toxic, the lethal dose usually being around one third to one half that for the organic mercurials. The toxicity of Hg^{++} is dependent on the blood pH, being least between 7.4 and 7.6, and increasing on either side, especially between 7.14 and 7.35 (Salant and Nadler, 1927). Since comparable experiments have not been run on isolated hearts, it is impossible to understand the mechanism for this sensitive pH dependence; it is difficult to accept that a direct effect of pH could

alter 2- or 3-fold the sensitivity of the heart to the Hg^{++} ion, and it is more likely that secondary changes due to the alteration of the pH are responsible.

The electrocardiographic changes in dogs are similar for all the diuretic mercurials tested and for $HgCl_2$, and are primarily the result of conduction disturbances. They may be summarized briefly as follows: depression and change of configuration of the *st* segment, increase in height of the *t* wave, widening and notching of the *qrs* complex, widening of the *p* wave, and increase of the *p-r* interval (McCrea and Meek, 1929; Farah *et al.*, 1951). In the rat there is an initial flattening of the *t* wave, and eventually the *p* wave may disappear (Gessler and Kuner, 1960). Most of these changes are, of course, simply due to the slowing of conduction throughout the myocardium. The *t* wave changes are different from those seen with most metabolic inhibitors and are perhaps related more to a membrane effect than a metabolic disturbance. It is interesting that *p*-MB acts differently than the mercurial diuretics in that no *qrs* changes are seen, even at doses 2–3 times the lethal doses of the other mercurials, and death is not due to fibrillation but to ventricular asystole (Farah *et al.*, 1951). The lethal dose of *p*-MB is also about 4 times as great as for the other mercurials.

(C) *Transmembrane potentials and ionic shifts.* The membrane characteristics of rat atria are changed markedly by 0.05 m*M* *p*-MB, although the rate of action is rather slow (this is probably not due to slow penetration into the atria since the potentials are recorded from cells at the surface) (Webb and Hollander, 1959). During the first 20–30 minutes there is no significant alteration of the contractile behavior, but there is a progressive reduction in the magnitude of the action potential, an acceleration of the repolarization rate, and a slowing of conduction. It is possible that these early effects arise from selective action on the cell membranes. During the next hour these changes continue but, in addition, contraction becomes impaired. At 1 hr the changes may be summarized as follows: no significant change in resting potential (+2.1%), a severe depression of the action potential magnitude (−29%), a faster repolarization (+51%) leading to a shorter action potential (−60%), a decrease of the developed tension (−48%), a slowing of conduction (−38%), and a prolongation of the latent period (+61%). Even during this later period it appears that the contractile depression is due mainly to the shortening of the action potential, and to some extent to its reduced magnitude, and there is little evidence for direct effects on the contractile systems. It may well be that *p*-MB penetrates into the cells rather poorly and that some of the other mercurials would not have so selective an action on the membrane. Stein *et al.* (1960) reported that mersalyl (0.2 m*M*) causes a faster repolarization and contractile depression in guinea pig atria, but no changes in either the resting or action potential magnitudes were observed. The failure of the mercurials

to affect the resting potential in either rat or guinea pig atria indicates that no marked changes in intracellular K^+ occur during the duration of the experiments, so that appreciable depression of ion pumps or increase in ion permeabilities seem not to be a characteristic of the action on the heart. It is difficult to interpret the cardiac ionic changes noted by Gessler and Bass (1960) in rats poisoned with $HgCl_2$, because the electrolyte changes resulting from the renal effects (either polyuria or anuria) probably complicate the picture. However, with a dose of $HgCl_2$ sufficient to produce a long-lasting polyuria there is only a minor fall in the myocardial K^+/Na^+ ratio (1.78 to 1.60), and, although plasma K^+ rises, the tissue/plasma ratio for K^+ certainly does not drop very much, although Gessler and Kuner (1960) felt that the *qt* changes are perhaps correlated with alterations of this ratio. The results on isolated atria support the concept that the major effect is on the ionic flux rates during membrane activation.

The various regions of the heart respond differently to the mercurials as they do to other inhibitors and drugs. Isolated pig ventricle fibers are not appreciably affected by 0.26 m*M* *p*-MB but the Purkinje fibers are more sensitive (Kleinfeld *et al.*, 1964). The action potential magnitude in the ventricle may fall around 10% within 15 min but there is little further change, while the resting potential and action potential duration are not significantly modified. In the Purkinje fibers, on the other hand, the magnitude of the action potential is rapidly depressed, falling approximately 25% within 10 min, after which another rapid fall occurs between 20 and 30 min. Since the resting potential is unchanged for 20 min, there is initially a marked decrease of the overshoot; the resting potential later falls gradually. The duration of the action potential is surprisingly not altered in contrast to the results in atria. Although no evident explanation for these differences is at hand, it was considered that the greater glycolytic activity of the Purkinje fibers might predispose them to inhibition. We have seen, however, that the glycolytic pathway is probably less sensitive than the cycle to the mercurials.

(D) *Cardiac innervation and responses to acetylcholine and epinephrine.* Salant and Kleitman (1922) noted that Hg^{++} exerts some vagal blocking action in the cat heart, but Jackson (1926 b) could find no acceleration of the heart and no evidence of vagal block by mersalyl in dogs. Salant and Brodman (1929 a) reinvestigated this question and established that Hg^{++} first sensitizes the heart to the vagus and later blocks the vagal endings. It is during the first sensitization phase that dysrhythmias are apt to occur, which is reasonable since acetylcholine is profibrillatory as a result of its marked shortening of the action potential duration. Hg^{++} and *p*-MB at 0.01–0.1 m*M* antagonize the effects of acetylcholine on the frog heart, the mercurials being allowed to act for 3–40 sec and then washed out (Pohle and Matthias, 1959). It was concluded that the acetylcholine receptors may

contain SH groups, a conclusion previously reached by Turpaev (1955). Nistratova and Turpaev (1959) titrated the SH groups in a frog ventricle homogenate and found that the presence of acetylcholine alters the shape of the titration curve, but not the total number of SH groups titrated — part of the SH groups becomes less reactive in the presence of acetylcholine. Inasmuch as cholinesterase is inhibited to some extent by mercurials (Table 7-13), it is possible that this can account for the vagal sensitization, a secondary blocking of the receptors for acetylcholine reversing this effect. There is no evidence for specific interference with the action of the catecholamines on the heart, but epinephrine potentiates the profibrillatory action of the mercurials (Jackson, 1926 a). Yet Salant and Brodman (1929 c) claimed that the cat heart is most sensitive to Hg^{++} when the sympathetics are blocked by ergotamine, and that high concentrations of epinephrine actually protect the heart against the mercurials. In any event, it is likely that the over-all effects of the mercurials on the heart, especially in the whole animal, must to some extent involve the sympathetic and parasympathetic innervation. Mercurials can release catecholamines from adrenal medulla granules (D'Iorio, 1957) but it is not known if such a release can occur in the heart or other tissue.

(E) *Consideration of some mechanisms of cardiac action.* There is little justification for discussing mechanisms by which the mercurials affect the heart because essentially no basic work to elucidate the cellular actions has been done. The interesting observations of Salant and Nagler (1930, 1931) on the relation of the response to Hg^{++} of the frog heart and the level of Ca^{++} in the medium may provide some clue. If the Ca^{++} is reduced to around one half normal, the heart is depressed much more readily by Hg^{++}, but if the Ca^{++} is reduced further (this in itself suppressing contractions), Hg^{++} may then actually stimulate the amplitude. High Ca^{++} somewhat antagonizes the action of Hg^{++}. Increasing the K^{+} slows the rate and then Hg^{++} accelerates the heart and seems to have less effect on the contraction. The authors suggested that the alterations in response to Hg^{++} might be due to permeability changes brought about by Ca^{++}, but we now know that Ca^{++} has other, perhaps more important, effects on the heart. It would be interesting to know how mercurials affect the positive inotropic action of Ca^{++}. It would also be worthwhile to determine if mercurials inhibit the various ATPases of the heart. Padykula and Herman (1955) showed histochemically that *p*-MB strongly inhibits cardiac ATPase, but it is not known if this occurs *in vivo*.

For the purpose of this volume it would be of some importance if the cardiac effects could be correlated with any of the well-known enzymic or metabolic inhibitions exerted by the mercurials, but this cannot be done because there are no investigations of metabolic changes during mercurial action. Even the results reported on respiratory inhibition, for example by

Ruskin and Ruskin (1953), where 5.8 m*M* meralluride depresses rat heart slices 57%, are scarcely pertinent to understanding how the mercurials act. We have postulated previously that the site of mercurial action on the heart is mainly at the membrane to alter ionic fluxes. This does not imply that the action is nonmetabolic, since enzymes in the membrane may be the ultimate vulnerable points of attack.

Smooth Muscle

It is necessary to consider briefly the effects of the mercurials on smooth muscle, if only because calomel was used for centuries as a purgative. We have not discussed Hg^{+} because little is known about its actions on metabolic systems. Hg^{++} salts are also capable of causing diarrhea, and it is likely that Hg^{+} is active after being oxidized to Hg^{++}. Hand *et al.* (1943) developed histochemical tests for Hg, Hg^{+}, and Hg^{++}, and found in various animals that within a few minutes of the intravenous injection of mercurous acetate they could detect both Hg^{+} and Hg^{++} in the parenchymal and endothelial cells of the kidney, the latter predominating. Whole blood oxidizes Hg^{+} to Hg^{++} quite rapidly. Many theories of the mechanism of the purgative action, several rather fanciful, have been advanced, but very few justify even serious criticism. $HgCl_2$ did not achieve its name of "corrosive sublimate" in vain; it is a direct irritant of tissues, by which is meant that it induces cellular damage of both metabolic and nonmetabolic origin, this initiating an inflammatory sequence, which in the intestine causes increased activity, depression of the ability to absorb water and various substances, and consequently colitis and diarrhea. In severe mercury poisoning there is a hyperemic and hemorrhagic appearance of the intestine, with erosion and necrosis. One is thus tempted to attribute the diarrhea to such a nonspecific action, but there is some evidence against this. Isolated intestine is stimulated in a characteristic way by Hg^{++} in low concentrations (0.004–0.02 m*M*), tonic contractions being markedly increased with suppression of rhythmic activity (Salant and Brodman, 1929 b). Organic mercurials apparently can stimulate similarly; e.g., merbaphen augments peristaltic activity of isolated cat intestine (Govorov, 1936), and *p*-MB at 0.0057 m*M* stimulates the rat intestine around 10% (Goodman and Hiatt, 1964), although *p*-MB was reported to inhibit rabbit intestine (Haley, 1945), perhaps because of too high a concentration (not given, but around 0.02 m*M*). The stimulations produced by both Hg^{++} and merbaphen are readily blocked by atropine, indicating that the action is to some extent mediated through the vagal nerves in the intestine. Govorov believed merbaphen to be a parasympathomimetic substance. There is also some increase in the sensitivity of the intestine to vagal stimulation when the tissue is treated with Hg^{++}, recalling similar actions on the heart, and it is possible that inhibition of cholinesterase is involved. However, it seems unlikely that

mercurials *in vivo* can produce intestinal stimulation by such a selective inhibition, and the subject needs further investigation.

Another mechanism which must be given serious consideration is histamine release. Bachmann (1938) showed that the isolated cat intestine exposed to Hg^{++} releases a substance which behaves like histamine pharmacologically, and felt that at least some of the action on the intestine can be explained by this release. It may be mentioned that Hg^{++} has been reported to release histamine from perfused dog liver (Feldberg and Kellaway, 1938) and *p*-MB to release histamine from rat mast cells (Bray and Van-Arsdel, 1961), but in both cases the concentrations used were too high to enable correlation with *in vivo* effects; it is quite likely that any substance at high enough concentration or any irritant histotoxic agent will release histamine.

Nervous System

Neurological dysfunction is common in mercury poisoning (page 951) but it is not known if the action is axonal or synaptic. One usually assumes that metabolic disturbances affect primarily junctional transmission. Halász *et al.* (1960) have shown that transmission in the cat superior cervical ganglion is rapidly and reversibly depressed by *p*-MB at 0.0056–0.02 m*M*, while simultaneously the effects of injected acetylcholine are potentiated. If the concentration is increased toward 0.028 m*M*, this potentiation of acetylcholine is lost. The stimulatory action of K^+ is unaffected by lower and depressed by higher concentrations. Inhibition of acetylcholine synthesis is apparently not involved since there is a store of acetylcholine and the depression of transmission is immediate, so the authors postulate a reduction of the response of the postganglionic cells to acetylcholine. However, at the time of the initial suppression of transmission there is actually a potentiation of the acetylcholine response, which is difficult to explain, particularly since cholinesterase inhibition is not a likely hypothesis for ganglia. It is possible that SH groups of the acetylcholine receptors are reacted at higher concentrations of the mercurial, as has been suggested for cardiac receptors. Recordings of the postganglionic membrane potential changes are needed to interpret these results.

Axonal conduction is also depressed by *p*-MB at low concentrations (H. M. Smith, 1958). Conduction in the frog sciatic nerve is blocked in 4 min by 0.002–0.02 m*M* *p*-MB and in lobster giant axon in 3 min by 0.045–0.07 m*M* *p*-MB. There is a gradual depolarization of the axon but block occurs long before the potential is lost. The post-tetanic hyperpolarization of sympathetic C fibers is more sensitive to metabolic inhibitors than the magnitude of the action potential, and is decreased by mersalyl at 0.34 m*M* (Greengard and Straub, 1962). However, the nature of such a hyperpolarization and its significance for conduction are not understood. The injection into the squid axon of 6×10^{-5} ml/mm of 7.5 m*M* *p*-MB is without effect on the action

potential (Brady *et al.*, 1958), which may indicate that the mercurial must act on the external surface of the membrane to block conduction. The changes in the structure of the myelin sheath of nerves brought about by Hg^{++} at high concentrations (around 10 m*M*) are certainly not relevant to acute experiments with low concentrations, but in chronic mercury poisoning it is possible that sufficient Hg^{++} is incorporated in the myelin to disturb nerve function (Millington and Finean, 1958, 1961). Thus at the present time we cannot decide whether the primary action of the mercurials is on the axon or on the synaptic regions, or on both, especially in chronic poisoning.

Skin

Various types of skin reaction to the mercurials administered both systemically and topically have been recognized for years. Some of these are undoubtedly of the allergic or sensitivity category and need not concern us. Mercurial diuretics, like mersalyl, when injected in small amounts into the skin cause blisters, and Hahn and Taeger (1931) concluded that there is a relationship between diuretic activity and vesication. Almkvist (1922) had claimed that mercurials cause vascular dilatation in the skin, with resulting edema, by a paralysis of the sympathetic nerves, but there is little evidence that this is a significant factor. Hellerman and Newman (1932) noted that alkyl mercurials are powerful vesicants and can cause a severe dermatitis. These early observations are of interest in the light of the relationship between SH group reaction in the skin and vesication established by work on the arsenical war gases, and one might postulate that the mercurials have a metabolic basis for their effects on skin, perhaps an inhibition of the cycle. Hg^{++} reduces frog skin potentials across both borders and increases the outer membrane resistance (Lodin *et al.*, 1963).

EFFECTS OBSERVED IN THE WHOLE ANIMAL

It is difficult to present the toxicology of the mercurials concisely because the effects depend on the type of mercurial, the species considered, whether the poisoning is acute or chronic, the route by which the mercurial is taken into the body, and many other factors. The symptoms of chronic mercury poisoning (mercurialism) in man are quite variable and usually not correlated with the blood or urinary levels of mercury. Frequently urinary mercury may be considerably higher than the normal range and yet no symptoms occur; however, definite symptoms may sometimes be observed in those whose level is in the normal range. This lack of correlation with urinary levels and the protean nature of the poisoning not only make diagnosis frequently difficult but indicate that the individual pattern of response must relate to a number of obscure factors, such as hereditary

constitution, vitamin intake, electrolyte balance, protein nutrition, and other imponderables. The concentrations of mercury in the blood or urine are, of course, not the critical determinants in poisoning when the mercury has been slowly taken into the body over a period of months or years. Mercury is picked up by the various tissues at different rates and to different degrees, and it is the eventual levels of mercury in these tissues which determine the toxic response. Such accumulation may occur over a long time and several weeks be required before a balance between intake and excretion is achieved. One factor which must be of importance, but about which little is known, is the concentrations of the various thiols in the blood, since this will not only alter the over-all tissue uptake but will modify the pattern of distribution in the body. Most mercurialism in adults is industrial in origin and due to the inhalation or ingestion of small amounts of metallic mercury or mercury compounds daily over a prolonged period.

General Symptoms of Mercury Poisoning

We have discussed the most important aspects of acute poisoning by the inorganic and organic mercurials, namely, the effects on the cardiovascular and renal systems, and little more need be added. Slow inhalation of mercury vapor produces typical poisoning of the kind commonly seen with the inorganic mercury salts, because the metallic mercury is oxidized during and after absorption. However, when the concentration of mercury vapor is high, absorption is faster than oxidation and unique symptoms are exhibited, e.g., hyperthermia, tachypnea, cough, nausea, dizziness, and weakness (Carpenter and Benedict, 1909). These may be due primarily to the greater uptake of mercury by the central nervous system under these conditions. It is important to emphasize that the character of mercury poisoning depends greatly on the tissue distribution, and hence on the physicochemical properties of the mercurial. Thus the more or less volatile, lipid-soluble, alkyl mercurials produce quite a different picture from the inorganic or diuretic mercurials (Müller *et al.*, 1911; Hunter *et al.*, 1940). Alkyl mercurials act rather selectively on the central nervous system to produce ataxia, paralysis, and depression — in higher concentrations they act much like certain anesthetics — and acutely these effects are possibly unrelated to mercury or reactions with SH groups.

The acute effects of mercurials on the central nervous system are often marked but have not been analyzed in detail. When $HgCl_2$ is injected subcutaneously into rats at the high dose of 17 mg/kg, there is progressive loss of the reflexes and all have disappeared after 54 hr, this being reversible upon administration of a Hg^{++}-binding thiol (Galoyan and Turpaev, 1958). Conditioned reflexes are suppressed partially at the much lower dose of 3.7 mg/kg (Galoyan, 1957). The respiration is usually affected and may be taken as an index of certain central actions. Respiratory stimulation by

Hg^{++} (Hanzlik, 1923 c) and mersalyl (Jackson, 1926 b) has been noted, this being attributed to a direct medullary effect at low mercurial concentration, but lethal doses of both $HgCl_2$ and PM cause dyspnea and depression of the respiration (Wien, 1939). The injection of certain mercurials can produce a very rapid fulminant type of reaction characterized by dyspnea, convulsions, and death, and this was also attributed to a central effect on the respiratory centers (Fourneau and Melville, 1931). Direct effects on the central nervous system were observed by Pentschew and Kassowitz (1932) following suboccipital injections of $HgCl_2$ at the minimal lethal dose (around 0.2 mg); tremors, convulsions, and other motor disturbances occur after 16 hr and last for several days. It would be interesting to know the form, or forms, in which Hg^{++} penetrates into the nervous system, whether mainly as the uncharged $HgCl_2$ or in combination with thiols and other substances in the blood.

The most characteristic symptoms of mercurialism, regardless of the type of mercurial responsible, may be summarized as follows (Hunter *et al.*, 1940; Cumings, 1959, p. 78; Noe, 1960; Kantarjian, 1961). (1) A fine intention tremor, starting in the fingers and hands, and progressing to the feet, eyelids, cheeks, tongue, and neck. The motor activity is primarily affected and usually there is little if any disturbance in sensation. (2) Insomnia, anorexia, and various emotional alterations, such as mood depression and timidity. There is generally little effect on intelligence or memory. (3) Erethism, or blushing, is often common, but whether it is due to emotional disturbances or alteration of the autonomic vascular control is unknown. Sometimes, especially in infants, the skin may become red; such erythema is most likely vascular in origin. (4) Stomatitis, salivation, and gingival swelling are frequent and possibly due to the secretion of mercurials in the saliva. Of these symptoms, and others less common, only the nervous system changes lend themselves to an analysis of the mechanisms which may be involved, but we shall see that regrettably little can be concluded.

Urinary mercury excretion in normal individuals is usually between 1 and 15 μg/day, but may be so low as to be undetectable or considerably higher without obvious symptoms. In patients with evident mercurialism, the urinary mercury may vary widely — excretions between 3 and 8000 μg/day have been reported — but it is generally above 250 μg/day. The level will depend on the daily uptake and whether the individual is in complete balance or not. The fact that the same degree of severity of symptoms may be observed in patients with very different urinary levels suggests that the susceptibility to mercury varies widely, but possibly the tissue concentrations of mercury are much more uniform than the urinary concentrations.

In view of the selective effect of iodoacetate on the retina and visual function, it is interesting to inquire as to whether other SH reagents, such

as the mercurials, possess this action. Since the mercurials seem to lack marked effects on glycolysis in intact tissues, one would not expect visual disturbances if these are indeed due to glycolytic inhibition. Sorsby *et al.* (1957) could detect no retinal degeneration in rats or rabbits given $HgCl_2$ or *p*-MB under conditions where lesions are produced by iodoacetate. However, in poisoning with certain organic mercurials there may be a marked constriction of the visual field (Hunter *et al.*, 1940). It is not known whether this is a retinal effect or due to nerve degeneration. The synthesis of rhodopsin from opsin and retinene$_1$ is blocked by 0.1 m*M* *p*-MB though an action on opsin (Wald and Brown, 1951, 1952), and the synthesis of iodopsin is likewise suppressed (Wald *et al.*, 1955). The mercurial does not alter rhodopsin directly but readily bleaches iodopsin. If this effect on the regeneration of visual pigments occurs *in vivo* it has not been reported.

For many years a rather uncommon disease of infants has been recognized and called pink disease, infantile acrodynia, or erythredema, and is characterized by a redness and swelling of the extremities and certain other skin areas, along with photophobia, irritability, loss of reflexes, and muscular hypotonia. Dr. Warkany of the Children's Hospital in Cincinnati in 1945 examined an infant suffering from this disease and found a urinary mercury concentration of 360 μg/liter. A summary of 20 cases showed that most infants with pink disease had definitely elevated mercury levels — 75% had more than 50 μg/liter and 10% more than 400 μg/liter — whereas most control infants showed undetectable levels (Warkany and Hubbard, 1948). It is now generally agreed that the majority of cases of pink disease are due to mercury poisoning, which manifests itself somewhat differently in infants than in adults although some of the neurological changes are similar. The source of the mercury is usually calomel or mercury ointments. Of the group of 54 studied by Zellweger and Wehrli (1951), around 80% had been given such drugs, but in the remainder there was no obvious source of mercury. The situation is probably more complex than believed originally. Some of the symptoms may not be directly due to the mercury but are predisposing conditions to mercury poisoning (Barrett, 1957). Thus a high intestinal alkalinity, due in infants to faulty acid secretion in the stomach, may accelerate the oxidation of calomel and increase its toxicity, and simultaneously reduce absorption of certain fatty acids, this latter possibly being responsible in part for the acrodynia. Since acrodynia in animals may be induced by pyridoxine deficiency, there is also some possibility that the mercury either inhibits some phase of pyridoxine metabolism or blocks a pyridoxal-P enzyme to produce symptoms similar to deficiency. Since typical pink disease can occur in the absence of excess mercury intake or significant urinary levels, one must assume that some basic metabolic disturbance is the basis of this malady and that it can be brought about in various ways. That all the symptoms are not immediately due to mercury seems to be

indicated by the fact that administration of dimercaprol is not remarkably successful, although certain clinical improvement has been noted (Bivings and Lewis, 1948).

Histological Changes

The neurological picture of fasciculations, hyperreflexia, tremor, and motor weakness, followed by muscular atrophy, often seen in chronic poisoning with the organic mercurials — as reported, for example, by Kantarjian (1961) in individuals eating bread treated with the fungicide Granosan M (ethylmercuri-*p*-toluene sulfonanilide) — accompanied by occasional numbness or paresthesias, may clinically resemble amyotrophic lateral sclerosis (Brown, 1954). Rats and monkeys chronically poisoned with MM show Wallerian degeneration of the peripheral nerves (Hunter *et al.*, 1940). The peripheral nerves and the posterior spinal roots are affected first, and later the posterior columns and granular layer of the middle lobe of the cerebellum. The ataxia and tremor could result from the cerebellar lesions. Bilateral cortical atrophy in the area striata was associated with the reduction of the visual field. A good review of the neurological changes has been provided by Noe (1960). The renal and intestinal changes have been described, and we shall only note that degeneration of the liver has also been observed (MacNider, 1918 b). Dogs given $HgCl_2$ orally (15 mg/kg) exhibit a deposition of fat in the cells surrounding the central vein, followed by cloudy swelling and necrosis, with eventual extension to the periphery of the lobule. Such hepatic changes could well be responsible for some of the over-all metabolic disturbances observed in animals.

Foulerton (1921) believed that Hg^{++} has an affinity for lipids, not only because of the solubility of $HgCl_2$ in fat but also due to the formation of oleates, and is transported to the liver in the circulating blood fat. The liver damage then results in a defective lipid metabolism. There are certainly definite disturbances in lipid metabolism — for example, Ogilvie (1932) found an immediate and considerable rise in blood lipid following administration of $HgCl_2$; subsequently there is a fall and a second rise — but no evidence to indicate the mechanism involved. Toxic doses of mersalyl in rats produce hypoglycemia and reduce the liver glycogen to essentially zero (Dzúrik *et al.*, 1963). It was stated that these effects are secondary to renal dysfunction and not a manifestation of a direct action of the mercurial on the tissues, but this seems unlikely and there are possibly several factors of importance, including epinephrine release as a result of a nonspecific stress reaction. Free amino acids in the livers of rats given $HgCl_2$ for 10 days were determined by Thoelen and Pletscher (1953), and it was shown that although serine, leucine, and phenylalanine do not change significantly, cystine rises to 3 times the control value. This was interpreted as a detoxification response. One must question the ability of animals to increase the synthesis of specific amino acids for the purpose of complexing with a non-

physiological metal ion. Might it not be assumed, with as little evidence, that reduction of the free cysteine-cystine concentration by the formation of Hg^{++} complexes would stimulate synthesis of these amino acids?

Toxic and Lethal Doses

A few of the results on different types of mercurial are summarized in Table 7-22. The lethal dose will depend on the time interval chosen for determination of the mortality, since death from mercurial poisoning may occur several days following the administration, and this accounts for some of the variability seen in the table. It is clear that the organic mercurials are generally less toxic than $HgCl_2$. It is difficult to estimate average doses, but roughly the LD_{50} for $HgCl_2$ is near 10 mg Hg/kg (0.05 millimole/kg) and for the organic mercurials around 40 mg Hg/kg (0.2 millimole/kg); there is so much species variation and differences between the organic mercurials that these figures are to be taken only as a crude basis for comparison. Differences between routes of administration are not as marked as one might expect. One of the most important factors determining the toxicity of mercurials given intravenously is the state of dissociation of the R—Hg—X bond, where X represents any ion or thiol either introduced with the mercurial or present in the blood. In other words, the concentration of the free $R—Hg^{+}$ ion, which is able to react with the SH groups of the tissue cell membranes, is a major toxicity determinant. If the mercurial is already complexed with a thiol, as in mercaptomerin, the dissociation of the Hg—S bond will be slow and little of the mercurial will be bound to SH groups, in either the blood or the tissues. A second factor of undoubted significance is the distribution of the mercurials, in both the R—Hg—X and $R—Hg^{+}$ forms. An R group or a slowly dissociating Hg—X bond will favor penetration into the central nervous system in some instances, and this may alter the pattern of toxicity from a rapid cardiovascular death to a slowly developing degeneration of certain nervous pathways.

Distribution, Metabolism, and Excretion of Mercurials

Certain aspects of the fate of the mercurials in the body have been discussed relative to the diuretic action, and these will be briefly summarized. (1) All mercurials are accumulated in the kidney and reach much higher concentrations in this tissue than in others, although the rate and degree of accumulation depend on the structure of the mercurial. (2) Mercurials are to a great extent bound to the plasma proteins and erythrocytes so that only a small fraction is free to enter the tissues or be filtered through the glomeruli. (3) Some of the mercurials may be secreted by the renal tubular cells. (4) Mercurials are excreted in the urine mainly complexed with thiols such as cysteine. (5) Some organic mercurials are split to form Hg^{++} in the body, but there is no agreement as to the degree to which this

TABLE 7-22

TOXIC AND LETHAL DOSES OF MERCURIALS

Mercurial	Animal	Route[a]	Parameter[b]	Dose (mg Hg/kg)	Reference
$HgCl_2$	Mouse	IV	LD_{50}	5.6	Wien (1939)
			LD_{50}	14	Lehman *et al.* (1950)
		SC	LD_{50}	17	Wien (1939)
			LD_{71}	15	Grunert (1960)
		IP	LD	5.9	Hagen (1955)
	Rat	SC	AT	2.7	Galoyan (1957)
		IP	LD_{50}	3	Turrian *et al.* (1956)
			LD_{90}	2.2	Aposhian and Pointer (1958)
			LD_{100}	3	Stocken (1947)
	Dog	IP	MLD	4.4	Barbour and Hjort (1921)
			MLD	11	Taylor and Austin (1918)
		Oral	LD	11	MacNider (1918 a)
	Cat	IV	LD	11	Macht (1931 b)
			LD	16	Modell and Krop (1944)
	Man	Oral	LD	2.5	Smith *et al.* (1936)
Hg-salicylate	Rabbit	IM	LD	30	Wakerlin and Loevenhart (1926)
$Hg(propionate)_2$	Cat	IV	LD	79	Müller *et al.* (1911)
$Hg(benzoate)_2$	Cat	IV	LD	73	Müller *et al.* (1911)
HO-Hg-propionate	Cat	IV	LD	19	Müller *et al.* (1911)

HO-Hg-benzoate	Cat	IV	LD	10	Müller *et al.* (1911)
Meralluride	Mouse	IV	LD_{50}	49	Lehman *et al.* (1950)
	Dog	IV	MLD	44	Chapman and Shaffer (1947)
Merbromin	Rat	IV	LD	15	Raiziss *et al.* (1926)
	Rabbit	IV	LD	5.6	Raiziss *et al.* (1926)
	Cat	IV	LD	51	Macht (1931 b)
Mercaptomerin	Mouse	IV	LD_{50}	178	Lehman *et al.* (1950)
	Cat	IV	LD_{50}	155	Lehman *et al.* (1950)
Mercurin	Cat	IV	LD	30	Modell and Krop (1944)
Mersalyl	Rat	IM	AT	20	Dzúrik *et al.* (1963)
	Dog	IV	AT	9.2	Long and Farah (1946)
	Cat	IV	LD	15	Modell and Krop (1944)
Methyl-HgCl	Mouse	IP	LD	16	Hagen (1955)
Nitromersol	Rat	IV	LD	4	Raiziss *et al.* (1926)
	Rabbit	IV	LD	1.7	Raiziss *et al.* (1926)
Phenyl-Hg-acetate	Chick	Oral	LD_{50}	36	Miller *et al.* (1960)
Phenyl-Hg-nitrate	Mouse	SC	LD_{50}	28	Wien (1939)
		IV	LD_{50}	17	Wien (1939)
Thimerosal	Mouse	SC	LD_{50}	33	Wien (1939)
		IV	LD_{50}	22	Wien (1939)

[a] Routes of administration: IV = intravenous, SC = subcutaneous, IM = intramuscular, IP = intraperitoneal.

[b] Parameters measured: AT = acute toxic dose, MLD = minimal lethal dose, LD = lethal dose (degree unspecified), LD_x = dose to kill x%.

occurs or whether it is important for the actions of the mercurials. (6) Hg^{+} is quite rapidly oxidized to Hg^{++} in the body and probably acts on the tissues in the oxidized form.

The results of distribution studies are shown in Table 7-23. Further data on the early distribution of several mercurials may be found in Table 7-19; rough values for tissues levels of Hg^{++} in fatal human poisonings were presented in Table I-8-1. Although quantitative comparisons are difficult due to the widely different doses and the various routes of administration, the general picture is clear. The concentrations in most tissues are not markedly different from those in blood, but there is slight accumulation in the spleen, moderate accumulation in the liver, and striking accumulation in the kidney. The central nervous system levels are generally low, as expected, and this must be due mainly to the small unbound fraction in the blood, since even the more lipid-soluble mercurials (e.g. MM) do not readily penetrate into the brain. Berlin and Ullberg (1963) gave single doses of $Hg^{203}Cl_2$ intravenously to mice and determined the changing tissue levels over 16 days. The greatest amount in the central nervous system occurs in the brain stem in the area postrema, in the hypothalamus, and in sites adjacent to the lateral ventricles, and the retention in these regions is greater than in other tissues. Essentially no Hg^{++} appears in the fetus so that the placenta presents a barrier to penetration, most of the Hg^{++} being bound to proteins and the cellular elements of the blood. With the exception of the kidney and brain there appears to be no obvious correlation between tissue levels and pharmacological or toxic actions, and it is possible that the acute effects, as on the heart, may be due to the initial binding to the cell membranes rather than the result of intracellular uptake. Most of the results in the tables were obtained with subtoxic doses, with the exception of those of Galoyan and Turpaev (1958) and the cases of human poisoning, so it is not possible to obtain a complete picture of the tissue levels during periods of toxic reactions, but it is evident that rather low over-all concentrations occur in most tissues. When it is considered that probably a major fraction of the tissue mercurial is bound to metabolically or functionally inert components, it appears that very little mercurial is required to alter tissue activity.

Loss of mercurials from the body by urinary excretion is usually slow. Rothstein and Hayes (1960) determined the total body content of Hg^{203} in rats given small doses of $HgCl_2$ over a period of 100 days, and found three distinct phases: 40% is lost in 5–10 days, 45% more during the next 40–50 days, and not over 5% more in the next 50 days, so that at 100 days there is still some 10–15% of the administered mercury in the body. When $Hg^{203}Cl_2$ is infused intravenously for periods up to 4 hr in rabbits, it is found that the renal excretion does not exceed 10% of the total amount of Hg^{203} passing through the kidneys. About 50% of the total dose is taken up in

TABLE 7-23

DISTRIBUTION OF MERCURIALS IN ANIMAL TISSUES

Mercurial	Animal	Route	Dose (mg Hg/kg)	Time (hr)	Tissue concentration (μg/g wet weight)										Reference[a]
					Kidney	Liver	Spleen	Adrenal	Lung	Muscle	Heart	Intestine	Brain	Blood	
$HgCl_2$	Rat	IV	2.2	24	75	14.7	21	—	1.5	0.39	0.63	0.79	—	1.2	(1)
	Rat	SC	12.5	54	144	35	—	—	—	—	—	—	1.4	4.6	(2)
	Rat	IV	0.2	4	1.7	0.5	0.43	0.15	0.05	0.22	0.07	0.12	0.01	0.1	(3)
				24	6.4	0.2	0.25	0.22	0.16	0.15	0.06	0.08	0.015	0.06	
	Rat	SC	7.0	192	118	9.4	2.0	—	—	—	—	—	0.36	0.67	(4)
	Rabbit	SC	2.0	24	35	3.4	1.54	—	—	—	—	—	0.07	0.44	(5)
				144	58	1.6	0.45	—	—	—	—	—	0.04	0.12	
$Hg(NO_3)_2$	Rat	IV	0.1	3	5.1	0.06	—	—	—	—	—	—	0.013	0.19	(6)
				24	61.5	0.04	—	—	—	—	—	—	0.06	0.17	
				96	17.5	0.07	—	—	—	—	—	—	0.012	0.005	
Methyl-HgOH	Rat	IV	0.1	3	0.74	0.15	—	—	—	—	—	—	0.12	0.65	(6)
				24	0.37	—	—	—	—	—	—	—	—	0.45	
				96	0.45	0.10	—	—	—	—	—	—	0.02	0.066	
Methyl-Hg-dicyandiamide	Rat	SC	7.0	192	51.7	14.3	24.8	—	—	—	—	—	2.96	48.3	(4)
Phenyl-Hg-acetate	Rat	IV	0.1	3	0.2	0.07	—	—	—	—	—	—	0.07	0.9	(6)
				24	25	0.7	—	—	—	—	—	—	0.03	0.74	
				96	6.6	0.12	—	—	—	—	—	—	0.03	0.21	
	Rat	IM	3.0	12	21	6	6	—	—	—	—	—	1	—	(7)
				24	10	5	6	—	—	—	—	—	0	—	
	Rabbit	SC	2.0	24	60	4.6	1.22	—	—	—	—	—	0.051	0.46	(5)
				144	47	2.9	0.63	—	—	—	—	—	0.055	0.11	
Mercaptomerin	Rabbit	IV	1.0	48	5.71	0.26	0.09	0.16	0.04	0.01	0.02	0.11	—	—	(8)

[a] References: (1) Surtshin (1957). (2) Galoyan and Turpaev (1958). (3) Rothstein and Hayes (1960). (4) Friberg (1959) (brain values mean of cerebrum, cerebellum, and brain stem; 1 mg Hg/kg/day administered for 1 week). (5) Friberg *et al.* (1957) (brain values mean of cerebrum, cerebellum, and brain stem). (6) Swensson *et al.* (1959). (7) Miller *et al.* (1960). (8) Aikawa *et al.* (1955) (intestine value from appendix).

the kidneys (Berlin and Gibson, 1963). Less than 1% of the plasma Hg^{203} is filtered, due to both protein binding and the fact that nearly 50% of the Hg^{203} is in the erythrocytes and is slowly exchangeable with the plasma. Thus much of the Hg^{203} found in the kidney tissue must come directly from the blood since the glomerular filtration cannot account for it. In the case of the mercurial diuretics, around 20–40% is excreted during the first day (Borghgraef and Pitts, 1956; Calesnick *et al.*, 1960), but progressively smaller amounts are lost each day. The kidney retains appreciable mercurial for many days; following injection of only 0.2 mg Hg/kg of $HgCl_2$ into rats, the renal level is around 12.4 μg/g at 52 days (Rothstein and Hayes, 1960), this constituting 90% of the body mercury. MM is surprisingly well retained in the kidney, at 32 days the level being only 30% reduced from that after 1 day (Swensson *et al.*, 1959). It is obvious that cumulation invariably occurs when a mercurial is administered daily. Indeed, $HgCl_2$ given intravenously every 21 days leads to a marked cumulation, the total body mercury after 5 doses being twice that from a single dose (Rothstein and Hayes, 1960). Rats given $HgCl_2$ subcutaneously daily cumulate mercury in several tissues and require 2 weeks for the rates of intake and excretion to be equal (Friberg, 1956).

Normal human tissues contain mercury because there is a daily intake in the food. Bread, flour, milk, pork, and beef contain 2–4 μg% mercury, and certain vegetables a good deal more, depending on soil conditions and sprays used (Szép, 1940). Forney and Harger (1949) reported a wide variation in kidney mercury levels in normal human subjects (from 0 to 12.7 mg% in 92 autopsies) with two thirds having concentrations greater than 0.1 mg%. Liver levels were less (from 0 to 1.72 mg%) with values greater than 0.1 mg% in one third. Those having received mercurial medication ranged from 0.94 to 27.5 mg% mercury in the kidney. Similar results were obtained by Griffith *et al.* (1954), the mean values in nonmercurialized cases being 0.45 mg% in kidney, 0.10 mg% in liver, and 0.026 mg% in spleen (these values are in terms of wet weight to compare with the results of others, and were calculated from the dry weight figures with the data in Table I-8-3). Patients receiving large amounts of mercurials for some time prior to death (mean of 4.7 g total) had much higher concentrations in the kidney (3.4 mg%) and liver (0.36 mg%). It is very interesting that normal human liver contains around 1 μg/g wet weight of mercury. From the values for liver in Table 7-23 it is seen that in many cases the levels are lower even though the animals had been given mercurials; since control concentrations have seldom been obtained, it is questionable how much of the mercury in most of the tissues is due to the administered mercurial and how much to other sources. Of course, this does not apply to studies with Hg^{203}. It would appear that human liver normally contains more mercury than the rodent liver, but whether this is of dietary origin or a species difference is not known.

The metabolism of the alkyl mercurials and PM in the body is of some importance in understanding the pattern of their effects. PM is one of the least stable mercurials in the body and much of it is split to inorganic mercury, little being retained in any tissue but the kidneys (Miller *et al.*, 1960; Gage and Swan, 1961). Very little MM, on the other hand, is split to inorganic mercury; it is slowly excreted and cumulates in certain tissues, such as the brain. Ethyl-Hg is also retained well by the tissues, but is apparently split to inorganic mercury at a moderate rate (i.e., faster than MM and slower than PM), so that by the seventh day only 21% of the total mercury in the kidney is ethyl-Hg (Miller *et al.*, 1961). It is odd that at the seventh day all the mercury in the liver is ethyl-Hg, so that one concludes that splitting does not occur in the liver. However, only 70% of the blood mercury is ethyl-Hg, so it seems that the liver takes up some inorganic mercury. It is possible, of course, that all the nonethyl-Hg mercury is not inorganic mercury.

Toxicity to Aquatic Organisms

A discussion of the effects of mercurials on animals would not be complete without mentioning briefly some of the interesting work done with marine invertebrates, mainly in connection with antifouling programs, and with fish. One would expect sea water not to be a favorable medium for the action of Hg^{++} because of the high concentrations of complexing anions and the elevated pH. The importance of the medium is apparent in the study of the amphipod crustacean *Marinogammarus marinus* by Hunter (1949). The minimal toxic concentration of Hg^{++} in sea water is 0.074 mM, while in distilled water it is only 0.0093 mM. Other factors, such as altered transport activity, may contribute to the increased susceptibility. Hg^{++} at 0.18 mM does not depress the respiration of this organism, indicating that the toxic effect is not to be attributed to a general metabolic inhibition.

Marine invertebrates often show marked changes in susceptibility to Hg^{++} during development. This is well illustrated by the results obtained on the barnacle *Balanus balanoides*, the sensitivity to Hg^{++} reaching a minimum during the free-swimming cyprid stage (see accompanying tabulation) (Pye-

	(Hg^{++}) for 50% lethality (mM)
Nauplii	
Stage III	0.00033
Stage IV	0.00085
Stage V	0.0011
Stage VI	0.0011
Cyprids	0.011
Barnacles	0.0026

finch and Mott, 1948). Although the cyprids are not killed so readily by Hg^{++}, their settlement on the substratum is reduced appreciably by 0.000037 m*M* and completely by 0.00018 m*M*. The lack of an open gut in the cyprids is suggested as a possible explanation for the reduced sensitivity. Support for the law that nothing is simple or predictable in the response of organisms to metal ions, although no further support is needed, is the fact that the cyprids are less sensitive to Hg^{++} in 50% diluted sea water relative to normal sea water, in contrast to the amphipod discussed above. Another interesting and complex phenomenon was discovered by Barnes and Stanbury (1948) in studying the effects of Hg^{++} and Cu^{++} on the harpacticid copepod *Nitocra spinipes*. Hg^{++} is over 1000 times more toxic than Cu^{++}, but when these metal ions are present together at certain concentrations the lethal effect is greater than would be expected on the basis of their actions alone (Table 7-24). An isobologram for 50% lethality provides a curve characteristic

TABLE 7-24

LETHAL EFFECTS OF MERCURY, COPPER, AND THEIR COMBINATIONS ON *Nitocra spinipes*[a]

Hg^{++} (m*M*)	Animals killed in 24 hr (%)				
	No Cu^{++}	Cu^{++} 0.0041 m*M*	Cu^{++} 0.041 m*M*	Cu^{++} 0.41 m*M*	Cu^{++} 4.1 m*M*
0	0	1.3	11.3	21.2	42.5
0.00026	0	9.1	11.9	—	—
0.00056	1.4	14.5	20.0	78	—
0.0011	10.0	12.7	45.6	82	—
0.0015	16.7	50.0	93.7	98	—
0.0022	50	61.8	100	100	—
0.0026	72	76.4	100	100	—
0.0056	78	87.3	100	100	—
0.011	84	100	100	100	—
0.016	100	100	100	100	—

[a] From Barnes and Stanbury (1948.)

of very definite synergism (see Fig. I-10-8), i.e., the curve is extremely concave upward. It was postulated that lowered vitality due to one metal ion may not allow the animal to deal effectively with the other metal ion. For example, Hg^{++} might impair the excretory system so that Cu^{++} would be retained, since it is known that certain crustaceans and mollusks excrete Cu^{++}. The synergistic effects indicate that Hg^{++} and Cu^{++} may act by dif-

ferent mechanisms, and possibly simultaneous attacks on different metabolic or functional systems would be particularly toxic.

Jones (1946) studied the effects of several metabolic inhibitors on the fresh-water stickleback *Gasterosteus aculeatus*. When Hg^{++} is present at 0.02 m*M*, there is a temporary stimulation of respiration (+ 20–30%) at 10–20 min, followed by a depression that reaches 50% at 55 min, the fish surviving for 110 min. During the phase of respiratory increase, there is accelerated motility, a greater opercular activity, and a faster heart rate; it is quite possible that the rise in respiration is associated with the greater functional activity. If the Hg^{++} is removed after the respiration has been depressed 50%, recovery is slow and erratic, and the respiration never recovers its normal level, although after 1 day the fish appear normal. The mechanism by which the fish are killed is unknown but possibly it is asphyxial.

EFFECTS ON MITOSIS, GROWTH, AND DIFFERENTIATION

If SH groups are particularly important in cell cleavage, as many have believed, the mercurials should be effective growth inhibitors and perhaps useful agents to determine if these SH groups are enzymic or involved in cytoplasmic structure. The rather potent inhibition of the proliferation of many microorganisms by mercurials has been known for almost 100 years and will be discussed in the following section, while here we shall attempt to analyze the mechanisms by which mitosis of plant and animal cells is disturbed by the mercurials.

Eggs and Embryos

Mercurials at concentrations in the range 0.001–0.01 m*M* usually interfere with cleavage, even in sea water and despite the fact that much of the mercurial in most work is removed from the medium because of binding. Thus Mathews (1904) showed that the formation of embryos from *Fundulus heteroclitus* eggs is 50% blocked by 0.0048 m*M* Hg^{++}, 90% blocked by 0.0095 m*M*, and completely blocked by 0.014 m*M*. The effects of mercurials on eggs and embryos at different stages of development may be quite complex. Hg^{++} may be parthenogenetic in that it induces membrane elevation in *Arbacia* eggs and initiates a form of cleavage at 0.01–0.1 m*M* (Heilbrunn, 1925). The membranes begin to rise 3–5 min after addition of Hg^{++} and after 12 min the cells may become constricted unequally or cleave, but the relation of this to normal division is not clear. Hoadley (1930) studied these effects more closely and observed that 0.025 m*M* Hg^{++} (a concentration several times that suppressing cleavage completely) caused, after membrane elevation, a clumping of the cortical pigment to one side of the egg, followed by an unequal constriction which pinches off a small fragment

containing all the pigment, this fragment later cytolyzing. Hoadley claimed that no true cleavage occurs and called the Hg^{++}-induced behavior *pseudocleavage*. Both Heilbrunn and Hoadley felt that the Hg^{++} acts primarily on the cortical region, and Hoadley, in addition, thought that the Hg^{++} may react with the pigment itself.

Kriszat and Runnström (1952) reported a strange phenomenon occurring in *Arbacia* eggs treated with 0.028 m*M* *p*-MB. This concentration of mercurial rapidly inactivates the spermatozoa, but some fertilization can occur before this is complete. Fertilization causes a strong contraction of the cortical layer, squeezing out the cytoplasm into a number of pigment-free lobes, the cortex, containing all the pigment, shrinking to a small folded sac. It was postulated that *p*-MB blocks rather specifically those processes reversing the surface contraction occurring during normal fertilization. It is quite possible that these effects are exerted directly on the SH groups of the protein components of the cortex (or plasma membrane, since it is difficult to differentiate them), rather than on enzymes. The concentration of mercurial is very critical. Hg^{++} at 0.02 m*M* acting for 20 min on *Arbacia* eggs prevents development beyond the early blastula stage, but acting for 6 min has no effect on motility or larvae; however, 0.025 m*M* acting for 3 min reduces cleavage and interferes with development (Hoadley, 1930). If the mercurial is added some time after insemination, the effects are modified. Thus 0.05 m*M* *p*-MB 30 min after fertilization scarcely interferes with cleavage, the delay in onset being only 1–2 min and 95% of the eggs dividing (Zimmerman *et al.*, 1957). Mersalyl is less inhibitory and at 2 m*M* the eggs cleave normally, although there is a 10–15 min delay; only 40% develop to the blastula stage. PM, on the other hand, is very potent, indicating possible permeability factors (Macfarlane and Nadeau, 1948). Development of *Tripneustes esculentus* (sea urchin) embryos, exposed at the 2-4 cell stage for 1 hr to 0.001 m*M* PM, is inhibited and only 2% reach a motile blastula stage. Many of the embryos are abnormal and partial cytolysis occurs. Even 0.00038 m*M* PM slows yolk absorption although cleavage is not affected. *Echinus miliaris* larvae exposed to 0.0005 m*M* Hg^{++} metamorphose, but there is dedifferentiation of the tissues so that the young echinus is often abnormal, perhaps possessing rudimentary tube-feet or spines (Huxley, 1928). Gastrulation is a process generally sensitive to toxic substances and this is true for the mercurials. For example, frog dorsal lip explants are depressed rapidly by 0.1 m*M* *p*-MB so that little further development takes place (Ornstein and Gregg, 1952; Gregg and Ornstein, 1953). The mercurial seems to prevent certain movements and spreading associated with gastrulation, e.g., the stretching of the mesoderm within the endoderm and the ectodermal flow over the endoderm. Not much has been done on later embryonic development, but *p*-MB injected into newborn mice brings about varying degrees of neuroblastic necrosis, chiefly in the

outer cortical zones, this to some extent simulating radiation injury (Hicks, 1953).

There is no evidence that mercurials disturb development by generally depressing metabolism. Ornstein and Gregg (1952) observed no effect of *p*-MB on dorsal lip explant respiration at a concentration blocking differentiation, and Brock *et al.* (1939) found that to inhibit sea urchin egg respiration requires 20 times the Hg^{++} concentration necessary for cleavage block. The latter concluded that this points to a nuclear effect as the basis for the inhibition of division, but this does not follow. However, the difference between SH reagents is well shown here, in that arsenite depresses respiration more readily than division. Haas (1941) also inclined to a primary nuclear effect, since Hg^{++} produces demonstrable damage to the nucleus at 0.00074 m*M*, while 0.037 m*M* is required for cytoplasmic damage in *Anodonta* (fresh-water clam) eggs. Without denying that Hg^{++} can damage the nucleus, one must be pessimistic as to the reliability of determining the site of action of an inhibitor by visual inspection; e.g., the action could have been on the plasma membrane and be microscopically undetectable, or a good deal of disturbance in the cytoplasm might have been caused without being immediately evident. Landau *et al.* (1954) stated that mersalyl is an effective ATPase inhibitor and hence was tried on the cleavage of *Arbacia* and *Chaetopterus* eggs. Fertilized eggs placed in 2 m*M* mersalyl complete the first 3–4 cleavages, but the furrowing strength is reduced, as measured by the pressure increases required to prevent furrowing, so an inhibition of the gelation of the cortex in the equatorial region by mersalyl was postulated, this presumably being mediated through an interference with ATP utilization. Since the ATPases from different sources vary a good deal in sensitivity to mercurials, one does not know what inhibition to expect in these eggs, and the mercurial concentration is so extremely high that many metabolic and functional processes must be affected. Heilbrun and Wilson (1955) explained the block of cleavage by mercurials as an inhibition of the proteolytic enzyme system involved in gelation, without obvious evidence. The direct effects of Hg^{++} on fibrous proteins extracted by sea urchin eggs by Sakai (1962) are meaningless because a concentration of 10 m*M* was used.

Plants

Many organic mercurials are applied to seeds, bulbs, or plants as fungicides, but occasionally the plant tissues may be damaged and growth depressed. The persistence of the mercurial in the plant is often remarkable. For example, carnation seeds treated with radioactive PM at a concentration causing growth abnormalities in the seedlings produces plants which at 8–9 weeks contain the mercurial in the cotyledon leaves, the hypocotyl, and the root adjacent to the hypocotyl (Robson and Fenn, 1961). A very interesting effect, and one illustrating that the mechanisms by which mer-

curials act may often be unexpected, is the zinc-deficiency disease of coffee trees in Kenya due to spraying with mercurial fungicides (Bock *et al.*, 1958). Not only do the plants exhibit typical signs of zinc deficiency — chlorosis, abnormal growth of shoots and leaves, and short internodes — but the zinc content is reduced to 25% of normal. In the promotion program for merbromin, Macht (1931 a) purported to show that organic mercurials are less toxic than Hg^{++} to plants, but his data are equivocal, since Hg^{++} is toxic around 0.1 m*M*, while merbromin inhibits growth slightly at 0.0013 m*M*, 50% at 0.043 m*M*, and 81% at 1.29 m*M*. The growth of *Avena* coleoptiles and of pea stems is readily inhibited by the mercurials, PM being much more potent than *p*-MB (see accompanying tabulation) (Thimann and Bon-

Tissue	Mercurial	Concentration for 50% inhibition (m*M*)
Avena coleoptile	*p*-MB	0.035
	PM	0.007
Pea stems	*p*-MB	0.4
	PM	0.02

ner, 1949). It is likely that the carboxylate group prevents the *p*-MB from penetrating as well as PM. These results will suffice to demonstrate growth inhibition by the mercurials, and we shall turn to what little evidence is available for the mechanisms involved.

Onion roots exposed to 0.0075 m*M* PM develop terminal swellings and growth is immediately stopped; however, after a day new growth starts distal to the swelling (Macfarlane and Nadeau, 1948). Doubling the concentration leads to 100% mortality of the roots. Hg^{++} even at 0.05 m*M* does not cause terminal swelling, inhibits growth only 15%, and does not kill any of the roots. Macfarlane (1951) pointed out that PM acts on onion roots cytologically like colchicine, in that spindles are abnormal, chromosome movement is impeded, and polyploidy results in the zone of cell enlargement proximal to the meristem. In addition, there is chromosome stickiness, fragmentation, and aggregation. Although mitotic and chromosomal disturbances certainly occur, there may be some question as to the validity of terming these effects mutagenic or radiomimetic (Macfarlane, 1953). Meyer (1948) also observed such changes in the root tips of *Crepis capillaris* exposed to 0.013 m*M* Hg^{++}, the sister telophase nuclei often being connected by chromatin bridges, with some breakage and recombination, leading to 80% diploid metaphases and 1.5% tetraploid metaphases. The formation of cell wall material in the microspores of excised *Lilium henryi* anthers is reversibly blocked by 0.01 m*M* *p*-MB and the progress of meiosis

is slightly retarded (Pereira and Linskens, 1963). Similar chromosomal changes induced by mercurials have not been reported, as far as I know, for animal cells. The growth stimulation by auxin applied to *Avena* coleoptiles is inhibited by *p*-MB at 0.3 m*M* (Cleland and Bonner, 1956), but the effects on auxin transport in sunflower stem section are complex in that 0.01 m*M* *p*-MB accelerates transport 125%, 0.1 m*M* depresses it 25%, and 1 m*M* blocks it completely (Niedergang-Kamien and Leopold, 1957). It is not known if interference with auxin transport or action is involved in growth inhibition.

The structure-action relationships of mercurials acting on the sporelings of the marine red alga *Plumaria elegans*, reported by Boney *et al.* (1959), were believed to demonstrate the importance of lipophilicity and penetration (Table 7-25). The alkyl mercurials are often 200–300 times more toxic than $HgCl_2$, the branched chain compounds being less toxic than the straight

Table 7-25

Lethal Concentrations for *Plumaria* Sporelings Exposed to Mercurials for 18 Hr.[a]

Mercurial	Concentration for 50% lethality (m*M*)	Potency relative to $HgCl_2$
$HgCl_2$	0.0115	1
HgI_2	0.000344	33
Methyl-HgCl	0.000176	65
Ethyl-HgCl	0.000097	119
n-Propyl-HgCl	0.000046	250
n-Butyl-HgCl	0.000043	268
n-Amyl-HgCl	0.000041	280
Isopropyl-HgCl	0.000099	116
Isoamyl-HgCl	0.000060	192
Phenyl-HgCl	0.000173	67
Phenyl-HgI	0.000260	45

[a] From Boney *et al.* (1959).

chain. Some correlation between potency and the distribution ratios between ether and water, and between methyloleate and water, was claimed, but discrepancies exist. Similar relationships have been reported for certain marine crustaceans (e.g., *Artemia salina*), but in others (e.g., *Acartia clausi*) there is little difference in toxicity between the mercurials. Inasmuch as

there has been no work on the relative inherent or direct toxicities to cellular processes, metabolic or functional, or adequate comparison of their abilities to react with relevant SH groups, it is impossible to be certain that the differences are due solely to variation in the penetration. Indeed, one is not sure that the mercurials all act by the same mechanism. One factor which is often ignored is the role of the size of the side chain in membrane processes, assuming that they all react with identical SH groups in the membrane. Nevertheless, such quantitative studies are valuable in establishing a necessary basis for understanding the mechanisms by which the mercurials act; further work will undoubtedly allow these results to be interpreted more readily.

Mammalian Cells and Tissue Cultures

We must note that growth, in common with many other processes, may be accelerated by low concentrations of the mercurials, as noted by Hirashima (1934) in chick fibroblast cultures, and by others. This has also been reported for plant tissues, *p*-MB at 0.001–0.005 m*M* stimulating the growth of *Avena* coleoptiles some 20–25% (Thimann and Bonner, 1949). It is interesting to recall that Mallus (1931) gave $HgCl_2$ at 0.25–0.3 mg/kg to atrophic but otherwise healthy children and observed certain changes — increased chest measurements, rise in erythrocyte count and hemoglobin, and elevation of urinary nitrogen — indicating a stimulation of growth and metabolism. The mechanisms by which mercurials can stimulate growth are unknown, but it is possibly not a specific action since many types of cells exhibit an increased proliferative activity when disturbed slightly by irritant substances (our terminology in this field is admittedly inadequate).

Inhibition of growth is invariable when the mercurial concentration is increased beyond a certain level, which is frequently quite low. Many of the experiments with tissue cultures have been done of necessity in complex media (e.g., embryo extract) which must bind a large fraction of the mercurial, so the true inhibitory potency must be much greater than is indicated by the concentrations used. Fibroblastic and leucocytic migration is depressed 50% by Hg^{++} at concentrations near 0.08 m*M*, and growth is somewhat modified even at 0.0037 m*M* (Meier, 1933). Chick embryo heart cultures fail to grow in 0.08 m*M* Hg^{++} (Salle and Lazarus, 1936) and here the organic mercurials are less toxic (Salle, 1943). Pulsations of the cardiac cells are stopped by Hg^{++} before growth is affected, but the organic mercurials (thimerosal and nitromersol) stop growth and cause cytolysis without previously interfering with the contractile activity. The concentration of Hg^{++} for 50% inhibition of Eagle's KB strain of human carcinoma cells is 0.037 m*M* (Smith *et al.*, 1959), and 75% reduction in the mitoses of mouse ear epidermis requires 0.01 m*M* *p*-MB (Gelfant, 1960). We may thus conclude that mercurials at concentrations around 0.005–0.05 m*M* seriously

restrict growth of mammalian cells in culture, higher concentrations usually killing the cells directly. If the mechanism of this growth inhibition is to be elucidated and correlated with metabolic alterations, it will be necessary to work within this range if the conclusions are to be valid.

Some General Aspects of the Effects of Mercurials on Mitosis and Growth

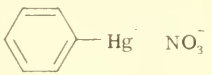
A few general comments on the localization of the sites of growth inhibition were made on page I-531 and reference to these will make it evident that at the present time we have little hope of explaining the actions of the mercurials. Essentially nothing is known of the possible effects of mercurials on protein, nucleic acid, or coenzyme synthesis, or whether the demonstrated inhibitions of active transport are in any way related to the growth depression. We have seen that respiration is not significantly depressed during growth inhibition in the few instances in which it has been determined; however, in view of the rather potent inhibition of the cycle, it would be worthwhile to pursue this question further. Mercurials are not efficient uncoupling agents and could scarcely act in this way. The significance as a possible metabolic mechanism of mercurial action of the observation by Hirashima (1935), that glucose reduces the toxicity of Hg^{++} for fibroblast cultures, cannot be evaluated.

A direct action on the sol–gel transformations and protoplasmic movements during furrowing, spindle formation, and cleavage has been postulated and discussed briefly in previous sections. Mazia (1959) believes that the mitotic apparatus may be an S—S bonded structure because of the ability of agents splitting S—S bonds to dissolve the structure, and states that *p*-MB and mersalyl bring about the dissolution of the freshly isolated spindle. The question is whether such an action can be exerted at the concentrations occurring within cells during mitotic inhibition. We have also mentioned that several workers favor a nuclear site for the mercurials but that the evidence is insufficient, as is that of Meyer (1960), who showed that the conidia of *Fusarium decemcellulare* incubated with 0.037 m*M* Hg^{++} accumulate mercury in some form either on or within the nucleus, since there is no necessary correlation between relative intracellular concentrations and the site of action. It is worth noting that *p*-MB interferes with the synthesis of RNA from nucleosides, as determined by the uptake of cytidine into the nuclear RNA of HeLa cells, and an inhibition of the RNA polymerase was suggested (Srinivasan *et al.*, 1964). HeLa cells are blocked in metaphase by 0.02 m*M* *p*-MB. In connection with the selective accumulation of mercurials, it is worth noting that merbromin appears to be localized in tumor tissues of both mouse and man, as indicated by fluorescence several days after initiation of intravenous or oral administration (Katsuya *et al.*, 1963). Although kidney exhibits the highest concentration of mercurial initially, it and other normal tissues lose the merbromin much faster than tumors. It is

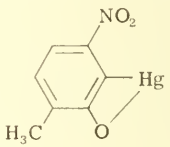
somewhat surprising that tumors could contain a component holding mercurials more tightly than in normal tissues, or at least more of a component binding the mercurial strongly, but this would support ideas which have been advanced relative to the abnormal state of SH group-containing substances in tumor cells.

EFFECTS ON THE GROWTH OF MICROORGANISMS

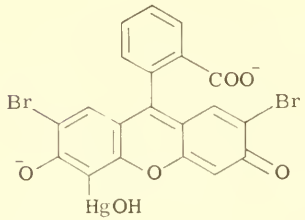
Mercurials have been used for years to control the growth of many types of microorganism, invertebrate, and plant, and have been applied commercially as fruit sprays, paint preservatives, mothproofers, grain insecticides, anthelmintics, as well as antiseptics and disinfectants, and in antifouling, crab grass control, bacterial plant diseases, and nematode control. One of the most important commercial uses at the present is as fungicides in the treatment of seeds, fruits, and plants, and for the most part certain organic mercurials have been developed for this purpose. The clinical use of mercurials as antiseptics, first popularized by Koch (1881), has declined somewhat due to the discovery of generally more selective antibacterial agents, but most of the experimental work has been done with this group of aromatic mercurials. Inasmuch as the relation between these actions and met-



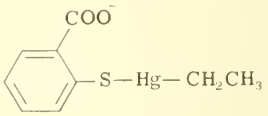
Phenylmercuric nitrate
(Merphenyl nitrate, Merphene)



Nitromersol
(Metaphen)



Merbromin
(Mercurochrome)



Thimerosal
(Merthiolate)

abolic interference is vague, only a cursory treatment of the mercurials as inhibitors of microorganism growth will be given.

Bacteria, Fungi, and Yeast

Koch originally claimed that Hg^{++} possesses the ability to kill various bacteria and their spores, but it was soon shown by Geppert (1889) that the proliferative activities of the treated bacteria can be restored by removing the Hg^{++} with sulfides, and that the action of the Hg^{++} is primarily bacteriostatic, a concept confirmed many times and extended to other organisms. Dilution or washing is not sufficient to extract the Hg^{++} bound to the cells (Chick, 1908), but sulfides and various thiols (Fildes, 1940) can readily reverse the bacteriostatic action. With high enough concentrations and prolonged exposure, of course, bacteria may be killed, especially at elevated temperatures. Yeast cells incubated with 0.93 mM Hg^{++} are killed progressively over an hour as determined by staining with methylene blue or Congo red (Rahn and Barnes, 1933). One of the first effects noted at minimal concentrations of the mercurials is a prolongation of the lag phase of proliferation (Cook and Steel, 1959). Increasing the concentration of mercurial progressively delays and slows the growth, and eventually stops it, at which point the cells can remain viable often for quite extended periods of time, as exhibited by the renewal of proliferation when the mercurial is removed with a thiol.

The sensitivities of various microorganisms to the mercurials are shown in Table 7-26, in which only a few of the reported results have been presented because the purpose is mainly to illustrate that proliferation is usually depressed at fairly low concentrations, and that the data often vary due to the conditions of the testing. One of the most important factors is the medium used, inasmuch as most growth media contain many substances capable of complexing with the mercurials and reducing their effective concentration. Claus (1956) demonstrated that the minimal inhibitory concentration of Hg^{++} varies over a 10-fold range depending on whether peptone media or simple nitrogen sources are supplied, and Cook and Steel (1959) also presented evidence that the usual culture media exert a protective action. Another important factor is the temperature, increase in temperature markedly enhancing the bacteriostatic effect (Cianci, 1940; Cook and Steel, 1959), the Q_{10} being 3–4 (Chick, 1908). There is unquestionably species variation in susceptibility but no correlation with bacterial metabolic or growth habits has been made. It should be noted that very low concentrations of the mercurials occasionally stimulate growth, this being perhaps more true for fungi than bacteria. Robertson (1943) noted that a mercurial used for preserving leather actually accelerated the growth of certain fungi within a particular concentration range, and Converse and Besemer (1959) reported that *p*-MB at 0.00028 mM stimulates the growth of *Coccidioides immitis*, although spherulation is definitely inhibited. This phenomenon would probably be seen more commonly if low concentrations of the mercurials were more frequently tested, and clinically and commer-

TABLE 7-26

STATIC AND CIDAL CONCENTRATIONS OF MERCURIALS ACTING ON BACTERIA, FUNGI, AND YEAST

Organism	Mercurial	Quantity measured [a]	Concentration (mM)	Reference
Bacteria				
Bacillus subtilis	*p*-MB	G_{97}	1	Falcone *et al.* (1959)
Clostridium histolyticus	Hg^{++}	S	0.0074	Cianci (1940)
Clostridium welchii	Hg^{++}	S_{100}	0.09	Albert *et al.* (1944)
Desulfovibrio desulfuricans	PM	S	1.28	Bennett and Bauerle (1960)
Escherichia coli	Hg^{++}	S_{100}	0.0081	Cooper and Mason (1927)
		S_{100}	0.045	Albert *et al.* (1944)
		S_{50}	0.0037	Loveless *et al.* (1954)
		S	0.0037	Clauss (1956)
		S_{100}	0.06	Cook and Steel (1959)
	p-MB	S_{50}	0.084	Loveless *et al.* (1954)
	PM	S	0.015	Thomas and Cook (1947)
Lactobaccilus casei	Hg^{++}	S_1	0.004	Madinaveitia (1946)
Proteus vulgaris	Hg^{++}	S_{100}	0.045	Albert *et al.* (1944)
Pseudomonas aeruginosa	PM	S	0.16	Bennett and Bauerle (1960)
Salmonella paratyphi	Hg^{++}	S_{100}	0.0037	Chick (1908)
Salmonella pullorum	Hg^{++}	S_1	0.012	Severens and Tanner (1945)
Salmonella schotmülleri	Hg^{++}	S_1	0.012	Severens and Tanner (1945)
Salmonella typhosa	Hg^{++}	S_1	0.012	Severens and Tanner (1945)
		C_{50}	0.00013	Smith *et al.* (1936)
		S	0.15	Thomas and Cook (1947)
Staphylococcus aureus	Hg^{++}	S_{100}	0.23	Salle and Lazarus (1936)

		C	0.1	Salle and Ginoza (1943)
		S_{100}	0.09	Albert *et al.* (1944)
		S_{100}	0.0093	Klimek *et al.* (1948)
		S_{50}	0.0014	Yamada and Yanagita (1957)
		C_{33}	0.062	Yamada and Yanagita (1957)
		C_{97}	0.13	Yamada and Yanagita (1957)
	Thimerosal	S_{50}	0.00032	Yamada and Yanagita (1957)
	PM	S	0.0015	Thomas and Cook (1947)
Streptococcus hemolyticus A	Hg^{++}	S_{100}	0.023	Albert *et al.* (1944)
Fungi				
Coccidioides immitis	*p*-MB	$Spher_{20}$	0.000028	Converse and Besemer (1959)
		$Spher_{75}$	0.00028	Converse and Besemer (1959)
		S	0.028	Converse and Besemer (1959)
Cochliobolus miyabeanus	Hg^{++}	G_{50}	<0.05	Tanaka (1961)
	PM	G_{50}	0.0007	Tanaka (1961)
Penicillium notatum	Hg^{++}	S_1	0.089	Partridge and Rich (1962)
	PM	S_1	0.048	Partridge and Rich (1962)
Rhizoctonia solani	Hg^{++}	S_{25}	0.185	Tolba and Salama (1962)
Sclerotinia fructicola	Hg^{++}	S_1	0.089	Partridge and Rich (1962)
	PM	S_1	0.048	Partridge and Rich (1962)
Stemphyllium sarcinaeforme	Hg^{++}	S_1	0.089	Partridge and Rich (1962)
	PM	S_1	0.048	Partridge and Rich (1962)
Yeast				
Saccharomyces cerevisiae	Hg^{++}	S_{70}	0.0185	Loveless *et al.* (1954)
	p-MB	S_{55}	0.00084	Loveless *et al.* (1954)

[a] The symbols represent the following: C = cidal or killing, G = germination inhibition, S = statie or growth inhibition, and Spher = spherulation inhibition. The subscript numbers indicate the per cent affected: S_1 indicates the minimal growth inhibitory concentration, and S_{100} the concentration to inhibit all growth.

cially it might be an important aspect of their use. Despite the many comparisons of the relative activities of different mercurials, very few interesting correlations between structure and effectiveness have emerged. Krahé (1924) found that the bacteriostatic activity of Hg^{++} is reduced by increasing the concentration of NaCl and postulated this to be due to the formation of $HgCl_3^-$ and $HgCl_4^=$ complexes, these being less lipid-soluble, and showed that the distribution coefficient between ether and water is reduced parallel with the antibacterial potency. Coleman *et al.* (1937) observed in the aliphatic mercurials that the antibacterial activity increases with the length of the side chain. Such relationships have been found for many actions of the mercurials and are probably based on differences in penetration into the cells rather than to fundamental differences in action on the susceptible cellular mechanisms.

The uptake and distribution of mercurials have been well studied and several facts relevant to the mechanism of their action have emerged. The amount taken up in any case will depend on the relative quantities of cells and mercurial present. Herzog and Betzel (1911) incubated 10 g of pressed yeast (2.6 g dry weight) with various concentrations of Hg^{++} and found the cellular Hg^{++} concentration to increase with the total amount of Hg^{++} present, but the percentage taken up falls (see accompanying tabulation).

Total Hg^{++} present (g/100 ml)	Hg^{++} taken up by yeast cells		
	Total (g)	Concentration (g/g dry weight)	%
0.092	0.077	0.030	84
0.460	0.168	0.065	37
0.921	0.219	0.084	24
1.341	0.304	0.117	16
3.683	0.449	0.173	12

Since there are roughly 10^{10} cells in 1 g of dry yeast, these uptakes would correspond to between 2×10^8 and 10^9 Hg^{++} ions/cell; they would also correspond to 0.065–0.38 g Hg^{++}/g yeast protein of molecular weight 100,000. The amount of Hg^{++} accumulated by yeast is thus very considerable and only a fraction is likely to be bound to SH groups. *E. coli* binds even more Hg^{++}, since Hahn and Remy (1922) found an uptake of around 0.5 g Hg^{++}/g dry weight (assuming around 75% water content) from a 3.7 m*M* solution. McCalla and Foltz (1941) calculated that *E. coli* possesses around 10^8 binding sites/cell, which is close to the figure to be estimated from the uptake found by Hahn and Remy. Steel (1960) claimed that a cell of *E. coli* contains about 10^8 SH groups, but that Hg^{++} does not react with all of them,

p-MB giving a much higher accumulation. It is more difficult to determine the distribution of Hg^{++} within the cells. Süpfle (1923) treated anthrax bacilli with Hg^{++} and then with H_2S, and showed black granules within the cells. However, the Hg^{++} enters much more slowly than the antibacterial action develops, which might be used as evidence for a primary membrane site. Ruska (1947) examined Hg^{++}-treated streptococci and *E. coli* with the electron microscope and observed mercury in the membrane and diffusely distributed in the cytoplasm, but most in small globular masses between the membrane and the cytoplasm. By a similar technique, Harris *et al.* (1954) found no mercury in the membrane or cell wall of *E. coli* — confirming the absence of electrophoretic change in cells treated with Hg^{++} — and most deposited as granules within the cytoplasm. Tröger (1959) localized mercury by the diphenylcarbazone method and found accumulation in certain areas. It should be noted that visualization either electron microscopically or histochemically is difficult in bacteria and, furthermore, that generally quite high concentrations have been used so that the pattern of distribution cannot apply directly to the bacteriostatic situation.

The possible mechanisms for the bacteriostatic action of the mercurials have been debated for years and many theories have been proposed without benefit of experimental evidence. The amount of valuable work on the mercurials from the standpoint of basic actions is almost negligible, due probably to the fact that when bacterial metabolism and proliferation began to be investigated seriously, attention was turned to the sulfonamides and antibiotics. From Tables 7-13 and 7-17 one might conclude that metabolism must certainly be depressed in some manner during the action of the mercurials on bacteria, and this may well be in many cases, but Yamada and Yanagita (1957) showed quite conclusively that the growth of staphylococci is 140 times and 57 times more sensitive than respiration to thimerosal and Hg^{++}, respectively. Indeed, it is possible to stop growth essentially completely without affecting respiration significantly. Despite the lack of critical experiments in other organisms, it is safe to say that the mercurials do not inhibit the growth of microorganisms by simply suppressing oxidative processes and the supply of energy for growth and division. Fildes (1940) on the basis of irrelevant evidence concluded that the antibacterial mechanism is based on reaction with SH groups, and the impression is gained that he was thinking of the smaller thiols rather than proteins and enzymes. Loureiro and Lito (1946) put this theory on a better basis by demonstrating some correlation between the fraction of bacterial SH groups reacted and the bactericidal activity. However, even here all one can do is to increase the mercurial concentration so that more and more SH groups are reacted, and it is not surprising that more and more cells are inhibited or killed; one cannot say what fraction of SH groups should be reacted before an effect on the bacteria is observed, and indeed it is very unlikely that it

requires a 1 : 1 ratio of mercurial to SH groups, as assumed by these workers. Nevertheless, in the face of no negative evidence, it is felt that, the mercurials do inhibit growth by reacting with some SH groups — the problem is with what SH groups, since there are many different SH-containing substances in the cell. Do the mercurials inactivate some SH enzyme, or enzymes, involved in an important metabolic pathway, or react with SH groups in the membrane to block active transport of necessary substances into the cell, or alter permeability so that intracellular components are lost, or directly stabilize the membrane to prevent division, or interfere with the utilization of ATP, or disturb metabolism by reacting with some thiol coenzyme? Experiments showing that certain substances protect against mercurials are not easy to interpret. Thus Pershin and Shcherbakova (1958) found that histidine, glutamate, methionine, and particularly thiamine protect *E. coli* against Hg^{++}, and interpreted this as indicating that the metabolism of these compounds is interfered with by Hg^{++}, but it is also possible that the protection is simply due to complexes formed with the Hg^{++}. Theories involving various physicochemical properties of Hg^{++} and other heavy metal ions — such as solution pressure, solubility products, electronegativity, and ionization potential (e.g., Shaw, 1954; Somers, 1959) — do not warrant serious consideration since they simplify the biological system beyond recognition and, even if true, would not help us appreciably to understand how the mercurials act.

Viruses

Most viruses and phages can be inactivated by the mercurials but it requires fairly high concentrations relative to those inhibiting bacterial growth (Table 7-27). In most work a virus suspension is incubated with the mercurial for a certain period and the infectivity is then tested. As with the effects on microorganisms in general, the degree of inactivation by the mercurials depends on many factors, particularly the medium in which the virus is suspended, the temperature, and the exposure time. The rates of inactivation are quite different for various viruses: ECHO 7 virus is 50% inactivated by 0.05 m*M* *p*-MB in 1 min and 99% inactivated in 6 min (Choppin and Philipson, 1961), whereas tobacco mosaic virus is not completely inactivated after 24 hr exposure to 18.5 m*M* Hg^{++} (Kassanis and Kleczkowski, 1944). This would be expected since the virus SH groups must vary widely in reactivity as do the SH groups of proteins in general. The inactivation is first order with respect to virus. Staphylococcus phage infectivity declines exponentially when exposed to Hg^{++} according to the equation:

$$dP/dt = k(HgCl_2)\,(P_0 - P_i)$$

where P_0 is the phage initially present and P_i the inactivated phage (Krueger and Baldwin, 1933). This equation holds fairly well over most of the range,

TABLE 7-27

INACTIVATION OF VIRUSES AND BACTERIOPHAGES BY MERCURIALS

Virus	Mercurial	Effect[a]	Concentration (mM)	Reference
Adenovirus type 5	*p*-MB	log TR = 5	1	Allison *et al.* (1962)
Bronchopneumonia	Hg^{++}	Inactivation = 100%	0.37	Gönnert and Bock (1956)
Common cold	*p*-MB	log TR = 3	1	Allison *et al.* (1962)
Coxsackie	*p*-MB	log TR = 0	1	Allison *et al.* (1962)
Echo 7	*p*-MB	log TR = 1.5	1	Allison *et al.* (1962)
		log TR = 3	0.1	Choppin and Philipson (1961)
E. coli phage	Hg^{++}	Inactivation = 99%	0.16	Moriyama and Ohashi (1941)
	p-MB	Replication < 80%	1	Shug *et al.* (1959)
		Burst size < 100%	0.2	Shug *et al.* (1960)
Ectromelia	Hg^{++}	Inactivation minimal	0.037	Gönnert and Bock (1956)
		Inactivation = 100%	0.37	
Encephalomyocarditis	Hg^{++}	Inactivation minimal	3.7	Gönnert and Bock (1956)
Fowl plague	Hg^{++}	log TR = 4	0.1	Allison (1962)
	p-MB	log TR = 3.4	0.1	Allison (1962)
		log TR = 2	0.1	Allison *et al.* (1962)
		log TR = 3.5	1	

TABLE 7-27 (*continued*)

Virus	Mercurial	Effect[a]	Concentration (m*M*)	Reference
Herpes	Hg^{++}	Inactivation minimal	3.7	Gönnert and Bock (1956)
	p-MB	Inactivation = 100%	0.1	Sery and Furgiuele (1961)
Influenza A	Hg^{++}	Inactivation = 100%	0.9	Klein *et al.* (1948)
	PM	Inactivation = 100%	1.1	Klein *et al.* (1948)
	Hg^{++}	Inactivation = 100%	0.18	Perêz *et al.* (1949)
		log TR = 7	0.74	Groupé *et al.* (1955)
		Inactivation = 100%	3.7	Gönnert and Bock (1956)
Influenza A Iksha	*p*-MB	log TR = 2	1	Allison *et al.* (1962)
Influenza A Lee	*p*-MB	log TR = 1.6	0.1	Allison *et al.* (1962)
		log TR = 3.5	1	
Influenza A MEL	*p*-MB	log TR = 2.8	0.1	Allison *et al.* (1962)
		log TR = 4.5	1	
Influenza A WS	*p*-MB	log TR = 1.5	0.1	Allison *et al.* (1962)
		log TR = 2.4	1	
Myxoma	*p*-MB	log TR = 4	1	Allison *et al.* (1962)
Newcastle	Hg^{++}	Inactivation = 100%	1.8	Tolba and Eskarous (1962)
	p-MB	log TR = 3.3	0.1	Allison *et al.* (1962)
		log TR = 4	1	

Pleuropneumonia (rat)	*p*-MB	log TR = 2	1	Allison *et al.* (1962)
Poliomyelitis	*p*-MB	log TR = 1	1	Allison *et al.* (1962)
		log TR = 0.7	0.1	Choppin and Philipson (1961)
Psittacosis	Hg^{++}	Inactivation = 100%	1	Burney and Golub (1948)
	p-MB	No inactivation	0.01	Burney and Golub (1948)
		Inactivation = 100%	0.1	
Psittacosis-lymphogranuloma (mouse)	*p*-MB	log TR = 4	1	Allison *et al.* (1962)
Staphylococcus phage	Hg^{++}	Inactivation = 100%	0.37	Krueger and Baldwin (1933)
		Inactivation minimal	0.37	Wahl (1939)
		Inactivation = 99%	0.37	Klein *et al.* (1948)
Tobacco mosaic	Hg^{++}	Inactivation = 41% (8 hr)	18.5	Kassanis and Kleczkowski (1944)
		Replication < 50%	4.4	Lindner *et al.* (1959)
	p-MB	No inactivation	1	Anson and Stanley (1941)
Vaccinia	Hg^{++}	Inactivation = 100%	0.1	C. Kaplan (1959)
		log TR = 4.6	0.1	Allison (1962)
	p-MB	log TR = 3.8	0.1	Allison (1962)
		log TR = 3.5	0.1	Allison *et al.* (1962)
		log TR = 5	1	
Western equine encephalitis	*p*-MB	log TR = 0	1	Allison *et al.* (1962)

[a] TR is the titer reduction resulting from incubation with the mercurial; log TR = 3 means that the infectivity titer is reduced 1000 times.

but there appears to be a small resistant fraction which remains infectious over several days (Krueger and Baldwin, 1934). Similar kinetics of inactivation have been reported by Moriyama and Ohashi (1941) for *E. coli* phage, and by Allison (1962) for fowl plague and vaccinia viruses.

The mercurials usually do not destroy the viruses or produce irreversible structural changes in them, since reactivation with thiols has been observed with staphylococcus phage (Wahl, 1939), influenza virus (Klein *et al.*, 1948; Perêz *et al.*, 1949), psittacosis virus (Burney and Golub, 1948), vaccinia virus (Kaplan, 1959; Allison, 1962), streptococcal phage (Kessler and Krause, 1963), and various enteroviruses (Choppin and Philipson, 1961). The results of Krueger and Baldwin (1933, 1934) are particularly impressive; reactivation with sulfide occurred even after exposure of phage to around 100 m*M* Hg^{++} for 9 days at 22°. It is remarkable that viruses, like certain enzymes, can be reactivated readily with thiols (especially dimercaprol) although no reactivation occurs by washing or dilution. Dimercaprol is able to reactivate influenza virus *in vivo* when injected after the treated virus in animals (Klein *et al.*, 1948) or chick embryos (Perêz *et al.*, 1949).

The reactivation with thiols does not prove that the mercurials react with virus SH groups, as has been concluded, but there is evidence for the importance of SH groups. The relative resistance of tobacco mosaic virus to the mercurials is probably due to the unavailability of the SH groups, since Anson and Stanley (1941) showed that *p*-MB reacts with all the SH groups of denatured virus but does not inactivate native virus. Some steric factor preventing reaction with *p*-MB was postulated by Fraenkel-Conrat (1959), since MM reacts stoichiometrically in a 1 : 1 ratio with the SH groups. The restriction may be imposed by hydrogen bonding to adjacent groups. Some plant viruses are structurally altered by mercurials. Solutions of potato virus X lose their flow birefringence when treated with *p*-MB, and sedimentation studies indicate disintegration into subunits (Reichmann and Hatt, 1961). It was concluded that the SH groups occur near the linkage sites holding the units together, rather than participating in the linkage, and that the bulky *p*-MB molecule splits the links. Turnip yellow mosaic virus is also split into subunits by *p*-MB, and RNA is liberated simultaneously (Kaper and Houwing, 1962 a). The artificial top component (empty virus protein shells) binds 645–660 molecules of mercurial per particle. As structural changes occur, new SH groups are unmasked and react with *p*-MB (Kaper and Houwing, 1962 b). Finally, one must consider the reaction of mercurials with the nucleic acid components of the viruses, since such complexes have been established (Katz, 1962). Tobacco mosaic virus RNA complexes with Hg^{++} (Katz and Santilli, 1962 b) but much of the infectivity remains in this case, although retention of specific infectivity was not demonstrated (Katz and Santilli, 1962 a).

We shall now inquire into the particular phases of virus multiplication

inhibited by the mercurials. It is clear that there is little or no selective action on viruses growing *in vivo*, and the mercurials have not been found to be effective virucidal or virustatic agents. Thus, although *p*-MB depresses psittacosis virus formation in chick embryo cultures, it also inhibits tissue growth, and it is quite possible that the effect on the virus is secondary to that on the host cells (Burney and Golub, 1948). Corneal infections with herpes virus are not benefited by application of *p*-MB, although the virus is readily inactivated *in vitro* (Sery and Furgiuele, 1961), and mercurials are not effective in preventing or treating plant virus infections. Kaplan (1959) concluded that mercurials react with SH groups on the surface of the virus and thus prevent attachment to the host cell. Certainly the hemagglutinating activity and the adsorption onto erythrocytes are depressed along with the infectivity (Choppin and Philipson, 1961). Adsorption of enteroviruses onto renal cells may also be reduced, but *p*-MB does not prevent adsorption of influenza virus onto the chorioallantoic membrane. Furthermore, *p*-MB does not prevent infection of *E. coli* by phage but inhibits the proliferation. Allison (1962) holds that the mercurials do not affect the primary attachment of the virus to the host cell, but may prevent the uncoating of the virus, an event which precedes multiplication. The studies of Shug *et al.* (1960) on T2 phage show that here the inhibition by *p*-MB is exerted early in the development, the maximal inhibition being 10–20 min after infection. This excludes the energy-yielding host metabolism as a primary site of action, since the energy requirements are greater after 25 min when the phage is being synthesized and assembled. They conclude that the site of attack is a protein concerned with the initial phases of replication and possibly involved in the assembly of the components into an intact phage. It would not be surprising if the site of mercurial action, or the phase disturbed, is different for the various phages and viruses.

Protozoa

Ciliates are immobilized and killed by the mercurials but the results reported are quantitatively discrepant. Nuhaus (1910) found that 0.15 m*M* Hg^{++} paralyzes paramecia in 50 min and kills them in 70 min, but Woodruff and Bunzel (1910) stated that 0.175 m*M* Hg^{++} stops all motion within 2 sec. Even greater sensitivity was reported by Gause (1933), as indicated in the accompanying tabulation. It should be noted that actually death

Hg^{++} (m*M*)	Duration of life (sec)
0.01	1080
0.015	360
0.02	116
0.03	30

was not the criterion, but cessation of movement, since no attempt to reactivate was made. When the log of survival time was plotted against log (Hg^{++}), two linear segments were obtained, which led Gause to conclude that two different processes are responsible for the paralysis. The treatment is based on an equation of the type I-12-89 and plotting as in Fig. I-12-38. A break in the curve would suggest a change of the exponent n and, since it is possible for one process or response to exhibit different values of n, it is not necessary to conclude that Hg^{++} kills by two processes. High sensitivity of paramecia to Hg^{++} was also noted by Calcutt (1950), who found 0.001 mM to paralyze within 6–14 min depending on illumination. Paralysis of *Colpidium colpoda* occurs in 3 min after exposure to 0.00087 mM PM (Walker, 1928). Some of the variations in sensitivity are due to the strains used, and probably some to the media in which the ciliates were suspended. It is probably safe to say that ciliary movement is very sensitive to mercurials and is stopped within a few minutes by concentrations in the range 0.001–0.01 mM. Reversal of ciliary beat by Hg^{++} is not observed (Oliphant, 1942). The classic death rate curves for *Colpidium* exposed to 0.2 mM Hg^{++} and their relation to population variation were discussed previously (page I-593 and Fig. I-12-36).

The effects of the mercurials on ameboid movement are interesting because of the possible bearing on muscle contractility. Reznikoff (1926) used rather high concentrations of Hg^{++} and consequently found only a pinching off of the region into which the Hg^{++} was injected (0.62 mM), or a break in the membrane (up to 10 mM), or an immediate gelation or coagulation of the protoplasm (up to 200 mM). Käppner (1961) used mersalyl since this mercurial has been a favorite with myologists, and the changes he observed in amebas with increasing concentration are worth describing briefly. With 0.001 mM mersalyl there are no immediate changes but after several days some damage is evident. At 0.01 mM there is restriction of normal pseudopodial response and fewer pseudopods are formed, while clumping of the cytoplasmic crystals occurs. At 0.1 mM the pseudopods withdraw, the cells soon form numerous small pseudopods at the end of which appear tiny spheres, the cortex appears to be thicker, and eventually the cells round up. At 1 mM the response is not so specific, the surface bubbles, the cells round up, and soon the membrane dissolves. Higher concentrations produce vacuolization and cytolysis. Abe (1963) reported a similar study but with *p*-MB to which amebas seem to be more sensitive than to mersalyl, since 0.1 mM causes cytolysis within 8 min. Further investigation of this interesting problem is warranted and a closer analysis of the effects of low concentrations on the sol-gel transformation might provide useful information on the role of SH groups in protoplasmic movement.

DEVELOPMENT OF RESISTANCE TO MERCURIALS

Most types of microorganism appear to be able to adapt to the presence of mercurials, but usually not as readily or to such a degree as to arsenicals, sulfonamides, or antibiotics. Some resistance factors are given in the accompanying tabulation, but it is likely that greater tolerance could have

Organism	Mercurial	Resistance factor	Reference
Staphylococcus aureus	Hg^{++}	>50	Benigno and Santi (1946)
		1.8	Klimek *et al.* (1948)
Escherichia coli	PM	2.6	Akiba and Ishii (1952)
Salmonella pullorum	Hg^{++}	12	Severens and Tanner (1945)
Salmonella typhosa	Hg^{++}	6	Severens and Tanner (1945)
Penicillium notatum	Hg^{++}	2.5	Partridge and Rich (1962)
Sclerotinia fructicola	Hg^{++}	2.5	Partridge and Rich (1962)
Yeast	Hg^{++}	>10	Imshenetsky and Perova (1957)
Candida utilis	Hg^{++}	7	Gérardin and Kayser (1959)
Treponema pallidum	Hg^{++}	75	Noguchi and Akatsu (1917)

been developed in some instances if training had been prolonged. There are also naturally occurring resistant strains and species. An interesting example is the relative tolerance of *Penicillium roqueforti* to PM, and this has bearing on the preservation of groundwood pulp (Russell, 1955). Most fungi fail to grow in 0.006–0.030 m*M* PM, but this species grows well in a concentration of 0.06 m*M* and furthermore accumulates sufficient mercurial to allow the less resistant organisms to grow. The number of serial cultures in increasing mercurial concentrations required to produce tolerance varies with the organism: It was 20 transfers for the fungi in the above table (Partridge and Rich, 1962), 70–100 transfers for the species of *Salmonella* (Severens and Tanner, 1945), and up to 500 transfers for yeast (Imshenetsky and Perova, 1957). Occasionally no transfers are required and the organism begins to grow normally after a prolonged lag period, as is the case with *Aspergillus glaucus* where hyphal inoculations fail to grow for periods of up to 14 days in 0.033 m*M* Hg^{++}, and then proliferate without loss of vigor (Briault, 1956). This indicates that resistance can develop in nonproliferating organisms.

Inasmuch as we do not understand how the mercurials depress growth, it is clear that we cannot immediately postulate logical mechanisms for the developed resistance. However, some interesting observations may contribute to the elucidation of the mechanisms of inhibition. The resistance is apparently not due to a reduction of permeability to the mercurials, as

occurs with the arsenicals, since Benigno and Santi (1946) found that staphylococci tolerant to Hg^{++} grow when they have taken up much more Hg^{++} than is required to prevent growth of the normal strain. The situation with respect to the thiol content of the resistant organisms is confused, since Akiba and Ishii (1952) showed that *E. coli* tolerant to PM have less SH groups than normally, and Gérardin and Kayser (1959) found that tolerant *Candida utilis* contained 6 times more SH groups than the normal strain. Zambonelli (1958 a,b) has obtained evidence that adapted yeast produces more H_2S and believed that this inactivates much of the Hg^{++}. Normal yeast produces H_2S only from sulfite, whereas adapted strains produce it from sulfate and hyposulfite in addition, although not from cysteine or glutathione. If the resistant strains are grown with only cysteine or glutathione as the source of sulfur, Hg^{++} readily inhibits their growth. If metabolic changes occur during adaptation, they are not marked. Thus resistant *Candida* respires normally (Gérardin and Kayser, 1959) and resistant yeast ferments glucose at the normal rate, although the respiration may be slightly higher (Imshenetsky and Perova, 1957). Claus (1956) has shown that the respiration of *Aerobacter aerogenes* is initially depressed by Hg^{++} but recovers after several hours and reaches normal levels (Fig. 7-46).

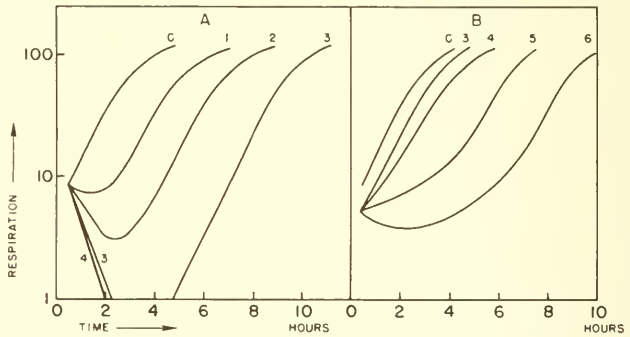


FIG. 7-46. Effects of Hg^{++} on the respiration of *Aerobacter aerogenes*. The O_2 uptake is given as mm^3 O_2/ml/30 min. A: Initial exposure to Hg^{++}; B: inoculation of organisms from 3 in A and re-exposure. C, control; 1, 0.0011 m*M*; 2, 0.0022 m*M*; 3, 0.0044 m*M*; 4, 0.0088 m*M*; 5, 0.0178 m*M*; 6, 0.037 m*M*. (From Claus, 1956.)

Adaptation to mercurials is apparently specific in most cases, since Severens and Tanner (1945) found that *Salmonella* sp. tolerant to Hg^{++} are not tolerant to Cu^{++}, and vice versa, while Launoy and Levaditi (1913) showed that spirochetes tolerant to antisyphilitic mercurials are not tolerant to arsenicals. However, Blumenthal and Pan (1963) noted that penicillin-resistant strains of staphylococci are more apt to be resistant to

Hg^{++} than the normal strains. Resistance to the mercurials is usually not lost during subsequent culturing in mercurial-free media, as shown for fungi (Partridge and Rich, 1962), yeast (Zambonelli, 1958 a), and spirochetes (Launoy and Levaditi, 1913). The most striking case of the retention of resistance is that of *Salmonella*, tolerance being unchanged during 55 transfers over a period of 18 months (Severens and Tanner, 1945). It has thus been concluded that the tolerance is inherited. Morphological changes during adaptation generally do not occur, but yeast cells tolerant to Hg^{++} are smaller, have lost their smooth contours, contain more vacuoles, and are deficient in lipid (Imshenetsky and Perova, 1957). Growth of tolerant organisms in normal media is usually not different from that of sensitive strains, and no instance of dependence on mercurials has been reported.

Development of resistance to mercurials is not confined to microorganisms but is observed in certain mammalian tissues. Gil y Gil (1924) claimed to have produced renal tolerance to Hg^{++} in rabbits, but his work was criticized by Hunter (1929), who repeated and extended this study using more appropriate dosages. Rabbits given nephrotoxic doses of mercurial exhibited a regeneration of the renal epithelium around the fourth day, the new cells being elongated, flattened, with hyperchromatic nuclei, and somewhat resistant to Hg^{++}. Tsurumaki *et al.* (1928) confirmed that the kidneys of rabbits surviving toxic injections of $HgCl_2$ are not damaged by the same doses if given 10–14 days after the disappearance of the original nephritis. During the period of adaptation, even though repeated subcutaneous injections are given, the nephritis disappears, but there is no alteration in the excretion of Hg^{++} (Miura, 1934). MacNider (1941) repeated such experiments in dogs and claimed that the newly regenerated renal epithelial cells are different both morphologically and chemically from the normal cells. The depressant effects of meralluride on intestinal transport of Na^+, Cl^-, and water also disappear with repeated administration of the drug (Blickenstaff, 1954), as does the suppression of conditioned reflexes in rats given $HgCl_2$ (Galoyan, 1957).

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