

METABOLITE ANTAGONISTS

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I. INTRODUCTION

The concept that substances chemically related to a metabolite may interfere with the normal function of that metabolite in living cells is attracting widespread interest among chemists and biologists. For the organic chemist, this concept frequently provides a unique starting point for the synthesis of biologically active compounds and the study of the relation between chemical structure and activity. The stimulus for many of the investigations under review came from the discovery of the striking relationship between *p*-aminobenzoic acid and sulfanilamide-type compounds. Not all important antagonists are structurally related to the affected metabolite, however, and the value of specific antagonisms in elucidating the function of metabolites and explaining the action of drugs has been recognized for some time.

It is the object of this review to summarize the pertinent background in several fields of investigation, and to review the available literature¹ relating to the direct

¹Because of current conditions complete coverage, particularly of the foreign literature, has not been practicable. I should greatly appreciate receiving references to papers which have been overlooked or were not available at the time this review was written.

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antagonism of a number of essential metabolites in living cells. Structural relationships have been emphasized whenever they appeared to be significant. Since the normal function of a metabolite is helpful in accounting for the effects of an antagonist, this aspect of the subject has been reviewed briefly wherever possible.

Broadly, a metabolite may be regarded as any substance involved in the chemical processes by which living cells are produced and maintained. But the present review is limited largely to substances of known structure which function as essential parts of cell catalysts (e.g., vitamins and hormones). Since these substances are usually present in minute amounts, only small quantities of an antagonist are required to produce distinctive effects.

Many types of antagonisms are also known. They may be divided roughly into direct chemical or physicochemical effects which frequently obey the law of mass action, and indirect or physiological antagonisms due to opposite but independent actions. Although there is no sharp line of demarcation, in this review the emphasis has been placed on antagonists having a direct, reversible effect on the synthesis or utilization of specific metabolites. In general, naturally occurring antagonisms (i.e., the antagonist is also a metabolite taking part in normal cell processes) and the numerous antagonisms between pairs of drugs have not been included.

II. HISTORICAL BACKGROUND

The current developments in metabolite antagonists can be understood most readily when viewed in the light of previous studies in several fields of investigation, particularly enzymology, pharmacology, and chemotherapy.

A. Enzymology

The use of inhibitors² and poisons has long been recognized as an important tool in the study of isolated enzyme systems. Although it is not within the scope of this review, the vast literature in this field forms a rich background for the observations on living cells. Present theories of the nature and specificity of enzymes are, to a considerable extent, founded on the effects of inhibitors. According to these theories, enzymes contain active centers or groups which combine with the substrates. The active centers are presumed to be oriented so as to confer a high degree of specificity.

Tammann (407) in 1891 and Armstrong (13) were among the first to observe that the addition of the products of an enzyme reaction produced an inhibitory effect on its velocity. In many instances, these results cannot be attributed to a mass action effect, but appear to involve a competitive inhibition. Similarly, Haldane (153) and others have called attention to the numerous cases of inhibition of enzymes by substances structurally related to their substrates. These effects are ascribed to a blocking of the active centers by the inhibitors. Haldane

²The terminology in different fields is sometimes inconsistent. "Inhibitor" is synonymous with "antagonist" in the discussion of isolated enzymes to conform to usage; elsewhere the term "antagonist" is preferred, to eliminate the situation in which an inhibitor inhibits an inhibitor. To avoid the confusion of ideas arising from a metabolite antagonizing the action of a drug, the drug is referred to as the antagonist and terms such as "preventing" or "reversing" are substituted to describe the effect of the metabolite.

(153) also listed a number of enzyme poisons most of which owed their activity to either a chemical reaction with a reactive group, or salt formation with acidic or basic groups of the enzyme. The plausibility of this explanation was illustrated by Myrbäck (305), who showed that sucrose, glucose, and fructose protected yeast saccharase from inactivation by nitrous acid. This result suggested the presence of an amino group in the active center of the enzyme, the amino group being protected from the nitrous acid by its combination with the substrate or the products of the reaction. Other substances such as maltose and lactose, which did not appear to combine with the enzyme, failed to protect it (153).

In general, inhibitors may affect the velocity of enzymatic reactions in two ways, resulting in either competitive or non-competitive inhibition. Those of the latter type reduce the velocity of the reaction, but the dissociation constant of the enzyme-substrate complex is unaltered. In other words, varying the concentration of substrate has little effect on the degree of inhibition. By contrast, competitive inhibition involves a direct relationship between inhibitor and substrate, so that increasing the concentration of substrate reduces the degree of inhibition, i.e., inhibitor and substrate appear to compete for the same active centers.

Because of the probability that the fundamental processes in the inhibition of isolated enzymes and the antagonism of metabolites in living cells are related, an examination of some of the more pertinent features of enzyme inhibitors reveals many interesting analogies. A few examples are listed in table 1, but further discussion has been deferred to a later section (*cf.* Section IV).

It is sometimes possible to account for the pharmacological action of drugs in terms of their effect on specific enzyme systems. Thus, Englehardt and Loewi (107) demonstrated that the inhibition of choline esterase by physostigmine resulted in a decreased rate of destruction of acetylcholine. The pharmacological actions of physostigmine are due entirely to its preservation of acetylcholine, the drug itself having no cholinergic activity (69). The Stedmans (399) showed

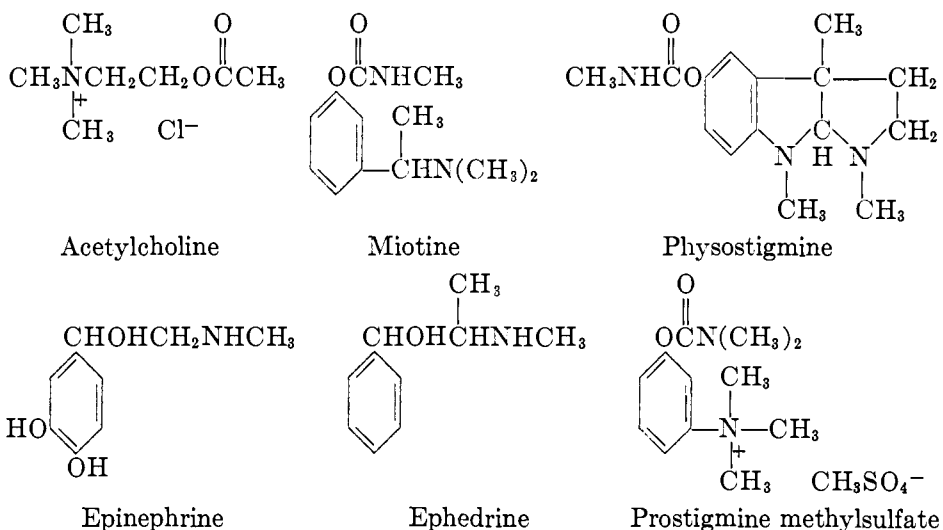


TABLE 1
Inhibitors of isolated enzyme systems

ENZYME	SUBSTRATE	INHIBITOR	OTHER OBSERVATIONS	REFERENCE
Choline esterase.....	Acetylcholine	Physostigmine (eserine)	Competitive inhibition; accounts for pharmacological activity of physostigmine (see text)	Loewi and Navratil (242)
Liver esterase.....	Tributylin	Octyl alcohol	$1.6 \times 10^{-6} M$ stimulates, but $2.5 \times 10^{-4} M$ or greater causes increasing inhibition	Sobotka and Glick (387)
Liver esterase.....	Tributylin	Sodium arsanilate	Much less susceptible to inhibition by quinine than serum esterase	Rona and Pavlovic (349)
Serum esterase.....	Tributylin	Quinine	Much less susceptible to inhibition by sodium arsanilate than liver esterase	Rona and Pavlovic (349)
Pancreatic lipase.....	Ethyl butyrate	Acetophenone	Competitive inhibition; other ketones also inhibitory but not the oxime of acetophenone	Murray (304)
Malt amylase.....	Starch (maltose production)	Glucose; maltose	Fructose and sucrose not inhibitory	Wohl and Glimm (439)
Yeast saccharase.....	Sucrose	β -Glucose; fructose	Competitive inhibition; α -glucose not inhibitory	Kuhn (204)
Aspergillus saccharase.....	Sucrose	α -Glucose	Competitive inhibition; β -glucose and fructose not inhibitory	Kuhn (204)
Arginase.....	Arginine	Ornithine	Urea not inhibitory, indicating that action of ornithine is not due to displacement of mass action equilibrium	Gross (150)

Histidase.....	<i>l</i> -Histidine	<i>d</i> -Histidine	Imidazole, guanidine, arcaine, and ethylenediamine also inhibitory, but guanidoacetic acid inactive	Edlbacher, Baur, and Becker (99)
Intestinal peptidases.....	Glycylglycine	Glycine; alanine	Competitive inhibition; glycine anhydride inhibitory but not benzoyl glycylglycine, which is also not hydrolyzed by enzyme	Euler and Josephson (114)
Intestinal peptidases.....	Glycyl- <i>m</i> -amino-benzoic acid	Glycyl- <i>o</i> -amino-benzoic acid	Glycyl- <i>p</i> -aminobenzoic acid also hydrolyzed by enzyme and inhibited by ortho isomer	Balls and Kohler (23)
Lactic dehydrogenase.....	Lactic acid	Pyruvic acid	Competitive inhibition; also produced by other substances with acidic groupings —CO—COH* or —CHOH—COH* (H* is labile)	Quastel and Wooldridge (328)
Succinic dehydrogenase.....	Succinic acid	Malonic acid	Competitive inhibition; other substances with —C—CH—COOH or —C—CH ₂ —COOH configuration also inhibitory	Quastel and Wooldridge (328)
Xanthine oxidase.....	Xanthine	3-Methyl-xanthine	Guanine, uric acid, 1(or7)-methyl-guanine also strong inhibitors	Coombs (76)
Monamine oxidase.....	Epinephrine	Ephedrine	Competitive inhibition; accounts for pharmacological activity of ephedrine (see text)	Blaschko, Richter, and Schlossmann (40)
Diamine oxidase (histaminase)...	Histamine	Guanidine	Competitive inhibition; <i>N</i> -methylation increases inhibitory action	Zeller (470)

that synthetic methyl urethans related to physostigmine, such as miotin, also inhibit esterases. They suggested that the inhibition could be explained by the ester-like structure of the urethans, which enables them to combine with and block the active centers of the esterases. Miotine and prostigmine (5) closely resemble physostigmine in their pharmacological effects. In an analogous fashion Gaddum and Kwiatkowski (138) considered that the pharmacological action of ephedrine is due to its sparing effect on the enzymatic destruction of epinephrine (table 1). Quastel and coworkers (326, 327) studied the effects of anesthetics and hypnotics on the enzymes of the brain. The action of many of these substances is attributed to the inhibition of a sensitive component of the brain respiratory system. Because of the intricate balance of many enzyme systems, antagonisms in living cells are obviously much more complex than the inhibition of isolated enzymes. A comprehensive review of the relation of drugs to the inhibition of specific enzymes has been written by Bernheim (34).

B. Pharmacology

Physiologists and pharmacologists for many years have employed antagonists to study the normal functions of metabolites and to determine the mode of action of drugs. An excellent survey of these investigations has been made by Clark (69), who classified the three chief forms of drug antagonism as follows:

“(a) *Chemical antagonism in vitro*. In this case the antagonists react together to form a product of reduced activity.

“(b) *Physiological antagonism*. In this case two drugs produce opposite but independent effects on cells or organs.

“(c) *Specific antagonism*. This term is reserved for cases in which one drug inhibits the action of another drug on living cells, although no reaction between the drugs occurs *in vitro*.”

In this classification Clark was considering antagonisms between pairs of drugs (e.g., pilocarpine and atropine) as well as metabolite antagonists. Presumably type a antagonisms can occur *in vivo* as well as *in vitro*, and the action of arsenicals may fall in this category (*cf.* Section III,D). Physiological antagonisms (type b) have not been considered in this review, since no direct relationship between metabolite and antagonist is involved.

One of the simplest systems of type c is the antagonism by carbon monoxide of the combination of oxygen and hemoglobin, i.e., $\text{CO} + \text{HbO}_2 \rightleftharpoons \text{O}_2 + \text{HbCO}$. This system was investigated from a quantitative standpoint as early as 1884 by Hufner (180) and later by Douglas, Haldane, and Haldane (93) and Hartridge and Roughton (164). Both oxygen and carbon monoxide form reversible compounds with hemoglobin and appear to compete for the same active centers or receptors. In dilute aqueous solution the distribution of hemoglobin between the two gases is given by the following formula:

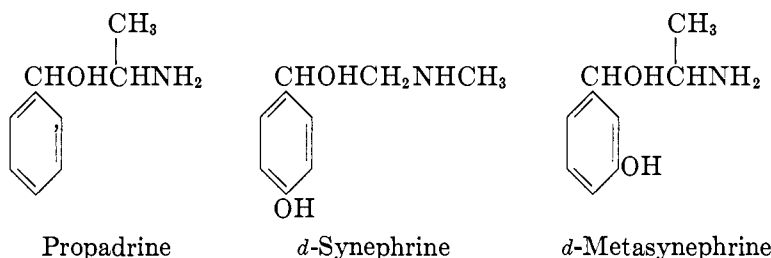
$$\frac{[\text{O}_2]}{[\text{CO}]} \times \frac{[\text{HbCO}]}{[\text{HbO}_2]} = K$$

The concentration of HbCO equals that of HbO₂ when the relative pressures of oxygen and carbon monoxide are approximately 200 to 1.

Külz (211) and Raventos (333) studied the action of a series of tetraalkylam-

monium salts on isolated frog muscle (rectus abdominis). They found that in the series $(\text{CH}_3)_4\text{NX}$ to $(\text{CH}_3)_3\text{N}(\text{C}_4\text{H}_9)\text{X}$ the salts, like acetylcholine, produced contractions. As the chain length increased, the effect decreased, so that $(\text{CH}_3)_3\text{N}(\text{C}_8\text{H}_{17})\text{X}$ not only failed to produce contractions, but in fact exerted a powerful antagonistic effect on the action of the lower members of the series. Both $(\text{CH}_3)_3\text{N}(\text{C}_8\text{H}_{17})\text{X}$ and $(\text{C}_2\text{H}_5)_4\text{NX}$ antagonized the action of acetylcholine and of the $(\text{CH}_3)\text{N}(\text{R})\text{X}$ type compounds on the frog's heart. In other tissues there were wide variations in the types of action and antagonism.

Modern and Thienes (297) investigated the antagonistic effects of a large group of compounds structurally related to epinephrine. They found that under certain conditions compounds such as propadrine and *d*-synephrine antagonized the action of epinephrine on smooth muscle, whereas *d*-metasynephrine was inactive.



The antagonists by themselves frequently had no effect on the motility of the muscle.

In 1937, Clark (69) pointed out that such results suggested the general hypothesis that substances are most likely to be antagonized by compounds of similar molecular configuration.

Quantitative studies of the acetylcholine-atropine (67) and epinephrine-ergotoxine (135, 287) antagonisms, in which the ratio of metabolite to antagonist remains constant over a wide range of concentrations, indicate a competition for the same tissue receptors (68, 69, 136). On this basis it is assumed that the union of drugs with specific receptors involves two processes: (a) fixation of drug, and (b) selective action after fixation. An antagonist either prevents fixation, or alters the pattern of receptors so that the active drug or metabolite can no longer produce its specific effect (69).

C. Chemotherapy

From the time when he founded modern chemotherapy, Ehrlich emphasized the importance of attempting to determine the mechanism of action of chemotherapeutic agents. Drawing upon his immunological studies and observations with drug-resistant trypanosomes, he developed the famous chemoreceptor or side-chain theory. This theory postulated the presence of chemical groups or side chains on the organisms with which the chemotherapeutic agents combined. The side chains were considered to be essential for the nutrition of the trypanosomes. Present concepts of the nature and structure of protoplasm have required major modifications in Ehrlich's original ideas, but the fundamental principle of interference with metabolic processes remains as a tribute to his brilliant imagination. Voegtlin (422) pointed out that if the —SH group is

regarded as the arsenic receptor, one finds a basis for Ehrlich's interpretation of the mode of action of arsenicals (*cf.* Section III,D). The observations of Yorke and coworkers (467) and Hawking (166), indicating that vulnerable trypanosomes concentrate arsenicals whereas resistant strains do not, has since helped to explain the differences in the susceptibility of trypanosomes and in their toxicity to host cells.

In the study of germicides several pioneer investigators attempted to relate the action of various agents to their effect on bacterial metabolism. While much of the early work was concerned with bactericidal action, Simon and Wood (378) in 1914 examined the mechanism of action of a group of aniline dyes which were primarily bacteriostatic. Like Ehrlich, these investigators believed that the growth-inhibitory action was probably due to a combination of the dyes with "nutriceptors" in the organism, the dye becoming anchored so that the organisms starved or were unable to multiply. Dye-resistant strains were developed, and this phenomenon was considered to be due to the formation of receptors which either did not attract the dye or were able to destroy it. Other investigators (49, 181, 401) carried these ideas as far as the limited contemporary knowledge of bacterial metabolism would permit. It was not until developments in other fields (e.g., biochemistry, enzymology, and nutrition) had filled in the background that a more complete picture could be drawn.

The theory that the bacteriostatic action of sulfanilamide-type compounds is due to the blocking of an essential enzyme system or systems was adumbrated by several investigators, notably Lockwood (237), McIntosh and Whitby (267), Stamp (393), and Green (148). It remained for Woods and Fildes (122, 443, 446) to provide a firm foundation for this theory and to extend it to the broad basis which has inspired so many subsequent investigations of other bacterial metabolite antagonists.

In 1940 Woods (443) isolated in a crude form from yeast cells a substance which prevented the antibacterial action of sulfanilamide and sulfapyridine. Stamp (393) and Green (148) had obtained similar extracts from other organisms. After concentrating his factor, Woods concluded from an examination of its chemical properties that the substance might be structurally related to sulfanilamide. The behavior of the material, resembling the competitive inhibition of enzyme reactions by compounds structurally related to the substrate or product, pointed to the same conclusion. Accordingly, *p*-aminobenzoic acid (PABA) was tried and found to be highly active. In order to reverse or prevent the bacteriostatic effect of sulfanilamide, a more or less constant molar ratio of PABA to sulfanilamide was required over a considerable range of concentrations. Both the unsubstituted amino group and the carboxyl group para to it proved to be necessary for antisulfanilamide activity. Woods considered that the results provided strong circumstantial evidence for the identity of the yeast factor, and suggested that PABA is an essential metabolite for the growth of microorganisms.

At the same time, Fildes (122) expanded the concept of metabolite antagonists in relation to bacterial growth in an illuminating paper entitled, "A Rational Approach to Research in Chemotherapy". He defined essential metabolites as the substances involved at "each stage in any synthesis necessary for growth,

without which, either synthesized or supplied from outside, growth cannot occur." A growth factor for a given organism is then an essential metabolite which it cannot synthesize. For example, *E. coli*, which grows on a medium containing only inorganic salts and glucose, can synthesize all its other requirements, whereas *P. vulgaris* needs preformed nicotinic acid. Given nicotinic acid, *P. vulgaris* can complete the synthesis of cozymase, which is also produced by *E. coli*. Nicotinic acid is regarded as a growth factor for *P. vulgaris* and an essential metabolite for *E. coli*, and cozymase an essential metabolite for both organisms. Fildes proposed the theory that antibacterial substances may inhibit growth by antagonizing or interfering with an essential metabolite in one of three ways:

"1. By oxidizing a substance which requires to be reduced.

"2. By molecular combination giving an inactive product.

"3. By competition for an enzyme associated with the essential metabolite."

Various oxidizing agents such as ferricyanide or methylene blue were presumed to act by the first mechanism, while the combination of mercuric chloride with essential —SH groups was regarded as an example of class 2. Fildes and Woods considered that, because of their structural similarity, sulfanilamide and PABA compete for an enzyme involved in the further utilization of PABA. On this basis, the sensitivity of an organism to sulfanilamide was considered to depend in part on its ability to synthesize PABA. More broadly, Fildes suggested that compounds closely related structurally to other known metabolites, but devoid of essential metabolic activity, might also be found to inhibit the growth of various microorganisms. Experimental verification for many of the ideas proposed by Fildes and Woods will be found in the subsequent sections of this review.

III. SPECIFIC METABOLITE ANTAGONISTS

The following sections, arranged by metabolites, cover most of those for which any extensive literature has accumulated. Other recognized metabolites which might have been included, such as acetylcholine and epinephrine, are described briefly in Section II. All but the recent literature relating to antagonists of these two metabolites has been discussed in detail by Clark (69). Some of the later studies in these fields are incorporated in Section IV. A number of articles summarizing a portion of the work with other metabolite antagonists, particularly in relation to antibacterial action, have appeared (6, 7, 94, 199, 205, 256, 257, 261, 425, 428, 451). The reader is referred to these summaries for brief discussions of this phase of the subject.

An attempt has been made to standardize terminology², so that the term "inhibition ratio" is used throughout to indicate the moles of antagonist required to nullify the function of one mole of metabolite. In certain instances, the ratio may be reported on the basis of a 50 per cent reduction, in which case these conditions are mentioned specifically. It is important to distinguish between these two criteria in making comparisons, because the ratio required for complete antagonism is frequently many times greater than at the 50 per cent level, and this relationship may vary with different antagonists or with the same antagonist in different cells.

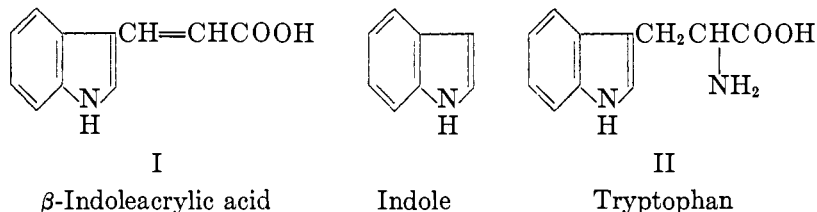
The criterion of antagonism has been based primarily on the reversal or pre-

vention of the biological effect of an antagonist by a specific metabolite, recognizing that this test can only be regarded as an important but not exclusive condition for the evaluation of direct antagonists. Other limitations of scope are outlined in Section I. The papers in each section have been reviewed in more or less chronological order, with some exceptions for the sake of continuity.

A. Amino acids

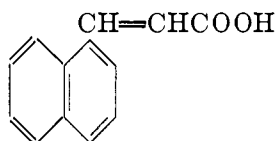
Many organisms are unable to synthesize one or more of the known amino acids. These essential metabolites are the building blocks of the cell proteins, and as such they are usually required in larger quantities than many of the other growth factors. Proteins are not only the basic units of cell structure but also, in conjunction with active prosthetic groups, assume the rôle of enzymes to catalyze many metabolic reactions (296). Finally, some of the amino acids probably serve as precursors of other essential metabolites (88).

In studying the production of indole from tryptophan, Fildes (121) noted that the growth of *E. coli* was inhibited by indoleacrylic acid. Following up this earlier observation, he found (124) that *M/8000* I prevented growth, while a

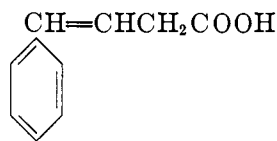


number of related substances, such as indoleacetic and propionic acids, produced very slight or no growth inhibition. Since indoleacrylic acid is closely related to II in several respects, Fildes reasoned that I might interfere with an enzyme system concerned with the metabolism of tryptophan. He demonstrated experimentally that, although not itself a growth stimulant, II counteracted the inhibition produced by I. The growth was proportional to the amount of tryptophan added, but there was no quantitative relation between the concentrations of I and II. Unlike the sulfonamide-PABA relationship, the response of the organisms was a function of the tryptophan concentration only, regardless of the amount of indoleacrylic acid present. These results, which were confirmed with *B. typhosum*, led to the suggestion that I interferes with the formation of tryptophan rather than its utilization. Under these conditions no quantitative relationship between I and II would be expected, and the minimum amount of tryptophan necessary for growth should reverse any reasonable amount of indoleacrylic acid. However, there should be a quantitative relationship between I and a precursor of tryptophan. Indole is a precursor for *B. typhosum*, but no interference with the action of I by this substance was found. To explain the lack of any reversing action with indole, Fildes assumed that either the blocking effect of I on tryptophan synthesis is not reversible or, because of the growth inhibition produced by indole itself at higher concentration, the effect could not be demonstrated.

Block and Erlenmeyer (42) reported that 1-naphthylacrylic acid and styrylacetate acid resembled indoleacrylic acid in their behavior. These substances ap-



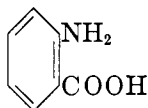
1-Naphthylacrylic acid



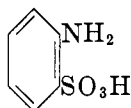
Styrylacetic acid

peared to antagonize the growth-stimulating action³ of tryptophan. While *trans*-cinnamic acid gave similar results, the authors concluded that tryptophan metabolism was not involved in this case. Dihydrocinnamic, benzoic, and fumaric acids produced no effect.

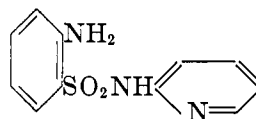
With several species of lactic acid bacteria, Snell (381) was able to substitute anthranilic acid for tryptophan. Since anthranilic acid is an isomer of *p*-aminobenzoic acid (PABA), he studied the corresponding isomer of sulfanilamide (orphanilamide) as well as orphanilic acid and 2-(orphanilamido)pyridine. None



Anthranilic acid



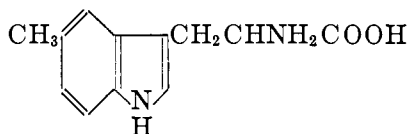
Orphanilic acid



2-(Orphanilamido)pyridine

of these substances inhibited growth promoted by anthranilic acid. Snell concluded that, if it existed at all, the antibacterial power of orphanilamide in relation to anthranilic acid was of a much lower order of magnitude than in the case of the corresponding sulfanilamide-PABA relationship.

Anderson (12) found that the bacteriostatic action of *dl*-5-methyltryptophan on *E. coli* in simple media was reversed by *l*-tryptophan, the inhibition ratio being approximately 1000:1 over a considerable range of concentrations. On



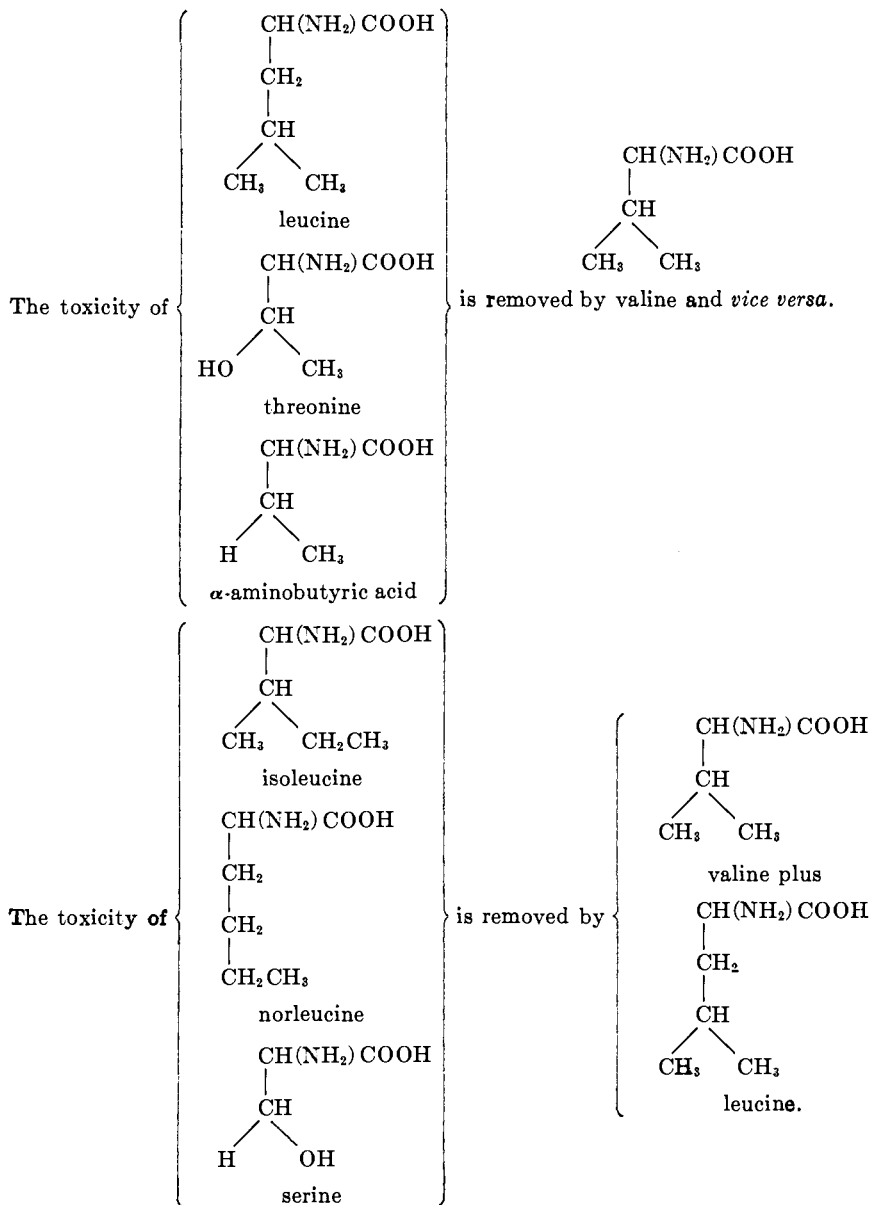
5-Methyltryptophan

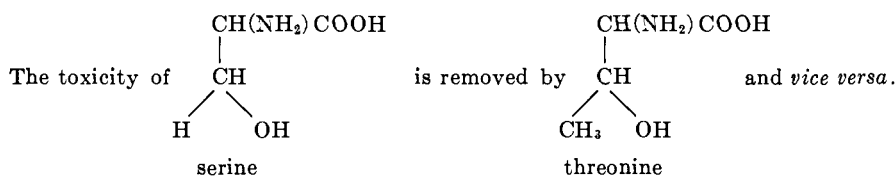
the other hand, he also demonstrated that the tryptophan requirements of certain *E. coli* bacteriophages could be replaced by the 5-methyl derivative. Earlier, Gordon and Jackson (145) had shown that the latter compound was toxic to rats on a tryptophan-deficient diet, but had no effect on animals receiving an adequate ration.

Gladstone (141), investigating the amino acid requirements of *B. anthracis*, observed a number of curious interrelationships among groups of chemically related amino acids. When leucine or valine was eliminated from the synthetic

³Growth stimulation usually indicates that the metabolite is a limiting factor for the growth of the particular organism, rather than a growth factor as defined by Fildes (122). Thus, the synthetic ability of the organism for the metabolite is limited, and addition of the preformed metabolite provides a more favorable medium for growth. Under these conditions, any growth inhibitor is likely to be less effective, regardless of whether it is a specific antagonist for the metabolite in question. Consequently, specific antagonisms are frequently more difficult to establish when the metabolite is a growth stimulant.

medium, the organisms failed to grow, and the removal of isoleucine resulted in delayed and incomplete growth. The absence of all three amino acids, surprisingly enough, allowed growth to occur, but the addition of any one of these amino acids to a mixture in which all three were originally absent completely inhibited growth. Isoleucine was the most effective, preventing growth at a concentration of $M/312,500$ under these conditions. The presence of the unnatural isomers did not account for the results, since the same effects were obtained with the purified natural isomers. Further study revealed several other similar relationships, which were summarized as follows by Gladstone:

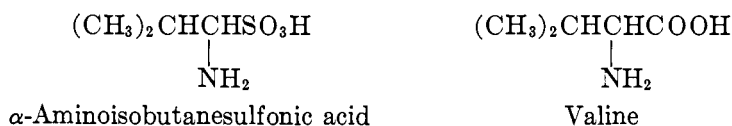




On the basis of the similarity in chemical structure of the growth inhibitors and their antagonists, Gladstone (141) suggested that some common reaction necessary for growth might be involved. Thus, an excess of one amino acid might block the enzymes necessary either for the synthesis of another chemically related, or for building it, when synthesized, into more complex substances.

Numerous other instances in which one amino acid may interfere with the utilization of another have been reported. The results of these studies are summarized in table 2. It is usually assumed in these cases that the antagonist is also an essential metabolite. However, an excess of one (the antagonist) blocks the function of the other (the metabolite), because of the structural similarity between them. Such effects are most frequently observed in relatively simple culture media. Results of this type do not come strictly within the scope of this review, but they have been included in this instance because of the numerous interesting structural relationships involved.

Several α -aminosulfonic acids structurally related to the natural α -aminocarboxylic acids were studied by McIlwain (251, 252). The sulfonic acid analogs of amino acids such as glycine, alanine, leucine, and valine were prepared by treating the corresponding aldehyde bisulfite derivatives with aqueous ammonia (251). It was found (252) that in a chemically defined medium organisms which required preformed amino acids were inhibited by the α -aminosulfonic acids. There appeared to be considerable overlapping in the reversing effect of the amino acids. Thus, the growth inhibition produced by α -aminoisobutanesulfonic acid was partly reversed by glycine or alanine, more effectively by valine, but not at



all by leucine. However, of the various metabolites tested, only the amino acids were capable of preventing the growth inhibition of the α -aminosulfonic acids. In general, bacteria synthesizing their own amino acids were not susceptible. McIlwain also investigated the action of cysteic acid, taurine and tauramide, the respective analogs of aspartic acid and β -alanine. These sulfonic acids were not inhibitory, and sometimes stimulated suboptimal growth.

Spizizen (391), investigating the multiplication of bacteriophage acting on *E. coli*, found that the reproduction of this bacterial virus could be stimulated by glycine or glycine anhydride. He demonstrated that the multiplication of phage stimulated in this manner could be inhibited by aminomethanesulfonic acid, the sulfonic acid analog of glycine. On the contrary, when xanthine was used as a stimulant, no inhibition was observed.

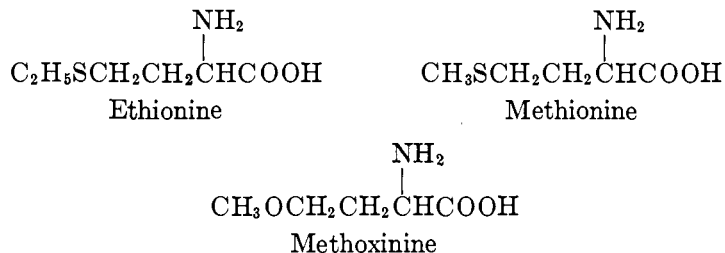
TABLE 2
Antagonistic relationships between amino acids

ANTAGONIST	METABOLITE	ORGANISM	OTHER OBSERVATIONS	REFERENCE
$\text{H}_2\text{NCH}_2\text{CH}_2\text{COOH}$ β -Alanine	$\text{H}_2\text{NCOCH}_2\text{CH}(\text{NH}_2)\text{COOH}$ Asparagine	Yeast	β -Alanine acts as growth stimulant only in presence of asparagine or aspartic acid	Nielsen and Hartelius (307)
$\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ Norleucine	$\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ Methionine	<i>Escherichia coli</i>	Norvaline also antagonistic; inhibition ratio norleucine: methionine approximately 1000:1	Harris and Kohn (159)
$\text{HOOCCH}_2\text{CH}(\text{NH}_2)\text{COOH}$ Aspartic acid	$\text{H}_2\text{NCOCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ Glutamine	<i>Lactobacillus casei</i>	Asparagine and glutamic acid also effective in preventing growth inhibition caused by aspartic acid	Feeney and Strong (117)
$\text{CH}_2(\text{NH}_2)\text{COOH}$ Glycine	$\text{CH}_3\text{CH}(\text{NH}_2)\text{COOH}$ α -Alanine	<i>Streptococcus fecalis</i> R	Bacteriostatic action of glycine also reversed by pyridoxine	Snell and Guirard (384)
$(\text{CH}_3)_2\text{CHCH}_2\text{CH}(\text{NH}_2)\text{COOH}$ Leucine	$\text{CH}_3\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}(\text{NH}_2)\text{COOH}$ Isoleucine	<i>Pasteurella pestis</i>	Valine also reverses antagonistic action of leucine; not due to growth-promoting action	Doudoroff (92)

Phenylalanine or norleucine or norvaline. . . .	Isoleucine and valine	<i>Neurospora crassa</i>	Antagonistic effect limited to mutant strain requiring preformed isoleucine and valine	Bonner, Tatum, and Beadle (43)
$\text{H}_2\text{NC}(=\text{NH})\text{NH}(\text{CH}_2)_3\text{CH}(\text{NH}_2)\text{COOH}$. . . Arginine	$\text{H}_2\text{NCH}_2(\text{CH}_2)_3\text{CH}(\text{NH}_2)\text{COOH}$ Lysine	<i>Neurospora crassa</i>	Only natural forms of amino acids showed effects—limited to mutant strain; inhibition ratio approximately 2:1	Doermann (89)
Norleucine.	Methionine	<i>Proteus morgani</i>	Norvaline and allothreonine also antagonistic but less specific; ratio norleucine:methionine 1000:1 (<i>cf.</i> Harris and Kohn (159))	Porter and Meyers (325)
Asparagine.	β -Alanine*	Yeast	Growth stimulated by pantothenic acid unaffected by asparagine (<i>cf.</i> Nielsen and Hartelius (307))	Sarett and Cheldelin (361)

* Other β -alanine antagonists are included under pantothenic acid.

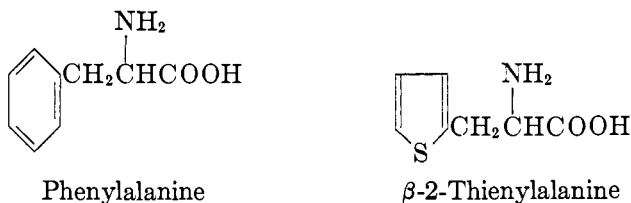
The preparation of ethionine was described by Dyer (96), who showed that it was toxic for rats on a methionine-deficient diet. She also demonstrated that the toxicity could be overcome by additional methionine. In the course of their



studies on sulfonamide inhibitors, Harris and Kohn (159) observed that ethionine inhibited the growth of *E. coli* on a synthetic medium. *M*/1000 ethionine produced a 50 per cent inhibition of growth, which was completely reversed by *M*/10,000 methionine.

Roblin, Lampen, English, Cole, and Vaughan (341) synthesized methoxinine and studied its action on *E. coli* and *Staph. aureus*. The growth-inhibitory effect of this compound was prevented by *l*-methionine but not by the *d*-isomer. One mole of *dl*-methionine reversed the antibacterial action of 500 to 1000 moles of *dl*-methoxinine or *dl*-ethionine. In combination with sulfonamides a synergistic bacteriostatic effect was produced by methoxinine. Similar results were obtained with ethionine, confirming the observations of Harris and Kohn (159). Other investigations involving a relationship between methionine and the sulfonamides are incorporated in the section on PABA antagonists.

An investigation of thienylalanine (469) was carried out by du Vigneaud, McKennis, Simmonds, Dittmer, and Brown (420). They found that the growth



of a strain of yeast, *S. cerevisiae*, was inhibited by thienylalanine. Although growth inhibition was not complete, phenylalanine reversed the maximum effect in a ratio of approximately 1:2. Other amino acids, including tyrosine, were ineffective in preventing the growth-inhibitory action. Preliminary experiments with thienylalanine in rats gave inconclusive results, although some evidence of a phenylalanine antagonism was obtained.

B. *p*-Aminobenzoic acid (PABA)

Because of the unconventional manner in which PABA emerged as a bacterial growth factor, its general acceptance developed slowly. The intensive studies which followed the reports of Woods and Fildes (443, 446; see Section II) have,

for the most part, provided strong supporting evidence for the validity of their suggestions concerning PABA and its relation to the mechanism of action of sulfanilamide-type compounds.

PABA or its derivatives have been isolated from yeast (38, 208, 355) and found to have growth factor activity for *Clostridium acetobutylicum* (213, 316, 355, 356) and several other microorganisms (182, 215, 232). Mutant strains of *Neurospora crassa* (409) and *E. coli* (348) requiring preformed PABA have also been obtained. By means of microbiological assays, the presence of the substance in many bacteria (220) and other natural sources has been demonstrated (170, 311), although it has been pointed out that this method of identification is not conclusive (170). Sulfonamide-resistant strains of some organisms have been shown to possess increased ability to synthesize PABA (bioassay) (221), although this is not the only means by which resistance can develop (183).

Much of the uncertainty regarding PABA has arisen from the conflicting reports of the action of related compounds. A number of these substances show considerable antisulfonamide activity (170, 311). Others, such as *p*-aminophenylacetic acid, have been reported to possess high growth factor activity but no reversing effect on sulfonamides (356). This observation has not been confirmed by other investigators (179, 214, 218, 222, 464). With few exceptions only those compounds which are converted to PABA, either by the organisms or spontaneously, can replace it as a growth factor or antisulfonamide⁴.

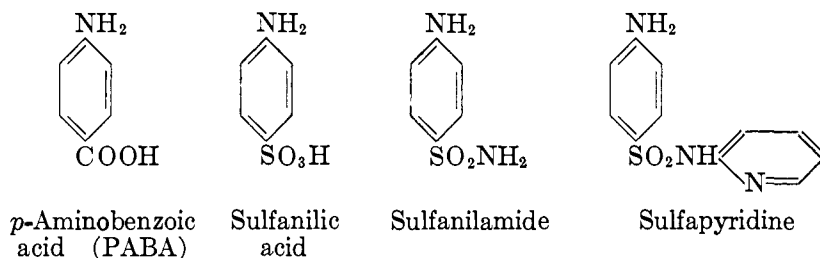
The so-called secondary sulfonamide-reversing agents (201), such as methionine (41, 159, 405) and the purines (160, 203, 280, 385) as well as peptone and other complex substances, represent another factor which has limited the widespread acceptance of the Woods-Fildes theory. However, if PABA represents only one stage in a complex series of metabolic processes, it seems reasonable that supplying precursors or products of subsequent stages may also prevent or limit the growth-inhibiting action of substances antagonizing the utilization of PABA. Moreover, under certain conditions proper supplements may promote the development of alternate routes to the same metabolic end-point and so render microorganisms insensitive to PABA antagonists.

Only some of the most pertinent results are included in this review. A complete and detailed account of the voluminous literature in this field can be found in more extensive reviews and books, such as those of Northey (310, 311) and Henry (170, 171). Since other explanations for the mode of action of sulfonamides are also discussed by these authors, these alternative theories are not included here. Moreover, in the opinion of this reviewer, they fail to explain more than a small fraction of the observations in this field as satisfactorily as the Woods-Fildes theory. It is also evident, however, that no single mechanism of action has yet been devised which will allow all of the multitudinous observations on the sulfonamides to be fitted into prescribed niches.

⁴In assessing results with substances which are active in extremely small quantities, the difficulty of avoiding contamination should be recognized. In one of our laboratories in which PABA was in common use, it was practically impossible to prepare a medium free of this factor. Using the same reagents in another laboratory, however, no difficulty was experienced in preparing a PABA-free medium.

1. PABA antagonists: sulfanilamide derivatives

Woods (443), in his original paper, described the more or less constant ratio (5,000–25,000:1 for *Strep. hemolyticus* or *E. coli*) associated with the reversal of the antibacterial activity of sulfanilamide by PABA. This effect was also observed with the yeast extract (*cf.* Section II). He attributed the ratio to a competition between sulfanilamide and PABA for an enzyme system associated with the utilization of PABA as an essential metabolite. Woods also pointed out the



close structural relationship between metabolite and antagonist. These considerations led him to examine PABA for antisulfonamide activity. Under the test conditions with *Strep. hemolyticus*, sulfapyridine had five times the antibacterial activity of sulfanilamide. PABA also prevented the action of sulfapyridine, although five times as much was required as for an equimolar concentration of sulfanilamide. The weaker growth-inhibitory action of sulfanilic acid was also reversed by PABA. After testing a number of other substances for antisulfonamide activity, Woods concluded that in general both carboxyl and free amino groups para to one another appeared to be necessary. Acetylation of the amino or esterification of the carboxyl groups of PABA resulted in a thousandfold or greater reduction in the antisulfonamide activity. Similar results were obtained with the yeast factor. Summing up the evidence, Woods presented the following hypothesis of the possible mode of action of sulfanilamide:

“In the first place it is suggested that *p*-aminobenzoic acid is essential for the growth of the organism. It is, however, normally synthesized in sufficient quantity by the present strain of streptococcus (and by coli) since it is not necessary to add it to a medium containing only known substances or preparations known to be free from anti-sulfanilamide activity (McIlwain unpublished). It can also be extracted from the streptococcal cell [Stamp (393)]. On the basis of the experimental work it is next suggested that the enzyme reaction involved in the further utilization of *p*-aminobenzoic acid is subject to competitive inhibition by sulfanilamide and that this inhibition is due to a structural relationship between sulfanilamide and *p*-aminobenzoic acid (which is the substrate of the enzyme reaction in question).”

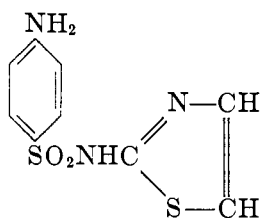
Selbie (368) demonstrated that PABA also prevented the chemotherapeutic action of sulfanilamide in mice infected with streptococci, indicating that the modes of action *in vivo* and *in vitro* are closely related, if not identical. Similar results were obtained by Findlay (126), who also reported that the effect of sulfanilamide on the virus of lymphogranuloma venereum in mice was reversed by PABA. He reasoned that PABA might be considered as an essential meta-

bolite only for those viruses which are susceptible to sulfonamides. Findlay's results with lymphogranuloma were not confirmed by Seeler, Graessle, and Dusen-berg (367). However, other investigators (230, 246, 275, 277, 303, 330) corroborated, in general, the *in vivo* reversal of various sulfanilamide derivatives and sulfones by PABA with a variety of infectious agents.

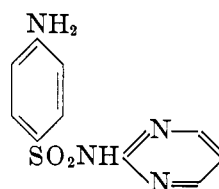
The *in vitro* observations of Woods (443) were confirmed by MacLeod (272), Rubbo and Gillespie (355), and in many subsequent studies. In addition to bac-teria, MacLeod (272) found sulfonamide-reversing agents in many body tissues and other natural sources. Some chemical differences between the naturally occurring substances and PABA, such as the extractability with ether below pH 4.5, were observed. MacLeod also showed that a sulfapyridine-fast strain of pneumococcus produced considerably more antisulfonamide than the parent strain.

Rubbo and Gillespie (355) first demonstrated that PABA is a growth factor for *Clostridium acetobutylicum*. These investigators isolated *p*-benzoylamino-benzoic acid from a benzoylated yeast extract. They also observed a close cor-relation between the growth factor and antisulfonamide activity of a number of related compounds. The molecular inhibition ratio between sulfanilamide and PABA was found to be 23,000:1.

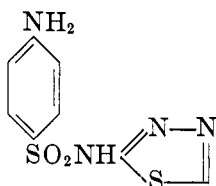
Numerous other investigators (84, 196, 222, 231, 245, 299, 302, 389, 405, 427, 431) extended Woods's observations to additional sulfonamides, sulfoxides, and sulfones, and to many other organisms. Employing pneumococcus type III,



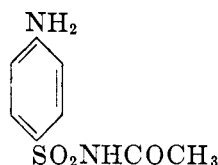
Sulfathiazole



Sulfadiazine



Sulfathiadiazole



Sulfacetimide

Strauss, Lowell, and Finland (405) demonstrated the roughly linear relationship between the concentrations of sulfonamides (sulfanilamide, sulfapyridine, and sulfathiazole) and the minimum concentration of PABA required to prevent their bacteriostatic action (figure 1). They also noted that the concentration of PABA required to reverse the sulfonamides was directly related to their anti-bacterial activity. Thus, under the conditions of the experiment, approximately

ten times as much PABA was required to prevent the action of 10 mg. per 100 cc. of sulfathiazole as was needed for the same concentration of sulfanilamide. Similar results were obtained by Spink and Jermsta (389) with *Staph. aureus* and various sulfonamides (sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine).

A number of sulfonamides, including a series of alkyl thiadiazoles, was investigated by Kimmig (196), who found that in every case their antagonistic action was prevented by PABA. No antisulfonamide activity could be demonstrated in gonococcal cells. Compounds related to PABA, such as the ortho and meta isomers and *p*-nitroaniline, showed no sulfonamide-reversing action.

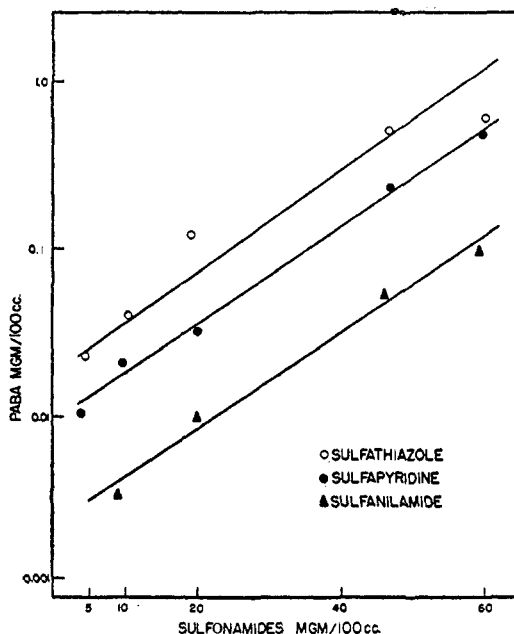
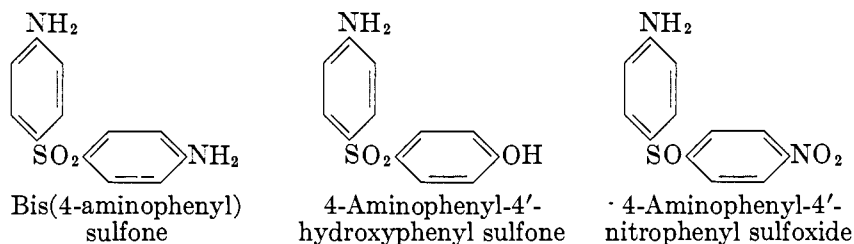


FIG. 1. Minimum concentrations of PABA required to reverse the bacteriostatic action of varying concentrations of three sulfonamides (semi-logarithmic scale). Strauss, Lowell, and Finland (405).

Lwoff, Nitti, Tréfouël, and Hamon (245) studied the action of sulfanilamide on the flagellate *Polytomella caeca*. The primary effect appeared to be on cell division, since the organisms increased to abnormal size but multiplied at a much reduced rate. At pH 4, five times as much sulfanilamide was required to produce the same result as that obtained at pH 7. PABA prevented the action of sulfanilamide, although the metabolite showed an even greater variation with pH. After correction for the change in the activity of sulfanilamide, the inhibition ratio was found to be approximately 1 from pH 9.10 to 7.5. From pH 7.0 to 3.65 the ratio increased to 245, and then declined gradually to 76 at pH 2.25. The maximum ratio corresponded closely with the isoelectric point of PABA. Lwoff and coworkers suggested that these results indicated that the unionized form of the metabolite penetrated the cells much better than the ionized form.

The action of sulfanilamide on *E. coli* and *Aspergillus niger* also varied somewhat with pH, the organisms being less sensitive in acid media. With these organisms, however, the sulfanilamide-PABA ratio (ca. 10,000:1 for *E. coli*) changed only slightly with pH.

The reversing action of PABA on the antibacterial action of sulfones and sulfoxides was investigated by Levaditi and Pérault (231). With *E. coli* and *Staph. aureus*, they found that PABA prevented the action of bis(4-aminophenyl)



sulfone and a number of related sulfones and sulfoxides. The action of antiseptics such as mercuric chloride, hydroxyquinolin, and thioflavine was not affected by PABA.

Bliss and Long (41) called attention to the fact that, in addition to PABA, other simple substances such as methionine also reverse the bacteriostatic action of sulfonamides under certain conditions. Of the ten amino acids tested with *E. coli*, only methionine consistently produced an antisulfonamide effect. With more potent sulfonamides, such as sulfapyridine and sulfathiazole, methionine was effective only against relatively small amounts of the sulfonamides. Even with sulfanilamide the reversal occurred over a relatively small range of concentrations, although very small amounts of methionine ($M/200,000$) prevented the bacteriostatic action of concentrations of sulfanilamide not exceeding $M/1000$. Above this concentration of sulfanilamide no amount of methionine was effective, so that, unlike the PABA relationship, no constant ratio was observed. The authors suggested that methionine might be a precursor of a substance with anti-sulfanilamide activity.

Of the many simple substances tested by Harris and Kohn (159), only methionine in addition to PABA was found to possess antisulfonamide activity. The *l*(-)-isomer was shown to be at least ten times as active as *d*(+)-methionine. In general, their results were in accord with those of Bliss and Long (41). Subsequently, Harris and Kohn (160, 203) reported that under certain conditions guanine and xanthine enhanced the effect of methionine. In the absence of methionine, however, these purines increased the antibacterial activity of sulfanilamide.

Snell and Mitchell (385) found that less PABA was required to reverse the action of sulfanilamide on certain lactic acid bacteria, if one of the purines (adenine, guanine, hypoxanthine, or xanthine) was added to the medium. Methionine was without effect in these experiments when *L. arabinosus* was the test organism. The purines and methionine were capable of at least partially replacing PABA as a growth factor for lactobacilli.

Kohn and Harris (159, 202, 203) extended the concept of Woods and Fildes

(122, 443) to incorporate the secondary reversing agents, such as methionine and the peptones. From quantitative data on *E. coli* obtained in both simple and complex media, they demonstrated that the size of the inoculum had no real effect on the bacteriostatic action of the sulfonamides. At normal bacteriostatic levels the primary inhibition appeared to be directed against growth rather than respiration. In synthetic media there was a simple relationship between rate of growth and sulfonamide concentration. The relationship in a proteose-peptone medium was more complex. These observations suggested that the sulfonamides interfere with the synthesis of substances necessary for growth. Kohn and Harris assumed that among these substances was a special group X requiring PABA as a catalyst. X was considered to represent a class of substances some of which were present in the proteose-peptone medium. Thus, low concentrations of sulfonamides were ineffective in this medium but higher concentrations produced bacteriostasis by interfering with additional syntheses. Methionine was assumed to act as a reversing agent, because the sulfonamides prevented its synthesis. The synthesis of methionine in turn was assumed to be dependent on the presence of PABA.

The competitive nature of the relationship between sulfonamides and PABA was investigated quantitatively by Wyss (461) and Wood (441). By mathematical analysis of his data, Wyss demonstrated that sulfanilamide and PABA appeared to compete for the same receptor site in the organism, the great disproportion between the molar concentrations of the metabolite and the antagonist merely indicating the greater affinity for PABA.

Using a different mathematical treatment, Wood (441) arrived at the same conclusions for sulfapyridine, sulfathiazole, sulfadiazine, sulfaguanidine, and bis(4-aminophenyl) sulfone as well as sulfanilamide. He also suggested that the variation in the antibacterial activity of the different compounds might be due to the difference in their affinity for the enzyme associated with PABA.

Ivanovics (183) examined the relation of drug resistance to the production of antisulfonamide substances. As indicated in table 3, he found that some resistant strains of *Staph. aureus* produced more sulfonamide-reversing agent than the parent strain. In other cases no marked differences could be detected. Where the differences were small, however, the inhibition ratio of sulfonamide to PABA changed considerably. Thus, while one molecule of PABA reversed the antibacterial action of only 14 molecules of sulfamethylthiazole with parent strain B, 220 molecules of the sulfonamide were reversed with the resistant strain B. On the basis of these observations, Ivanovics suggested that sulfonamide resistance could result either from an increased production of PABA (or a related substance), or by an increased efficiency in the utilization of PABA. In some instances, e.g., strain VI, a combination of both mechanisms might be involved.

In studying the bacteriostatic effect of sulfanilamide and four of its derivatives (sulfaguanidine, sulfapyridine, sulfathiazole, and sulfadiazine) on *E. coli*, Rose and Fox (350) observed that a constant concentration of PABA was required to reverse the minimum effective concentration (M.E.C.) of the various sulfonam-

ides. Thus, although the M.E.C. of the different derivatives varied over 500-fold (i.e., from $2.5 \times 10^{-3}M$ for sulfanilamide to $4 \times 10^{-6}M$ for sulfathiazole and sulfadiazine), the same minimum concentration of PABA ($5 \times 10^{-7}M$) was required to prevent the bacteriostatic action of each at its M.E.C. Rose and Fox also found that the relationship of PABA to sulfathiazole was independent of the number of bacteria. The organisms appeared to undergo a definite limited num-

TABLE 3
*Relationship of PABA to sulfamethylthiazole with various strains of Staph. aureus**

<i>Staph. STRAIN</i>	TITER†	INHIBITION RATIO‡	ANTISULFONAMIDE ACTIVITY OF EXTRACT§
B (parent).....	M/323,000	14	--
B (resistant).....	M/10,000	220	--
VI (parent).....	M/550,000	7	--
VI (resistant).....	M/15,000	133	±
IX (parent).....	M/480,000	31	
IX (resistant).....	M/21,400	66	
X (parent).....	M/60,000	21	--
X (resistant).....	M/8,300	35	++

* Adapted from Ivanovics (183).

† Minimum effective concentration of sulfamethylthiazole.

‡ Moles of sulfamethylthiazole ($M/1000$) reversed by one mole of PABA.

§ -- = absence of detectable amounts of PABA or other antisulfonamide; ± and ++ = relative antisulfonamide activity of extracts from various strains.

TABLE 4
Relationship of PABA to sulfanilamide (SA) and sulfathiazole (ST) with various organisms (463)

ORGANISM	MOLES SA/PABA	MOLES ST/PABA	EFFICIENCY ST/SA
<i>E. coli</i>	2,000	27	74
<i>A. aerogenes</i>	3,220	45	72
<i>Staph. aureus</i>	4,660	53	88
<i>Ps. aeruginosa</i>	13,330	184	73
<i>Sal. typhimurium</i>	6,650	92	72
<i>L. acidophilus</i>	8,000	133	60
<i>P. vulgaris</i>	4,000	55	73

ber (six to seven) of cell divisions, regardless of the size of the original inoculum. They attributed these results to the inability of the organism to synthesize a substance, possibly involving PABA, and necessary for normal reproduction. The presence of the sulfonamide was assumed to prevent this synthesis so that, after a certain number of cell divisions, the concentration per individual cell was insufficient to permit further reproduction.

Wyss, Grubaugh, and Schmelkes (463) examined the specificity of various sulfonamides for different bacteria. These investigators found no indication

that the relative effectiveness of sulfanilamide and sulfathiazole changed from one organism to another (table 4). Similar results were obtained when other sulfonamides were compared.

Fox and Rose (130) and Schmelkes, Wyss, Marks, Ludwig, and Strandkov (364) pointed out a relationship between the degree of dissociation of sulfonamides and their antibacterial activity. With four sulfonamides, Fox and Rose demonstrated that while the molar ratio of PABA to sulfonamide varied over

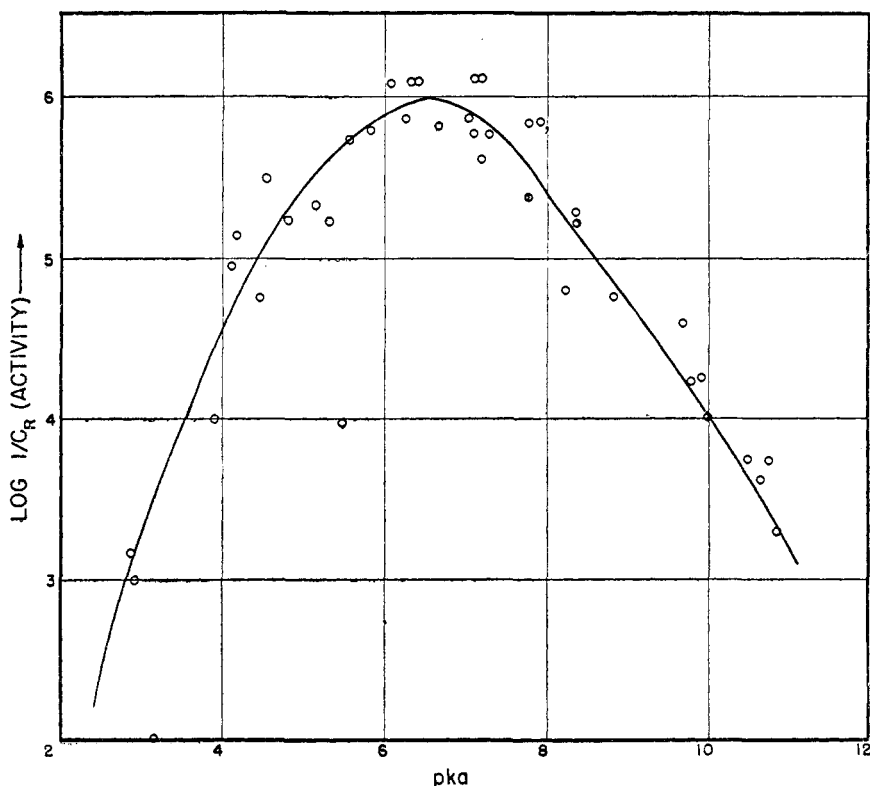


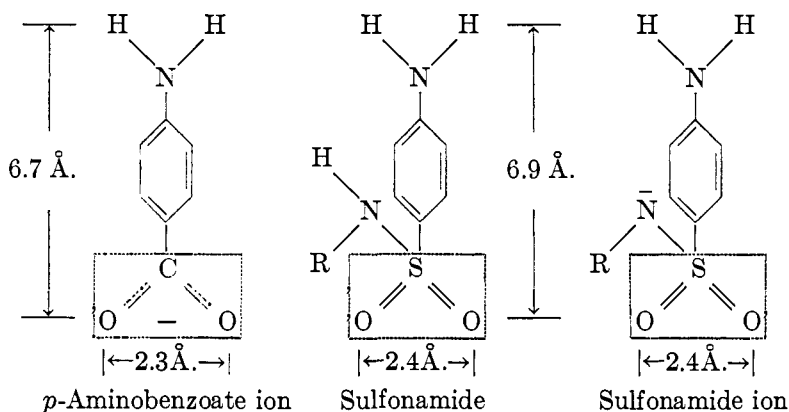
FIG. 2. The relationship between bacteriostatic action and acidity of N^1 -substituted sulfanilamides. Bell and Roblin (32).

600-fold, the ionic ratios were 1.4 for sulfanilamide and 1.4, 4.9, and 6.4 for sulfapyridine, sulfathiazole, and sulfadiazine, respectively. At pH 7.8 sulfanilamide was found to be about ten times as active as at pH 6.8, corresponding to the increase in the ionic form at the higher pH level. These observations led to the suggestion that only the ionic form of the sulfonamides possessed antibacterial activity. Similar results were obtained by Schmelkes *et al.* They also pointed out the relative activity of sulfonamides at different pH levels and concluded that the anionic form was the effective agent, since it might be expected to compete with PABA ions on a more favorable basis.

For several sulfonamides, Cowles (78) and Brueckner (51) found that antibacterial activity was at a maximum when the sulfonamide was approximately

50 per cent ionized. Cowles suggested that, like a number of other substances, the sulfonamides might penetrate the bacterial cell effectively only in the unionized form. Once inside the cell, the ionized form was considered to be responsible for the antibacterial action. Thus, the maximum activity of any sulfonamide would be expected to fall near the point at which $pK_a = \text{pH}$ of the culture medium. At the point of 50 per cent ionization, all sulfonamides were regarded as essentially equally active. With the exception of sulfaguandine⁵, Cowles's experimental data appeared to be consistent with these views⁶. Brueckner confirmed Cowles's results and the earlier observations of Lwoff *et al.* (245), indicating that the efficiency of PABA in reversing sulfonamides decreases with increasing alkalinity of the culture medium in the range pH 6.0–8.9. He pointed out that this effect would explain the observation that the sulfonamide–PABA ratios decreased in this pH range for weakly acidic sulfonamides but remained relatively constant over most of the range for the more strongly acidic derivatives.

Bell and Roblin (32, 340) proposed a theory which related the structure of sulfanilamide-type compounds to their *in vitro* antibacterial activity. This theory was based on the experimental observation that when the acid dissociation constants of *N*¹-substituted sulfanilamide derivatives were plotted against activity a smooth curve was obtained which passed through a maximum as the acid strength increased (figure 2). The numerical values for some representative sulfonamides are recorded in table 5. It was suggested that if the bacteriostatic action of sulfonamides is due to a competition with PABA, then the more closely the competitor compound resembled the metabolite, the greater should be its blocking or bacteriostatic effect. The authors pointed out the similarity in the geometric configuration of sulfonamides and of *p*-aminobenzoate ion, as indicated:



⁵The values for sulfaguandine reported by Cowles ($pK_a = 11.2$) and Brueckner ($pK_a = 10$) do not appear to be correct (32). The acid strength is probably considerably weaker than these values indicate.

⁶While the experimental observations of Cowles (78) and Brueckner (51) are in accord with the idea that all sulfonamide ions are essentially equally active, the results of Fox and Rose (130) and Schmelkes *et al.* (364) indicate that as pK_a decreases the ratio PABA/sulfonamide ion also decreases (32, 340).

At pH 7, PABA exists almost exclusively in the dissociated form carrying a formal ionic charge. Since the CO_2 ion is a strong negative group, the more negative the SO_2 group, the more closely it should resemble the CO_2 ion. The theory was stated as follows: *The more negative the SO_2 group of an N^1 -substituted sulfanilamide derivative, the greater the bacteriostatic activity of the compound.* Bell and Roblin showed that the acid constants provided an indirect measure of the negative character of the SO_2 group. They demonstrated that, although the SO_2 group in the ionized form is more negative, the negative character of both forms decreases with decreasing $\text{p}K_a$. The net result of these two opposing factors resulted in a maximum in the experimental curve (figure 2). The theory was also

TABLE 5

Dissociation constants and bacteriostatic activity of sulfanilamide-type compounds (32)

COMPOUND	$\text{p}K_a^*$	K_a	$C_R(M \times 10^3)^\dagger$
N^1 -Methylsulfanilamide.....	10.77	1.7×10^{-11}	30
Sulfanilamide.....	10.43	3.7×10^{-11}	20
N^1 - <i>o</i> -Tolylsulfanilamide.....	9.96	1.1×10^{-10}	10
N^1 -Phenylsulfanilamide.....	9.60	2.5×10^{-10}	3.0
5-Sulfanilamido-2-aminopyridine.....	8.82	1.5×10^{-9}	2.0
Sulfapyridine.....	8.43	3.7×10^{-9}	0.6
3-Sulfanilamidopyridine.....	7.89	1.3×10^{-8}	0.2
Sulfathiazole.....	7.12	7.6×10^{-8}	0.08
Sulfadiazine.....	6.48	3.3×10^{-7}	0.08
Sulfapyrazine.....	6.04	9.1×10^{-7}	0.08
Sulfamethylthiadiazole.....	5.45	3.5×10^{-6}	0.2
Sulfanilylurea.....	5.42	3.8×10^{-6}	10
Sulfacetimide.....	5.38	4.2×10^{-6}	0.7
Sulfathiadiazole.....	4.77	1.7×10^{-5}	0.6
3-Sulfanilamido-4-methylfuran.....	4.10	7.9×10^{-5}	1.0
N^1 -Chloroacetylsulfanilamide.....	3.79	1.6×10^{-4}	10
N^1 -Ethylsulfonylsulfanilamide.....	3.10	7.9×10^{-4}	1000
Sulfanilylcyamide.....	2.92	1.2×10^{-3}	100

* $\text{p}K_a = \text{Log } 1/K_a$.

† C_R = minimum effective molar concentration for complete bacteriostasis of *E. coli* in synthetic medium buffered at pH 7.

developed mathematically and a theoretical curve obtained which agreed very well with the experimental results over a large part of the pH range. In general, from the known relative electronegative character of the N^1 -substituents, it was possible to predict the *in vitro* activity of new sulfanilamide derivatives of this type. The theory also implied that the optimum in bacteriostatic action of sulfanilamide-type compounds appeared to have been reached.

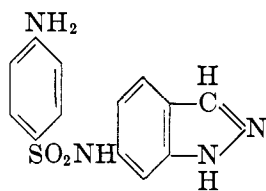
Klotz (197) applied the law of mass action to a mathematical analysis of equilibria between PABA-enzyme complex and sulfonamide-enzyme complex with the dissociated forms of the metabolite and antagonists. Utilizing the data of Bell and Roblin (32), he arrived at similar conclusions regarding the optimum activity and the effect of changes in the pH of the culture medium. From quantitative

studies with *E. coli*, Klotz and Gutmann (198) calculated the dissociation constants of the theoretical enzyme-sulfonamide complex for several derivatives. They considered that the deviations between predicted and observed bacteriostatic activities for sulfonamides of low pK_a might be due to extensive binding of the ionized forms of the compounds to extraneous bacterial proteins.

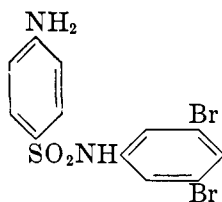
Although PABA usually produces a complete reversal of the growth-inhibiting action of practically all sulfonamides with a wide variety of microorganisms, several cases have been reported which constitute exceptions to this generalization. Thus, Feldt (119) and Hawking (167) demonstrated that the protective action of sulfapyridine and sulfathiazole on treponema infections in mice was not reversed by PABA. A number of other sulfonamides were ineffective as therapeutic agents. Hawking concluded that the effect of sulfapyridine and sulfathiazole on these infections was atypical.

Tamura (408) found that PABA failed to prevent the *in vitro* antibacterial action of sulfathiazole, sulfadiazine, or sulfapyrazine for *Bacterium tularensis*. In fact, PABA itself inhibited the growth of this organism in concentrations exceeding $M/10,000$. Although lower concentrations did not produce a reversal, the usual action of PABA was observed with the same sulfonamides in the same medium when *E. coli* or *P. pestis* were the test organisms.

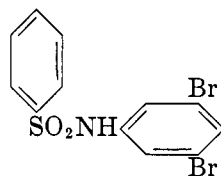
During the study of a group of isomeric sulfanilamidindazoles (212), Lawrence and Goetchius (223) found that PABA only partially reversed the antibacterial action of these sulfonamides on *Brucella melitensis*. There was over a thousand-



6-Sulfanilamidoindazole



Sulfanilyl-3,5-dibromoanilide

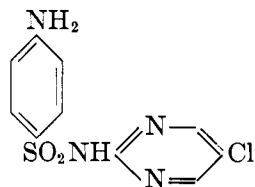


Benzenesulfon-3,5-dibromoanilide

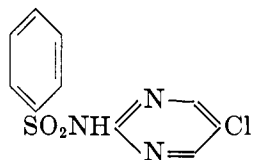
fold decrease in the effect of PABA on these sulfonamides compared with sulfathiazole. Similarly, Goetchius and Lawrence (144) reported that the antibacterial activity of at least two members (sulfanilyl-3,5-dibromoanilide and benzenesulfon-3,5-dibromoanilide) of a series of halogen-substituted sulfonamides (193) was not appreciably affected by PABA⁷.

Brackett, Waletzky, and Baker (48) demonstrated that the antimalarial activity of 2-sulfanilamido-5-chloropyrimidine and of the corresponding bromo compound (109, 344) was only partially prevented by PABA, while the lesser activity of 2-benzenesulfonamido-5-chloropyrimidine was entirely unaffected by

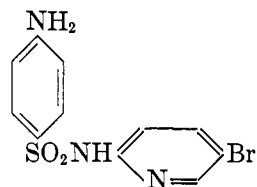
⁷White (432) found that these results require qualification, since the antibacterial action of sulfanilyl-3,5-dichloroanilide and the corresponding bromo derivative against certain Gram-negative organisms is completely reversed by PABA. The failure of PABA to prevent the growth inhibition caused by these compounds appears to be limited primarily to Gram-positive species.



2-Sulfanilamido-5-chloropyrimidine



2-Benzenesulfonamido-5-chloropyrimidine



2-Sulfanilamido-5-bromopyrimidine

this metabolite. On the other hand, PABA completely reversed the activity of other heterocyclic halogen compounds such as pyridine (343) and thiazole (109) derivatives. The authors concluded that two modes of action might be involved: one attributed to the anti-PABA effect of the sulfanilyl group, the other to the sulfonamidochloropyrimidine.

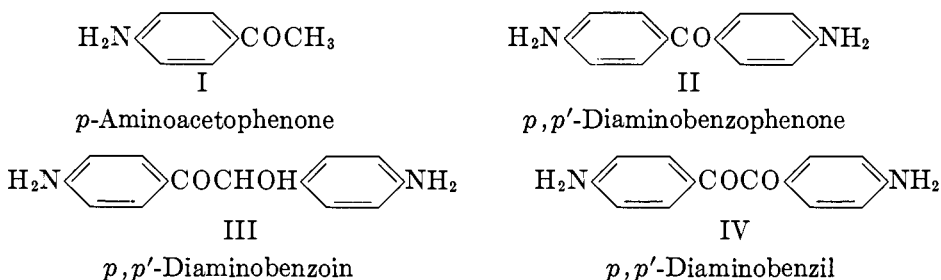
2. Other PABA antagonists

In addition to the sulfanilamides and other sulfur derivatives, a number of sulfur-free compounds chemically related to PABA have been found to inhibit the growth of microorganisms. The metabolite also reverses the antibacterial action of these compounds.

During a study of the action of sodium *p*-nitrobenzoate on a strain of *Strep. viridans*, Miller (292) observed that small concentrations (10–100 mg./100 cc.) of this substance were bacteriostatic, while larger amounts (200–400 mg./100 cc.) produced little or no effect. He suggested that part of the *p*-nitrobenzoate might be reduced to PABA by the organisms, and showed that the amount of diazotizable amine formed was proportional to the concentration of nitro compound. A filtrate from organisms grown in the presence of *p*-nitrobenzoate prevented the bacteriostatic action of sulfathiazole, whereas a similar filtrate lacking the nitro derivative did not (293). Miller (292) also demonstrated that the antibacterial activity of *p*-nitrobenzoate could be prevented by small amounts of PABA. This reversing action was confirmed by Ivanovics (184) with *Staph. aureus*, but he was unable to eliminate the bacteriostatic action of the nitro compound with PABA when *E. coli* was the test organism.

Kuhn, Möller, Wendt, and Beinert (206, 207) investigated the effects of a number of *p*-aminophenyl derivatives on *Streptobacterium plantarum*, an organism which requires preformed PABA for growth in a synthetic medium. Several derivatives could, to some extent, replace PABA, while *p*-aminobenzamide was either a growth factor or an inhibitor depending on the concentration. Hirsch (175) also found that the growth of *E. coli*, inhibited by *p*-aminobenzamide, could be restored by PABA. The corresponding thioamide was a growth inhibitor, but Kuhn *et al.* (207) concluded that the thioamide competed with the organisms for iron, since its bacteriostatic effect could be reversed by iron salts but not by PABA. Some of the results obtained by Kuhn and his associates are summarized in table 6.

It is evident that there are marked differences in the bacteriostatic activity of the various ketones. As the authors pointed out (207), the introduction of a



second carbonyl group (*cf.* II and IV) produces a thirtyfold increase in effectiveness. Over a short period of time III was also bacteriostatic. However, the activity dropped off rapidly with time, and Kuhn *et al.* (207) suggested that a "self-inhibition" caused by the formation of PABA might occur. This effect

TABLE 6

Relative activity of some p-aminophenyl derivatives as PABA antagonists (206)

CONCENTRATION FOR 50 PER CENT INHIBITION	COMPOUND	REVERSAL
<i>mg./100 cc.</i>		<i>moles per mole PABA</i>
150	2-(<i>p</i> -Aminobenzamido) pyridine	48,000
30	Phosphanilic acid	12,000
25	<i>p</i> -Aminoacetophenone	12,700
10	Sulfanilic acid	5,000
7	<i>p</i> -Nitroacetophenone	2,900
5	<i>p,p'</i> -Diaminobenzophenone	1,600
0.3	Sulfanilamide	150
0.15	<i>p,p'</i> -Diaminobenzil	50*
0.1	<i>p,p'</i> -Diaminodiphenyl sulfone	28

* Approximate value from reference 207.

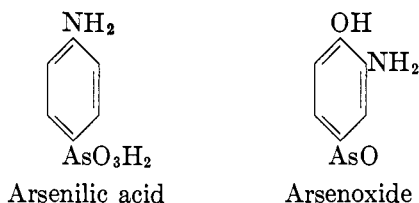
is similar to the results obtained by Miller (292), and analogous to the formation of benzoic acid by the autooxidation of benzoin in alkaline solution.

Auhagen (21) corroborated the results of Kuhn and coworkers with I and found that *p*-aminobenzophenone was also a growth inhibitor. From the PABA reversal ratios the latter appeared to be the less active. He reported that II was slightly effective in the treatment of experimental streptococcal infections in mice.

Green and Bielschowski (149) reported that the antibacterial action of 4,4'-diaminodiphenyl diselenide was partially reversed by PABA. *p*-Aminophenyl-seleninic and selenonic acids appeared to be too unstable to be prepared in sufficient purity for testing. Under certain conditions, PABA completely prevented the growth inhibition produced by 4,4'-diaminodiphenyl disulfide, but in some respects this substance seemed to differ from the sulfonamides. The authors concluded that the disulfide might act by two mechanisms, one of which was related to PABA metabolism.

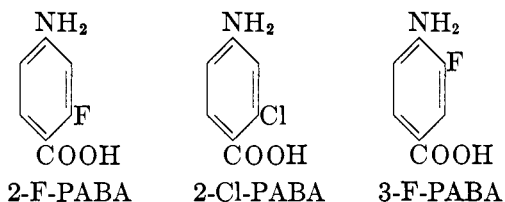
Arsenilic acid was studied by Hirsch (175), who reported that it reduced the speed of growth as measured by oxygen consumption. PABA counteracted this

effect. Later, Peters (319) showed that $M/1000$ arsenilic acid was bacteriostatic for *E. coli*, and that this action could be prevented by $M/10,000,000$ PABA. Methionine also reversed the activity of arsenilic acid, as it does in the case of the sulfonamides. Peters found that PABA did not prevent the bacteriostatic action of arsenoxide, nor the trypanocidal action of either arsenilic acid or arsen-



oxide. He concluded that the trypanocidal action of arsenilic acid was exerted through the arsenoxide by a different mechanism from the bacteriostatic effect (*cf.* Section III, D).

A series of nuclear-substituted *p*-aminobenzoic acids prepared by Schmelkes and Rubin (363) was investigated by Wyss, Rubin, and Strandkov (464). 2-Fluoro-4-aminobenzoic acid (2-F-PABA) was found to be one-third as effective as PABA in growth factor tests with *Clostridium acetobutylicum*. This same sub-



stance reversed the bacteriostatic activity of sulfanilamide when *E. coli* was the test organism. In contrast to the 2-fluoro derivative, 2-Cl-PABA inhibited growth, and this inhibition was prevented by PABA or methionine. Similarly, the bacteriostatic action of 3-F-PABA was reversed by PABA. A number of other halogen-, amino-, and carboxy-substituted PABA derivatives were studied by Wyss and coworkers. 2-Cl-PABA appeared to be the most potent antibacterial agent, although it showed very little antipneumococcal activity in mice. The growth factor and antisulfonamide action of several other derivatives such as 2-Br-PABA was probably due to the presence of traces of PABA as a contaminant (39). However, it is unlikely that the high growth factor activity of 2-F-PABA could be explained on this basis.

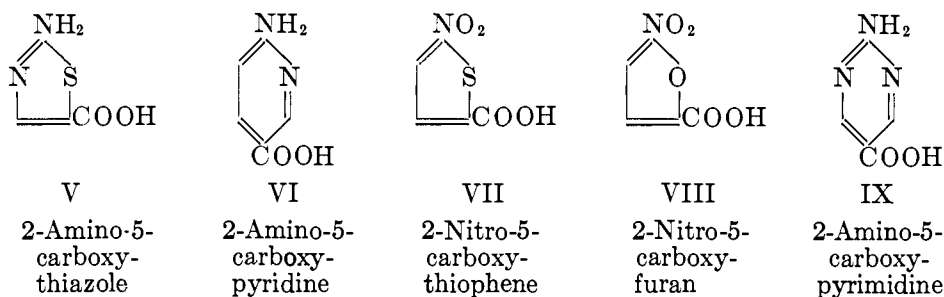
Results with a number of PABA derivatives were described by Johnson, Green, and Pauli (191). These investigators found that nuclear methyl and methoxy substituents resulted in compounds with bacteriostatic activity which PABA reversed. In general, they confirmed the observations of Wyss *et al.* (464) with other nuclear substituents, although they reported that 2-Cl-PABA exhibited antisulfonamide action with some microorganisms⁸. The effects of modifications

⁸The possibility that traces of PABA may have been formed in the synthesis of this compound does not appear to have been excluded.

of PABA in the benzene series were summarized as follows: (1) Monosubstitution by neutral or weakly electropositive groups yielded compounds with bacteriostatic properties. (2) Disubstitution in general resulted in compounds exhibiting no antibacterial action. (3) Replacement of the amino group, with the exception of the nitro group, produced inactive compounds. (4) Variation in the carboxyl gave antisulfonamide, bacteriostatic, or no activity. (5) Simultaneous modification of amino group and nuclear substitution, or changing both the amino and the carboxyl groups, yielded inactive compounds.

A similar group of nuclear-substituted derivatives, and of other compounds related to PABA, was investigated by Martin and Rose (278). Of the thirty-three compounds studied, 3-Cl-PABA and 3-HO-PABA had the highest anti-streptococcal activity. This activity was considerably reduced but not completely abolished by PABA. In addition, 2-methyl-PABA and 2-Cl-PABA were reported to have antisulfanilamide activity, while the 3-methyl derivative was inert. A slight chemotherapeutic effect in mice infected with streptococci or pneumococci was demonstrated for 3-HO-PABA.

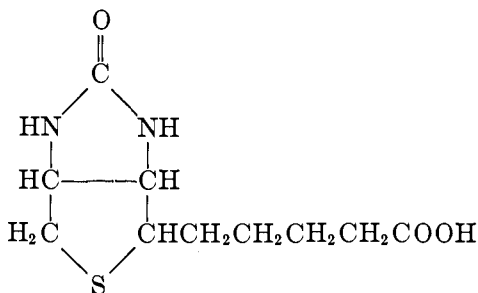
Johnson and associates (191) also studied a group of heterocyclic analogs of PABA. Compound V was inactive, but VI was bacteriostatic for *E. coli* and streptococci in approximately the same concentrations as sulfapyridine. VII



and its amide were active, whereas the corresponding furan derivative, VIII, showed no antibacterial action. The action of all the active heterocycles was reversed by appropriate concentrations of PABA. With *Strep. pyogenes*, Martin, Rose, and Swain (279) found IX to be without antibacterial activity, but it prevented the action of sulfanilamide on the same organism. PABA was approximately 2000 times as effective in this respect.

C. Biotin

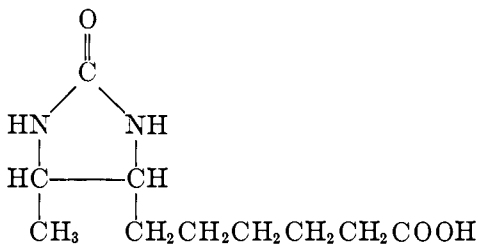
Although biotin as a constituent of "bios" was long recognized as a yeast growth factor, its identity with the anti-egg-white-injury factor (vitamin H) (152), its chemical constitution (419), and its synthesis (161, 162) are more recent developments. Alternative formulas have been proposed for biotin from various sources (200), but the total synthesis has established the structure of liver biotin as follows:



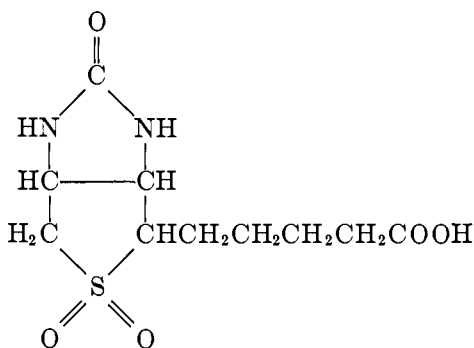
Biotin

The substance in egg white responsible for biotin deficiency has been shown to be a protein (98), to which the graphic name avidin has been assigned. This material forms a stable complex from which biotin cannot be readily dissociated. By the use of avidin it has been demonstrated that many bacteria probably require preformed biotin for growth (219).

Desthiobiotin was first obtained from biotin by cleavage with Raney nickel (421), although the total synthesis of the racemic derivative has also been described (440). *d*-Desthiobiotin was reported (286) to replace biotin for a strain of yeast but not for *Lactobacillus casei*. Subsequently, Dittmer, Melville, and



Desthiobiotin

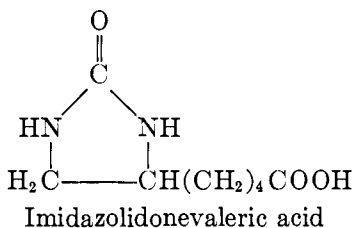
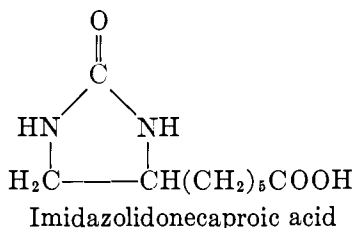


Biotin sulfone

du Vigneaud (87) found that the yeast strain appeared to synthesize biotin from the desthio derivative. These investigators and Lilly and Leonian (233) also found that desthiobiotin inhibited the utilization of biotin by *L. casei*. Dittmer and coworkers showed that growth due to 0.82×10^{-10} molar biotin was reduced

to one-half of this value by 2.3×10^{-6} molar *d*-desthiobiotin. This growth inhibition was completely reversed by increasing the concentration of biotin to 4.1×10^{-10} molar. The results obtained by Lilly and Leonian were similar over a wide range of concentrations when *L. casei* was employed as the test organism. A number of other biotin-requiring organisms, most of which were found to utilize desthiobiotin as a growth factor, were also studied (233). In only one or two other cases did desthiobiotin exhibit antibiobiotin activity.

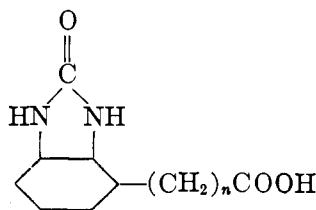
Dittmer and du Vigneaud (85) investigated the sulfone of biotin, which had been shown to possess slight biotin activity for a strain of yeast (86). With *L. casei*, *L. arabinosus*, and *Staph. aureus*, however, biotin sulfone inhibited growth. The antibiobiotin activity of this compound for *L. casei* was such that the molar inhibition ratio (moles of antagonist required to reduce growth stimulated by one mole of biotin to one-half of normal) was approximately 280. The growth inhibition was completely reversed by the addition of more biotin. Two analogues of desthiobiotin, 4-(imidazolidone-2)caproic acid and 4-(imidazolidone-2)valeric acid, were also prepared by Dittmer and du Vigneaud (85).



The caproic acid derivative was found to have antibiobiotin activity for both *L. casei* and a yeast, *S. cerevisiae*. The molar inhibition ratio, which was 9,100 for *d*-desthiobiotin with *L. casei*, was 126,000 with this organism for imidazolidonecaproic acid. In contrast to this result, imidazolidonevaleric acid stimulated the growth of yeast slightly, but it neither stimulated nor inhibited *L. casei*.

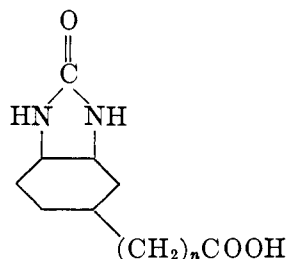
Rubin, Dreker, and Moyer (357) found that *dl*-desthiobiotin had about one-half the growth-inhibitory activity of the *d*-isomer for *L. casei*, suggesting that the *l*-desthiobiotin derivative was without antibiobiotin activity. The oxygen analogue of biotin, "oxybiotin" (177), also reversed the antibiobiotin activity of *dl*-desthiobiotin (358). In this instance, the inhibition ratio for half normal growth was 1200, as compared with 17,000 when biotin itself was the reversing agent.

A series of ureylenecyclohexane derivatives was synthesized by English, Clapp, Cole, Halverstadt, Lampen, and Roblin (108). In the type I series the cyclohexylvaleric acid derivative differed from biotin only in that the sulfur atom was replaced by two methylene groups, while in the type II compounds the side chain also was in a different position with respect to the urea ring. With few exceptions these compounds possessed antibiobiotin activity for both *L. casei* and yeast. With the former organism, γ -(3,4-ureylenecyclohexyl)butyric acid was the most active, 6×10^{-7} M producing a 50 per cent inhibition of growth. On the other hand, δ -(2,3-ureylenecyclohexyl)valeric acid was 100 times more effective as a growth inhibitor for yeast than the 3,4-butyric acid derivative. The molar inhibition



I

ω -(2,3-Ureylencyclohexyl)-
butyric or valeric acid



II

ω -(3,4-Ureylencyclohexyl)-
butyric or valeric acid

ratio in this case was 1500 for one-half normal growth. In all instances the growth-inhibitory action was completely reversed by appropriate concentrations

TABLE 7

Relative antibiotin activity of ureylenebenzene and ureylencyclohexane derivatives†*

TYPE	<i>n</i>	CARBOCYCLIC RING	<i>L. casei</i>	YEAST
I.....	3	Phenyl	1	20
I.....	3	Cyclohexyl	4	4000
I.....	4	Phenyl	4	2.5
I.....	4	Cyclohexyl	800	2000
II.....	3	Phenyl	16	1
II.....	3	Cyclohexyl	6000	40
II.....	4	Phenyl	32	4
II.....	4	Cyclohexyl	800	40

* Arbitrary values based on least active derivative = 1 for each organism.

† Adapted from English *et al.* (108).

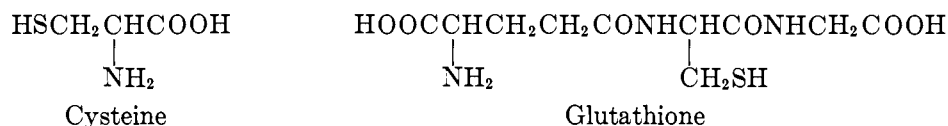
of biotin. Geometrical isomers of the type I analogues were separated, but there appeared to be no significant difference in antibiotin activity between them. The analogous ureylenebenzene derivatives were also studied by English *et al.* The antibiotin activity of these compounds was considerably lower than that of the corresponding derivatives in the cyclohexane series. None of the products produced any growth stimulation with either of the organisms studied. The relative growth-inhibitory activity of a number of the compounds is summarized in table 7.

D. Glutathione and other mercapto compounds

Enzyme proteins very often contain mercapto groups which are essential for enzymatic activity. Compounds of arsenic or selenium, the heavy metals, oxidizing agents, reactive alkyl halides such as iodoacetic acid (in short, most substances which react readily with —SH groups) have been employed to determine their presence in and necessity for the action of isolated enzymes. Several excellent reviews are available on this subject (34, 35, 168, 169). It is evident

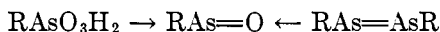
from these, and from more recent studies (28, 29, 379), that numerous enzymic systems involved in carbohydrate, nitrogen, and fat metabolism contain essential mercapto groups.

Simpler metabolites such as cysteine and glutathione (γ -glutamylcysteinylglycine) also owe many of their biochemical properties to the presence of a mercapto

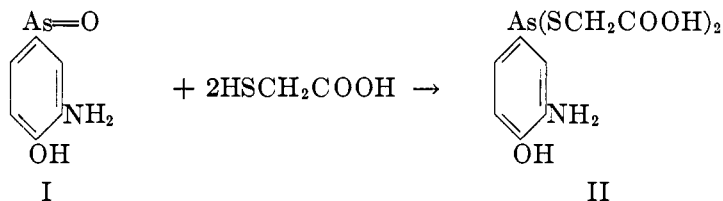


group. In fact, it has been suggested that the primary function of glutathione in cellular systems is the continuous reactivation of $-\text{SH}$ enzymes. Glutathione is itself a coenzyme for the coglyoxalase enzyme system (406), although other similar substances, such as isoglutathione (α -glutamylcysteinylglycine) or asparthione (β -aspartylcysteinylglycine), can also serve in its place (31). Many microorganisms require an outside source of potential organic mercapto groups, and glutathione has been shown to be a growth factor for variant strains of the gonococcus (146).

Ehrlich (110) first suggested in 1909 the possibility that the toxic action of arsenicals might be due to their affinity for mercapto groups. About the same time, Chick (65) demonstrated that the action of mercuric chloride on bacteria was reversed by excess hydrogen sulfide. It was not until 1923, however, that the importance of $-\text{SH}$ compounds in relation to the trypanocidal action of arsenicals was established experimentally by Voegtlin, Dyer, and Leonard (423). The basis for their work was the concept, now generally accepted, that arsenicals are directly toxic only in the arsenoxide form. Compounds in other stages of oxidation were presumed to be converted to arsenoxides before exerting trypanocidal action, i.e.,

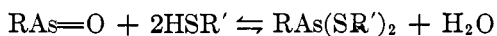


Voegtlin *et al.* (423) applied the known reactivity of arsenoxides with hydrogen sulfide to 3-amino-4-hydroxyphenylarsenoxide (arsenoxide) and mercaptoacetic acid, which were shown to react as follows:



Trypanosomes and other cells with an active metabolism were found to contain $-\text{SH}$ groups, as indicated by the characteristic nitroprusside test. *In vitro* tests demonstrated that, whereas $M/20,000$ arsenoxide (I) killed trypanosomes in less than 15 min., the organisms retained their motility for a period comparable with the controls in $M/200$ arsenoxide together with $M/20$ mercaptoacetic acid.

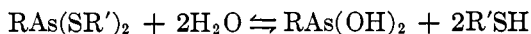
Cysteine, glutathione, and other mercapto derivatives also prevented the toxicity of I, but a number of other amino acids not containing sulfur were without effect. The disulfides of the active —SH compounds were either much less effective or devoid of reversing action. Experiments carried out *in vivo* with infected rats gave similar results. In this case, a ratio of mercapto compound to I of about 100:1 was required to abolish the action of the latter. A considerable lag in therapeutic effect was observed in tests with the thioarsenite (II). The reversible nature of the reaction



was suggested as an explanation for these observations. Voegtlin and coworkers also demonstrated that the mercapto compounds were readily oxidized to the less active disulfides under their experimental conditions. This factor would also increase the amount of antagonist required.

Rosenthal and Voegtlin (352) found that glutathione protected rats from an otherwise lethal dose of arsenoxide. They also demonstrated that trypanosomes exposed to arsenoxide plus glutathione for an hour retained their infectivity for animals as well as their motility, whereas with arsenoxide alone the organisms lost both infectivity and motility after 5 min. exposure. Similar results were obtained by Reiner and Leonard (335) with sodium mercaptoacetate.

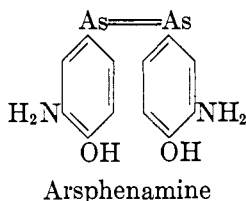
The trypanocidal action of several thioarsenites synthesized by Cohen, King, and Strangeways (72, 73) was investigated by Strangeways (403). The thioarsenites, prepared from aromatic arsenoxides and glutathione or cysteine, were equally as toxic as the parent arsenoxides. Dilutions as high as 1:25,000,000 killed trypanosomes in 6 hr. This activity was attributed to the hydrolysis of the thioarsenites, which had been shown to be favored by alkali (72). Strangeways (403) pointed out that dilution would also favor the right-hand side of the equation,



whereas excess mercapto compound should shift the equilibrium to the left. Thus, an excess of glutathione had no preventive effect on the trypanocidal action of high dilutions (1:1,000,000) of either the arsenoxides or thioarsenites, even when 2000 moles of glutathione was employed per mole of arsenical. When the concentration of the arsenicals was increased to $M/1000$ or greater, however, complete reversal was observed at molar ratios of only 10:1. The trypanocidal action of tartar emetic and acriflavine was not affected by glutathione or cysteine.

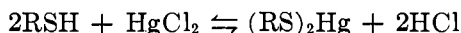
The reaction of glutathione and cysteine with copper sulfate and auric chloride was examined by Voegtlin, Johnson, and Dyer (424). In addition to the formation of copper and gold derivatives, part of the —SH compounds appeared to be oxidized, with the formation of cuprous and aurous salts. It was suggested that the toxic action of the metallic salts might be due to a disturbance of the equilibrium, $2\text{RSH} \rightleftharpoons \text{RSSR} + \text{H}_2$. Rats could be protected from a lethal dose of sodium cupritartrate with sufficient amounts of glutathione or cysteine. The toxic action of copper sulfate on spirogyra was also prevented by excess glutathione.

Eagle (97) extended the earlier observations in a study of the effect of mercapto compounds on the antispirochetal activity of arsenic, bismuth, and mercury compounds *in vitro*. He found that the immobilization of *T. pallidum* by substances



such as arsenoxide, arsphenamine, and mercuric chloride was prevented by an excess of glutathione, cysteine, and sodium mercaptoacetate. Methionine and thiamine had no effect. In the case of glutathione and arsenoxide, 5–12 moles was required to inactivate one mole of arsenoxide, but Eagle considered that the hydrolysis of the thioarsenite (72, 403) probably explained the discrepancy.

The action of mercuric chloride on bacteria (*E. coli*) was examined by Fildes (123) from the same general point of view. He found that mercaptoacetate, cysteine, and glutathione effectively prevented the bacteriostatic action of mercuric chloride, whereas oxidized cysteine (cystine) and methionine as well as a number of other metabolites such as thiamine, nicotinamide, riboflavin, and pantothenic acid were ineffective. A molar ratio of approximately 4:1 for glutathione and 25:1 for mercaptoacetate was required to produce complete reversal. These differences were explained on the basis of the relative chemical stability of the products formed when these two mercapto compounds combined with mercuric chloride, i.e.,



Even when the organisms were exposed to mercuric chloride ($2 \times 10^{-7} M$) for 4 days at room temperature, the addition of excess —SH compound allowed growth to proceed normally. On the other hand, higher concentrations ($2 \times 10^{-6} M$) and temperatures (38°C.) for 17 hr. appeared to produce an irreversible effect on the bacteria. Fildes concluded that within limits the only function of mercuric chloride was to inactivate mercapto groups without other demonstrable injury to the cell. Several organic mercurials have also been shown to be inactivated by sodium mercaptoacetate (Nungester, Hood, and Warren (312)). Under the same conditions, other disinfectants such as phenol and iodine were not affected.

Albert, Falk, and Rubbo (8) studied the effect of arsenoxide and other arsenicals on several bacteria. They found that arsenoxide possessed considerable antibacterial action comparable to mercuric chloride, and that its bacteriostatic effect could also be reversed with an —SH compound, in this case mercaptoacetate. On the other hand, the pentavalent arsenical, *m*-acetylamino-*p*-hydroxyphenylarsonic acid, was ineffective, and neoarsphenamine showed only slight activity. Thus, at least one arsenoxide could be shown to possess con-

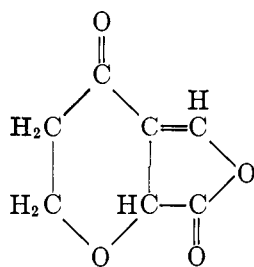
siderable antibacterial action of the same type as that of the mercurials, while closely related arsenicals at higher or lower levels of oxidation were inactive.

Chen, Geiling, and MacHatton (64) found that cysteine reduced the trypanocidal activity and toxicity of trivalent antimonials such as tartar emetic, fuadin, anthiomaline, and antimony sodium thioglycolate, but not of the pentavalent derivatives, stibamine, stibenyl, and neostibosan.

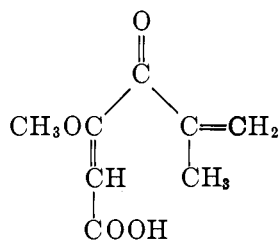
Substances preventing the action of an antibiotic agent called penicidin (derived from a penicillium but apparently not penicillin or clavacin) were investigated by Atkinson and Stanley (20). In addition to a slow-acting, dialyzable substance in serum, several mercapto compounds rapidly inactivated the antibacterial action of penicidin. Glutathione was the most effective, $M/1000$ reducing the activity approximately thirtyfold. Cysteine and mercaptoacetate were less effective, while other types of reducing agents showed no antagonistic action. When a mercapto compound and penicidin were mixed in solution and the nitroprusside test applied, the $-SH$ group was found to disappear very quickly. This result favored the conclusion that a chemical reaction took place between the two substances, and that the antibiotic activity was due to the inactivation of essential mercapto compounds.

A number of other antibiotic agents were tested for bacteriostatic action in the presence of cysteine by Cavallito and Bailey (59). In every case examined (penicillin, citrinin, gliotoxin, clavacin, pyocyanine, and the active principles of several plants) cysteine diminished or completely prevented the antibacterial activity. The methyl and ethyl esters of cysteine were also effective in this respect, but *S*-methylcysteine, methionine, alanine, and serine were not. In some instances the inactivation was considered to be due to an irreversible reaction of cysteine with the antibiotic. Other $-SH$ compounds, such as glutathione and mercaptoacetic acid, were much weaker or entirely ineffective. The action on Gram-positive organisms appeared to be more susceptible to cysteine inactivation than the Gram-negative activity. It was suggested that possibly the fundamental mode of action of certain classes of antibiotics involves their ability to interfere with the normal function of mercapto groups in bacterial metabolism.

A more detailed study of the preventive effect of mercapto compounds on the antibacterial activity of clavacin and penicillic acid was carried out by Geiger and Conn (139). Attention was called to the fact that these antibiotics are α,β -

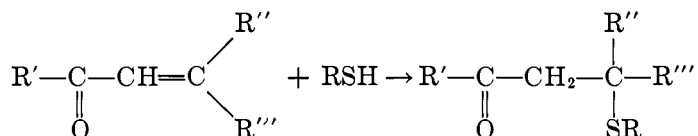


Clavacin



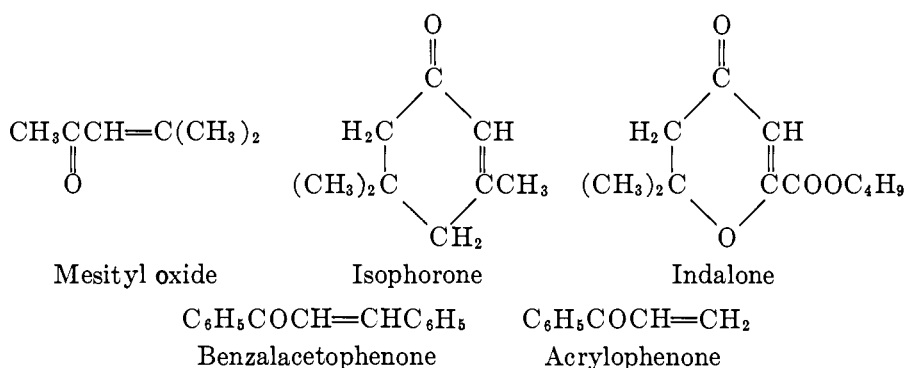
Penicillic acid

unsaturated ketones, which in general react with —SH compounds as follows:



This reaction takes place most readily when R' is an aromatic radical and R'' or R''' is H.

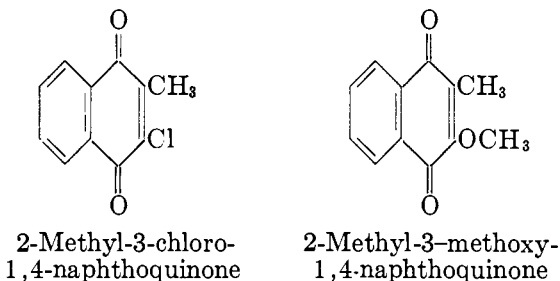
Both clavacin and penicillic acid were inactivated when incubated for 24 hr. with an excess of mercaptoacetate or cysteine. Only a slight excess of mercaptoacetate (10 per cent) was required to inactivate clavacin almost completely. Thiosulfate also prevented the action of clavacin but not of penicillic acid. Tests were carried out with a number of different microorganisms, and the activity against the Gram-negative *E. coli* seemed to be more easily abolished than the effect on several Gram-positive organisms. Other α, β -unsaturated ketones, such as mesityl oxide, isophorone, indalone, benzalacetophenone, and acrylophenone, were tested for bacteriostatic activity.



Of these, only acrylophenone showed a high degree of activity. The action of this compound was also prevented by mercaptoacetate and cysteine, whereas the lower activity of compounds such as benzalacetophenone was not. This difference was explained on the basis of the chemical reactivity of the unsaturated ketones. Clavacin, penicillic acid, and acrylophenone reacted rapidly and completely with mercaptoacetic acid, but benzalacetophenone reacted much more slowly and incompletely.

Chow and McKee (66) found that the inactivation of penicillin by cysteine was a function of the time of exposure. Thus, 0.02 mg. of penicillin was inactivated in 48 hr. by 0.08 mg. of cysteine, while 1.2 mg. was required to destroy the same amount of antibiotic in 5 hr. On the basis of these observations, it was concluded that the inactivation of penicillin was due to a chemical reaction rather than interference with an essential —SH enzyme system. Other amino acids—such as glycine, methionine, serine, and cystine—were inactive. Mercaptoacetic acid showed some effect, but simple mercaptans in general were not effective.

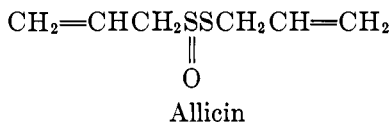
Colwell and McCall (74) investigated the effect of mercapto compounds on the bacteriostatic activity of naphthoquinones. They showed that sodium mercaptoacetate and cysteine hydrochloride prevented the antibacterial action of 2-methyl-1,4-naphthoquinone and its 3-chloro derivative, with *E. coli* as the test



organism. On the other hand, the —SH compounds failed to affect the bacteriostatic action of 2-methyl-3-methoxy-1,4-naphthoquinone, indicating that an interference with essential mercapto groups might not completely explain the growth-inhibitory mechanism of this class of compounds.

Streptomycin, but not streptothricin, was found to be inactivated by cysteine (Denkewalter, Cook, and Tishler (82)). 2-Aminoethanethiol was also effective in preventing the antibiotic activity of streptomycin, but mercaptoacetic acid produced no significant effect. Cysteine-inactivated streptomycin could be quantitatively reactivated by iodine. The authors concluded that a mechanism postulating either a reversible chemical reaction between the antibiotic and certain —SH compounds, or a competitive effect on metabolic processes would be consistent with their observations.

A group of antibiotics, and other antibacterial agents which were inactivated by —SH compounds, were investigated by Cavallito, Bailey, Haskell, McCormick, and Warner (60). They concluded that the inactivation might be due to one of several mechanisms, and distinguished between specific and non-specific effects. Thus, penicillin was inactivated by only a few of the mercapto compounds tested, whereas allicin appeared to be inactivated by almost any —SH



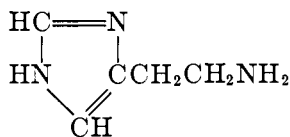
derivative. The mechanisms of reaction between antibacterial agents and mercapto compounds were enumerated as follows: (1) Oxidation—e.g., inorganic oxidizing agents, allicin, quinones, and certain dyes. (2) Formation of heavy metal complexes—such as the action of mercury, silver, and other metals forming mercaptides or complex addition products. (3) Metathesis—e.g., the reaction with alkylating agents such as iodoacetic acid. (4) Condensation—as in the formation of thioacetals or ketals with aldehydes and ketones⁹. (5) Addition to

⁹Although the authors did not mention it, the formation of thioarsenites with arsenoxides would presumably fall in this category.

unsaturated structures,—such as clavacin and penicillic acid, and other unsaturated ketones or esters.

E. Histamine

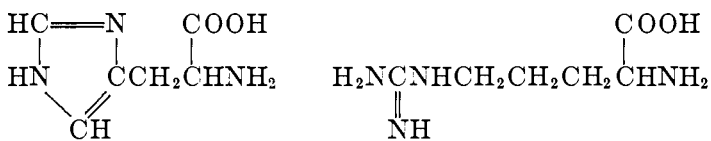
Histamine is present in appreciable quantities in most body fluids and tissues. It is a substance of multifarious actions, minute amounts exerting a powerful vasodilator effect while contracting other smooth muscle. To account for the absence of these effects under normal conditions, histamine is generally assumed to occur in a bound form *in vivo*. Although its rôle has not been completely elucidated, the liberation of histamine appears to be an important factor in anaphylactic shock and many allergic conditions such as asthma and hay fever



Histamine

(36, 118, 151). Most vasoconstrictor substances counteract the vasodilator effect of histamine, and spasmolytic agents such as atropine neutralize the contracting action of histamine on smooth muscle (173). However, other agents have been found which, at least in some cases, appear to be more direct and specific histamine antagonists.

Edlbacher, Jucker, and Baur (100) first demonstrated that arginine, histidine, and cysteine inhibit the effect of histamine on isolated intestinal strips of guinea



Histidine

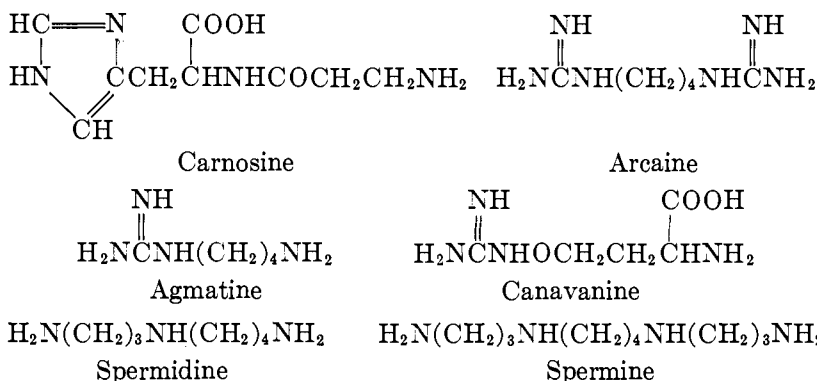
Arginine

pigs. They reported that Rothlin found the contracting action of acetylcholine on intestinal strips was not inhibited by arginine, indicating the specificity of the histamine antagonism. The other amino acids tested did not counteract the effect of histamine. The molar inhibition ratio for arginine appeared to be approximately 28,000:1, when the histamine action was completely prevented. Histidine and cysteine were reported to be somewhat less effective. In all cases the reaction was reversible, since, after removal of the antagonist, exposure to histamine again produced the usual contraction of the intestinal strips.

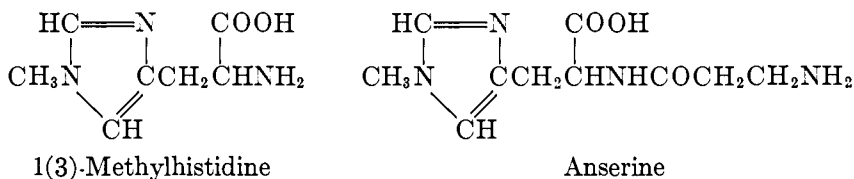
The observations with histidine were confirmed by Mackay (268), who found that this substance inhibited the action of histamine *in vitro*. Histidine did not appear to influence the depressor action of histamine in the cat¹⁰. Mackay reported that the effect of acetylcholine on isolated gut strips was also prevented by histidine.

¹⁰The action of histamine may be studied either *in vitro* using isolated smooth muscle or in intact animals. Unless otherwise stated, the observations are with intact animals.

In an effort to correlate structure and activity, Ackermann and Wasmuth (3) carried out an extensive study of compounds which antagonized the action of histamine on isolated intestinal strips of guinea pigs. They reported that, in addition to arginine and histidine, combined forms of these substances such as arginine-rich proteins and carnosine were active inhibitors. Other guanidine derivatives and polyamines, such as arcaine, agmatine, γ -guanidobutyric acid, canavanine, spermidine, and spermine, also prevented the action of histamine.



Since creatine, creatinine, and *asym*-dimethylguanidine were inactive, it was suggested that the presence of a free hydrogen atom on the side-chain nitrogen was necessary for activity. An acid group near the substituted hydrogen of a guanidine derivative, as in glycoyamine and β -guanidopropionic acid, appeared to eliminate activity. Similarly, methylation of the imino group, as in 1(3)-methylhistidine and anserine, destroyed the antagonistic action of the parent compounds.



On the basis of these studies, Ackerman and Wasmuth (1, 3) suggested that the NH group in the guanidines and secondary amines competed on a mass action basis with the ring NH of histamine for the receptor groups in smooth muscle. The ring NH was assumed to anchor the molecule in the tissues, while the toxic action of histamine was attributed to the primary amino group. Using gut strips from sensitized guinea pigs, they demonstrated that arginine prevented the contraction which would normally have been produced by the liberated histamine, without interfering with the desensitization of the strips. Ackerman and Wasmuth (2) reported that neither choline nor acetylcholine was inhibited by arginine.

Histidine hydrochloride was also studied by Halpern (155), who found that

10 mg. of histidine completely and immediately counteracted the contraction of guinea pig gut produced by 1γ of histamine—a molar inhibition ratio of approximately 6500:1. He reported that histidine caused a considerable immediate reduction in the tone and diminished the amplitude and frequency of the contraction of the isolated intestine of the rabbit. Histidine also prevented, although in a more irregular fashion, the action of acetylcholine, eserine, and barium chloride.

Landau and Gay (216) conducted a careful investigation of the influence of the monohydrochlorides of arginine and histidine on histamine and anaphylactic reactions of isolated smooth muscle and in intact guinea pigs. They also summarized the results in an earlier paper by Linneweh (236) on this subject. Linneweh extended the previous studies to other isolated smooth muscles, and found that arginine inhibited the histamine effect in every case. The effects of acetylcholine and epinephrine were not influenced by arginine. The action of pitocin was inhibited, although to a lesser extent than that of histamine. Guinea pigs were protected from histamine toxicity and anaphylactic shock with large doses of arginine monohydrochloride.

The investigations of Landau and Gay (216) also confirmed, in general, the earlier observations with both arginine and histidine. They found that the action of acetylcholine was not suppressed by concentrations of arginine or histidine which prevented histamine contractions of intestinal strips. In some cases, however, the response to acetylcholine was decidedly weaker in the presence of the antagonists, and it was concluded that absolute specificity for arginine or histidine as histamine antagonists for smooth muscle was not established. In some instances Landau and Gay also observed a relaxation of the tone of isolated muscle in the presence of arginine or histidine. They confirmed Linneweh's results with arginine by protecting guinea pigs from fatal doses of histamine, but were unable to extend the protection to anaphylactic shock. Higher doses, which might have produced positive results, were found to be too toxic.

The results of Gredner and Schumrick (147) and of Jadassohn, Fierz-David, and Vollenweider (187, 188) did not corroborate some of the observations in the preceding studies. The latter investigators reported that the antihistamine activity of arginine and the polyamines did not parallel their effect on the anaphylactic contraction of the guinea pig uterus. The antihistamine activity decreased in the order: arcaine, spermine, spermidine, and arginine. Triethylenetetramine showed only slight antihistamine activity, but it was a more potent inhibitor of anaphylactic contractions than arcaine. Both groups of investigators reported that the effect of these substances was not limited to histamine, since they also prevented the action of pitocin, choline, and acetylcholine. Gredner and Schumrick considered that the antihistamine activity was due to a slight antispasmodic effect, which counteracted any contractions regardless of the nature of the causative agent.

Rocha e Silva (345) prepared several simple peptide-like derivatives of histamine which he considered might serve as models for bound histamine, e.g.:

Young and Gilbert (468) and Gilbert and Goldman (140) demonstrated that although theophylline-ethylenediamine (aminophyllin) alone had little effect on thin sections of rabbit or dog lung tissue, it had a definite protective effect against the constrictor action of histamine. The former authors found that a solution of 1:1000 aminophyllin in many of the trials completely prevented the bronchiole constriction produced by 1:50,000 histamine. Neither the sodium salt of theophylline (1:2000) nor the free acid (1:5000) showed any antihistamine activity. Ethylenediamine and alkalinity corresponding to that of the aminophyllin solution (pH 8.5) were also without effect. *In vivo* some degree of protection was obtained against fatal doses of histamine in guinea pigs, as well as a slight effect on anaphylactic shock in these animals.

TABLE 8

Relative amounts of different compounds required to inhibit the action of histamine on the guinea pig ileum (346)

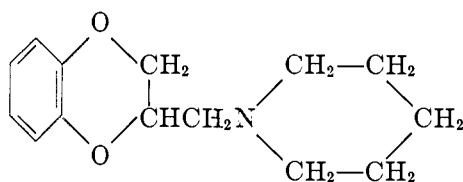
COMPOUND	AMOUNT OF M/20 SOLUTION	HISTAMINE ADDED	MOLAR INHIBITION RATIO†
	cc.	γ	
Arginine hydrochloride*	0.94	0.036	145,000
Benzoyl-L-arginine.....	0.35	0.02	97,000
Benzoyl-L-arginineamide hydrochloride*..	0.18	0.033	30,000
Histidine hydrochloride*.....	0.18	0.03	33,000
Acetyldehydrophenylalanylhistamine hydrochloride*.....	0.29	0.043	37,500
Acetyl-DL-phenylalanylhistamine hydrochloride*.....	0.25	0.045	31,000

* Average of two or more values on different intestinal strips.

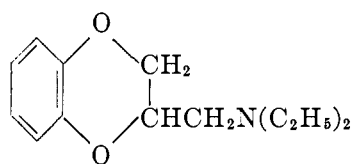
† Moles of antagonist required to inhibit one mole of histamine; calculated from Rocha e Silva's data (346).

Another group of substances, mainly derivatives of ethanolamine and ethylenediamine, has received considerable attention as histamine antagonists. While these compounds may not be specific in their action, some of them are powerful antagonists of many of the complex effects of histamine.

In 1937 Unger, Parrot, and Bovet (414) reported that a spasmolytic agent, piperidinomethylbenzodioxan, in a concentration of one part in 100,000, in-



2-N-Piperidinomethyl-
1,4-benzodioxan



2-Diethylaminomethyl-
1,4-benzodioxan

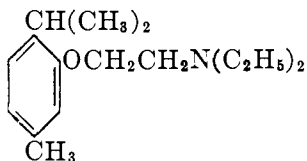
hibited the action of histamine (1 γ /10 cc.) on the isolated intestine of the guinea pig. The action appeared to be specific and reversible (after washing, the gut

strip regained its normal reaction toward histamine). Later Vallery-Radot, Bovet, Mauric, and Holtzer (417, 418) studied the effect of this compound and the related diethylaminomethylbenzodioxan on anaphylactic and histamine shock in the rabbit. Under certain conditions, these substances protected the animals, the diethyl derivative acting more regularly than the corresponding piperidine.

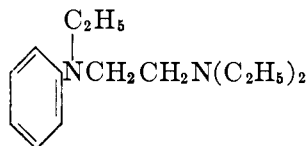
Several compounds in the phenoxyethyldiethylamine [$C_6H_5OCH_2CH_2N(C_2H_5)_2$] series were investigated by Bovet and Staub (47), who found that 2-isopropyl-5-methylphenoxyethyldiethylamine (thymoxyethyldiethylamine), in particular, regularly protected guinea pigs from toxic doses of histamine. The parent compound, its *p*-methoxy derivative, β -naphthoxyethyldiethylamine, and diethylaminomethylbenzodioxan were less active, while α -naphthoxyethyldiethylamine and piperidinomethylbenzodioxan were reported to be almost inactive. Thymoxyethyldiethylamine was also the most active in antagonizing the effect of histamine *in vitro*. No close relationship between histamine antagonism and spasmolytic activity was found in this series of compounds. Thus, thymoxyethyldiethylamine had only a slight spasmolytic action and β -naphthoxyethyldiethylamine, none. The phenolic ethers possessed pharmacological properties more closely related to those of histamine itself. Bovet and Staub suggested that their action as histamine antagonists might be due to their respiratory stimulation. In another series, diethylaminoethylaniline, which was without marked pharmacological properties, showed antihistamine activity. The effect of thymoxyethyldiethylamine on anaphylactic shock in guinea pigs was studied by Staub and Bovet (398), who demonstrated a definite protective action with this compound both *in vitro* and *in vivo*.

Staub (396, 397) carried out a comprehensive investigation of a number of compounds in the phenoxyethyldiethylamine and diethylaminoethylaniline series. In comparing the relative activity of compounds, she preferred histamine shock to the isolated-gut method because of the variability of the latter. Thymoxyethyldiethylamine, the most active of the phenolic ethers, protected guinea pigs against 2 M.L.D.'s (minimum lethal doses) of histamine in doses of 5 mg./kg., although symptoms of shock were present. An equal dose of *N*-phenyl-*N*-ethyl-*N'*-diethylethylenediamine protected the animals against 3-4 M.L.D.'s and no shock symptoms were observed. In the same test atropine (5 mg./kg.) was effective against only 1 M.L.D. of histamine. Some of the relationships between structure and activity as recorded by Staub (396) are indicated in table 9.

A number of other structural modifications were also studied. Thus, in the thymoxyethyldiethylamine series (I) removal of one *N*-ethyl group eliminated



I

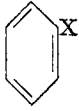
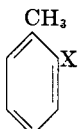

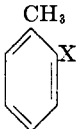


II

the activity, while replacing the diethylamino group by piperidine decreased the antihistamine index (A.H.I.=2). Variations of *N*-phenyl-*N*-ethyl-*N'*-diethyl-ethylenediamine (II), such as removal of the one *N*-ethyl or of both the *N'*-ethyl groups, destroyed activity. Replacing the *N*-ethyl by methyl reduced the A.H.I. to 1.5, and the *N*-isopropyl derivative was also less active than the

TABLE 9

Antihistamine index of various diethyl derivatives of phenoxyethylamine and N-phenyl-ethylenediamine (396)

FORMULA	A.H.I.*		
	X = OCH ₂ CH ₂ N(C ₂ H ₅) ₂	X = NHCH ₂ CH ₂ N(C ₂ H ₅) ₂	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{X} = \text{NCH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2 \end{array}$
	1	1	4
	1	1	1
	2	2	1.5
	3	3	1

* A.H.I. = antihistamine index expressed as the number of M.L.D.'s necessary to kill an animal protected by 5 mg. of compound injected intravenously. Thus, A.H.I. = 1 indicates no activity.

N-ethyl derivative (A.H.I. = 3). The antihistamine activity of compounds I and II on smooth muscles of various organs was investigated by Staub (397). She found that 5-10 parts by weight of these derivatives prevented the action of one part of histamine on the isolated guinea gut. They also prevented the effect of histamine on the isolated guinea pig uterus and lung as well as the bronchi *in situ*, but were ineffective, in general, against the vascular and local skin reactions. A certain amount of parallelism between the relative activity against these different effects of histamine was found, but marked divergencies were also observed, particularly in going from one series to another.

Staub (397) attempted to determine the mode of action of I and II. She reported that, while I suppressed the action of other substances capable of contracting smooth muscle, such as acetylcholine and barium chloride, II diminished the action of these substances only slightly or not at all. Some correlation between the spasmolytic action of the phenolic ethers and their antihistamine effect was found, but in the *N*-phenylethylenediamines there was a considerable divergence in these activities. The action of the compounds *in vivo* was attributed to some unexplained phenomenon by virtue of which only a part of the injected histamine reached the lungs of treated animals. No appreciable effect on histaminase and no pronounced evidence of a chemical reaction between II and histamine could be demonstrated.

Numerous investigations have verified the antihistamine activity of I and II (71, 353, 354, 434), but there appears to be no general agreement as to the specificity of these antagonists. Both compounds have been reported to be in-

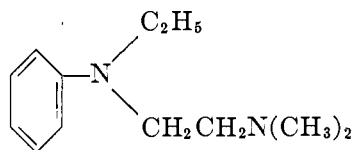
TABLE 10
Relative antihistamine activity of N-phenylethylenediamines on isolated intestine of guinea pigs (156)

COMPOUND	AMOUNT*	CONCENTRATION	RELATIVE ACTIVITY
	$\gamma/10\text{cc.}$	<i>M</i>	
II.....	15	1.5×10^{-5}	0.1
III.....	1.5	1.5×10^{-6}	1
IV.....	0.1	1×10^{-7}	15

* Amount of antagonist required to counteract 90-100 per cent of the action of $2-5 \times 10^{-8}$ *M* histamine.

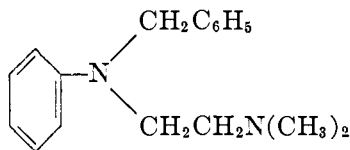
effective or non-specific in reducing the gastric secretion in dogs following histamine stimulation (44, 53, 154, 238).

Following up the work of Staub and Bovet, Halpern (156) carried out an extensive study of the *N*-phenylethylenediamine derivatives III and IV. Both



III

N-Phenyl-*N*-ethyl-*N'*-
dimethylethylenediamine



IV

N-Phenyl-*N*-benzyl-*N'*-
dimethylethylenediamine

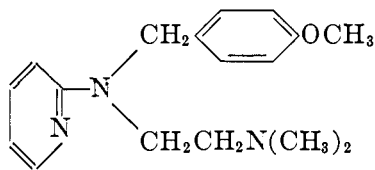
of these compounds appeared to be much more active as histamine antagonists than the *N'*-diethyl derivative (II), as shown in table 10.

Halpern (156) studied the effect of III and IV on bronchial spasms in the intact guinea pig by employing histamine aerosols. He found the same order of activity for these compounds and II as in the *in vitro* experiments. Comparing IV with atropine and epinephrine, he reported that 1 mg./kg. of IV

produced an effect equivalent to 25 mg./kg. of atropine and 0.2 mg./kg. of epinephrine. On the other hand, using acetylcholine aerosols 50 mg./kg. of IV was without effect, while 0.025 mg./kg. of atropine, and the same amount of epinephrine which was effective for histamine, controlled the bronchial spasms. These results, together with numerous *in vitro* studies, appeared to establish the specific nature of the action of the histamine antagonists. Compounds III and IV showed remarkable activity in protecting guinea pigs against the toxic action of histamine, as little as 5–10 mg./kg. of IV preventing the death of animals injected with 40–50 M.L.D.'s of histamine. Similarly, in anaphylactic shock experiments the compounds protected the sensitized animals against multiple shocking doses of serum. In many cases practically all of the symptoms of shock were eliminated. Halpern concluded that the compounds neutralized the effects of histamine by a pharmacodynamic mechanism similar to the pharmacological antagonism between atropine and acetylcholine. He suggested that the normal affinity of cells for histamine was modified in some unknown fashion by the histamine antagonists.

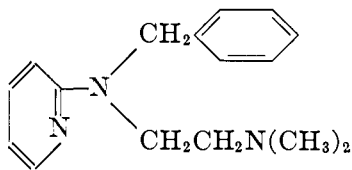
The antihistamine activity of IV was confirmed by other investigators (2, 61, 63, 284, 331). The Chauchards (63) found that IV alone produced pharmacological effects opposite to those of histamine. In different tissues the actions of the two substances individually were sometimes the reverse, but together they still neutralized each other. Thus, on cervical preganglionic fibers histamine increased chronaxia while IV caused a decrease, whereas on postganglionic fibers it was the latter which increased chronaxia and histamine decreased it. In both cases, the two substances in combination neutralized each other.

Bovet, Horclois, and Walthert (46) demonstrated that *N*-(2-pyridyl)-*N'*-(*p*-methoxybenzyl)-*N'*-dimethylethylenediamine (V) also possesses a high degree



V

N-(2-Pyridyl)-*N*-(*p*-methoxybenzyl)-*N'*-dimethylethylenediamine



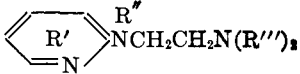
VI

N-(2-Pyridyl)-*N*-benzyl-*N'*-dimethylethylenediamine

of antihistamine activity in guinea pigs. A dose of 1 mg./kg. protected these animals against 75 M.L.D.'s of histamine (45 mg./kg.) and 0.1 mg./kg. was effective in preventing experimental bronchial spasms produced by histamine aerosols. *In vitro* 10^{-7} to 10^{-8} V prevented the contraction of isolated guinea pig gut produced by histamine in concentrations of 10^{-7} . The authors suggested that the action of this type of histamine antagonist was due to a competition for specific tissue receptors. In dogs, Bovet, Horclois, and Fournel (45) found that, although the results were less striking, 0.1–1.0 mg./kg. of V diminished or abolished the effects of relatively small (10–150 γ /kg.) doses of histamine.

A series of closely related compounds was investigated by Mayer, Hutterer, and Scholz (284, 285), who found that *N*-(2-pyridyl)-*N*-benzyl-*N'*-dimethylethylenediamine (VI) was the most active member of the group (table 11). Secondary amines were all inactive or almost inactive. A concentration of VI one hundred times greater was required to abolish acetylcholine contractions. This result was contrasted with antispasmodics such as β -diethylaminoethyl-diphenylacetate (Trasentin), which is one hundred times more active against acetylcholine than it is against histamine. With histamine aerosols in guinea pigs, VI was reported to be twice as active as IV. The same animals were also protected from anaphylactic shock by doses (of 0.1-1.0 mg./kg.) of VI.

TABLE 11

Comparative antihistamine activity of compounds of the type ,
(285)

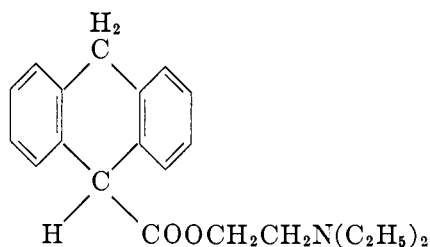
COMPOUND			ACTIVITY*
R'	R''	R'''	
			γ /cc.
Pyridyl.....	Benzyl	Methyl	0.02
Pyridyl.....	Benzyl	Ethyl	>5
Pyridyl.....	Phenyl	Methyl	>5
Pyridyl.....	Phenyl	Ethyl	>10
Pyridyl.....	Isopropyl	Methyl	>10
Pyridyl.....	Propyl	Ethyl	>10
Pyridyl.....	β -Pyridyl	Methyl	>10
Pyridyl.....	α -Pyridyl	Ethyl	>10
β -Picolinyl.....	Benzyl	Methyl	2
α' -Picolinyl.....	Benzyl	Methyl	1
γ -Picolinyl.....	Benzyl	Methyl	0.2

* *In vitro* activity expressed in concentrations antagonizing the action of 1 γ /cc. of histamine diphosphate on isolated intestinal strips of guinea pigs.

Additional studies by Yonkman and coworkers (336, 465, 466) indicated that VI (25-50 γ) antagonized the action of equal concentrations of histamine in the perfused lungs of guinea pigs. Histamine skin wheals produced in rabbits were also prevented by VI. In anesthetized cats VI alone produced no observed effects; histamine salivation was prevented but not mydriasis. Anaphylactic reactions in dogs were not consistently controlled by VI.

An extensive series of spasmolytic agents¹¹ prepared by Burtner and Cusic (54, 55) were investigated for antihistamine activity by Lehmann and Knoefel (224, 225). These compounds included esters of diphenylacetic, fluorene-9-carboxylic, 9,10-dihydroanthracene-9-carboxylic, and a number of related acids.

¹¹ Many general spasmolytic agents may fall in the category of physiological antagonists (i.e., effects due to opposite but independent actions). They are included in these instances for completeness and because the mechanism of action of most of the antihistamine agents is still largely unknown.

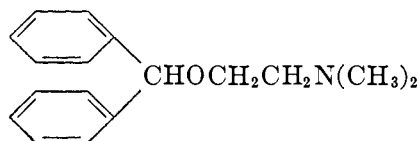


VII

β -Diethylaminoethyl 9,10-dihydroanthracene-9-carboxylate

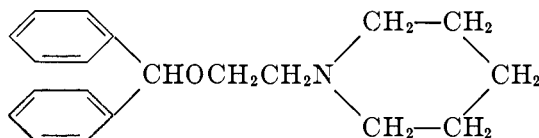
Although these derivatives also antagonized the action of acetylcholine and other spasmogenic agents, VII in particular showed a marked antihistamine effect both *in vitro* and *in vivo*. No close relationship between the antagonistic action toward histamine and acetylcholine was observed among these types of compounds. Thus, replacing the methylene group of VII with oxygen (β -diethylaminoethyl xanthene-9-carboxylate) led to a tenfold drop in antihistamine activity but a corresponding increase in acetylcholine antagonism, whereas replacement by an NH group decreased the former activity but left the latter practically unchanged. A subcutaneous dose of 50 mg./kg. of VII protected guinea pigs from 1.7 mg./kg. (3 M.L.D.'s) of histamine phosphate. Lehmann and Young (226) found that these animals were also protected against death from anaphylactic shock, but against only one fatal dose of antigen. The actions of histamine other than those on smooth muscle were not affected by VIII.

Another large group of antispasmodics (benzohydril ethers) was studied by Loew, Kaiser, and Moore (241). These compounds were synthesized by Rieveschl and Huber (338). Employing a histamine aerosol technique similar to that used by Halpern (156), these investigators reported that many of the benzohydril ethers were much more effective than theophylline-ethylenediamine (aminophyllin) in protecting guinea pigs from otherwise fatal doses of atomized histamine. Compared with II, VIII and IX appeared to be ap-



VIII

Benzohydril β -dimethylaminoethyl ether



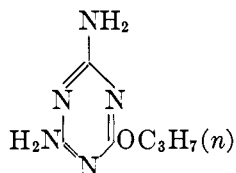
IX

Benzohydril β -piperidinoethyl ether

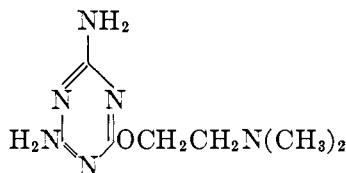
proximately twice as effective. In general, compounds with longer or branched aliphatic chains were less active. The character of the nitrogen atom affected activity so that the order of effectiveness was tertiary amine > secondary > primary. Smaller groups on the nitrogen usually produced more active compounds, while replacement of the oxygen atom by NH led to inactive compounds. Loew and Kaiser (239) also found VIII and IX to be effective in alleviating experimental anaphylactic shock in guinea pigs. The potency of VIII was at least equivalent to that of II, while IX was less than one-half as active.

The antihistamine activity of VIII was confirmed by other investigators. Ellis (101), employing perfused guinea pig lungs, noted that not only did VIII control histamine bronchial spasms, but it also caused a significant amount of dilatation. The quantitative relationship between VIII and histamine was studied by Wells, Morris, Bull, and Dragstedt (429, 429a). These investigators found that the amount of histamine antagonized bore a constant relationship to the amount of histamine injected, and concluded that VIII might compete with histamine for its site of action.

Controulis and Banks (75) prepared a number of 2-alkoxy-4,6-diamino-*s*-triazines. These compounds were examined for antihistamine activity by Loew, Kaiser, and Anderson (240), employing the histamine aerosol method in guinea pigs (241). The most active members of this series were the propoxy derivative (X), some of the butoxy derivatives, and the thio ether corresponding to X, which were approximately four times as active as aminophyllin. XI was



X

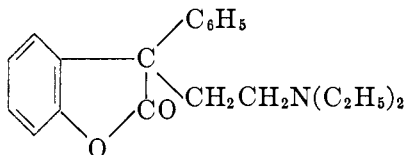


XI

2-Propoxy-4,6-diamino-*s*-triazine 2-(β -*N,N*-Dimethylaminoethoxy)-
4,6-diamino-*s*-triazine

considerably less potent than the alkyl derivatives, while substitution on the 4- and 6-amino groups did not appear to alter activity appreciably.

Richards, Everett, and Kueter (336a) investigated the antihistamine effect of XII, one of a series of benzofuranone antispasmodics. This compound was



XII

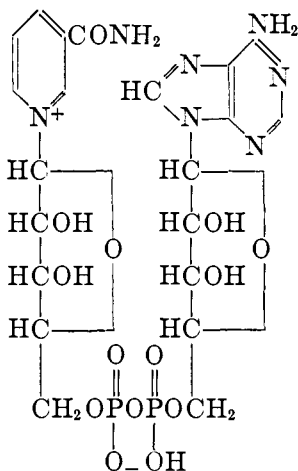
3-(β -Diethylaminoethyl)-3-phenyl-2-benzofuranone

found to antagonize histamine contractions of the isolated guinea pig uterus. It also counteracted bronchial spasms induced by histamine more effectively

than theophylline. Other studies with XII indicated that it usually reduced anaphylactic symptoms and prevented histamine shock in guinea pigs.

F. Nicotinic acid; cozymase

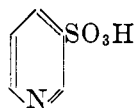
Nicotinic acid and its amide are more or less equivalent for most organisms which are unable to synthesize them (321). As a constituent part of coenzymes I and II, nicotinamide is generally recognized as an important factor in bacterial metabolism as well as animal nutrition. The coenzymes with various apoenzymes (specific proteins) function as dehydrogenases in a number of fundamental respiratory processes.



Cozymase (coenzyme I)

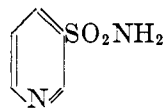
Coenzyme II differs from cozymase only in having an additional phosphoric acid residue in the molecule (104). Despite the known functions of these systems, many of the results obtained with antagonists have been inconclusive. Some of the inconsistencies may be due to the complex nature of the enzyme systems. Under the proper conditions an antagonist could presumably interfere directly or indirectly with (a) the synthesis of nicotinic acid or its conversion to the amide, (b) the production of coenzymes from either the acid or amide, (c) the combination of the coenzyme with a specific apoenzyme, or (d) the action of the holoenzyme (coenzyme + apoenzyme) on its substrate.

In 1940, McIlwain (249) reported that *M/100* pyridine-3-sulfonic acid (I) inhibited the growth of *Proteus vulgaris* when nicotinic acid was used as a growth-promoting agent, but not when corresponding concentrations of nicotinamide



I

Pyridine-3-sulfonic acid



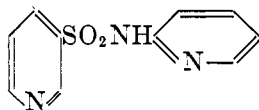
II

Pyridine-3-sulfonamide

were employed. He postulated that the action of I might be localized at the enzymatic reaction converting nicotinic acid to its amide or at a similar stage of amide formation from a more complex derivative. Growth stimulated by cozymase was even more strongly inhibited by I, suggesting that nicotinic acid was not used by *Proteus* solely for the synthesis of cozymase. With *Staph. aureus*, II ($M/100$ – $M/500$) inhibited growth promoted by nicotinamide more effectively than that promoted by the acid. Inhibition of nicotinamide-stimulated growth by I was differentiated from II in that it appeared to change with time and was not directly related to the concentration of growth factor. Only temporary and incomplete effects were observed with II on *E. coli*, which does not require preformed nicotinic acid or amide. McIlwain (249) distinguished three types of growth inhibition, depending on the effect of time and changes in the ratio of growth factor and antagonist.

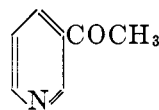
Matti, Nitti, Morel, and Lwoff (282) did not observe any growth inhibition by II ($M/80$) with their strains of proteus and coli. Employing *Proteus vulgaris* and *Streptobacterium plantarum* as test organisms, Möller and Birkofer (298) found I and II to have slight growth-promoting activity, although inhibition was sometimes produced at higher concentrations. In a careful study these investigators (298) concluded that, since I and II were bacteriostatic only in concentrations ($M/10$) at which nicotinic acid or nicotinamide also produced growth inhibition, the action under their conditions appeared to be non-specific with respect to these growth factors. They also demonstrated that ferric citrate reversed the antibacterial activity of high concentrations of II.

On the basis of the analogy between sulfanilamide and sulfapyridine, McIlwain (250) also prepared 2-(3'-pyridinesulfonamido)pyridine (III). In this case, however, III was less active as an antagonist of nicotinic acid or its amide



III

2-(3'-Pyridinesulfonamido)pyridine

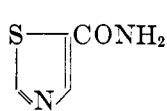


IV

3-Acetylpyridine

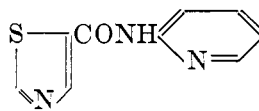
than I or II. Auhagen (21) found that 3-acetylpyridine failed to inhibit the growth of *Streptobacterium plantarum* except at high concentrations where the action could not be prevented by nicotinic acid.

Erlenmeyer, Bloch, Kiefer, and Würgler (111, 112) investigated a series of compounds structurally related to nicotinic acid. When *Proteus vulgaris* was the test organism, Erlenmeyer and Würgler (112) confirmed McIlwain's results



V

5-Thiazolecarboxamide



VI

2-(5'-Thiazolecarboxamido)pyridine

(249) with I. They also reported that V reversed the growth inhibition produced by I. On the other hand, with *Staph. aureus* V was bacteriostatic at $M/1000$ and the effect was reversed by $M/25,000,000$ nicotinamide (111). With this same organism I, in combination with nicotinic acid, produced a slight growth promotion while alone it appeared to have no effect. The bacteriostatic action of $M/5000$ II was prevented by nicotinamide at a concentration of $M/12,500,000$. III appeared to be a growth factor alone at $M/1000$, while VI produced growth inhibition which could be reversed with nicotinic acid.

Euler (113) pointed out that by working with the isolated enzyme systems it might be possible to decide which enzyme was being inhibited. The system substrate-apoenzyme-coenzyme could be inhibited either through the substrate or the coenzyme. An antagonist might also block the active group of the coenzyme or inactivate or occupy groups which confer specificity to the apoenzyme. Using glucose and lactic dehydrogenases, Euler investigated a number of substances and found that many of them inhibited these enzymes. Nicotinic acid produced about the same degree of inhibition as pyridine-3-sulfonic, benzenesulfonic, benzoic, or *p*-aminobenzoic acid. Salicylic acid, adenosine, and yeast tetranucleotide were somewhat more effective inhibitors.

In a subsequent paper Euler and Ahlström (115) reported a more complete study of salicylate [S], since it had been demonstrated that the inhibiting effect of this substance could be reversed by cozymase. Assuming that the inhibition depended on a displacement of cozymase on the apoenzyme, the authors set up the following displacement equilibrium:

$$K = \frac{[\text{cozymase-apoenzyme}][\text{NaS}]}{[\text{S-apoenzyme}][\text{cozymase}]}$$

At 50 per cent inhibition the concentration of both molecular complexes should be equal, so that,

$$K = \frac{[\text{S}]}{[\text{cozymase}]}$$

With 100 mg. of glucose and 400 mg. of apoenzyme the following data were obtained:

γ -COZYMASE	MILLIMOLES OF COZYMASE	SALICYLATE	MILLIMOLES OF SALICYLATE	K
10	0.00503	1600	3.33	662
30	0.01515	5000	10.40	686
43	0.02163	7000	14.56	673
50	0.02515	8400	17.60	692

Varying the concentration of apoenzyme had practically no effect on the degree of inhibition. Moreover, preliminary incubation of cozymase and apoenzyme for 2 hr. had no effect on the per cent inhibition. Euler and Ahlström considered that the equilibrium constant might also be explained by the

reaction of salicylate with another group in the apoenzyme. They concluded that, since so many different substances produced an inhibition of the same system, because succinic dehydrogenase which does not require cozymase was inhibited by some of the same compounds, and since salicylate inhibited both cocarboxylase and cozymase, it was improbable that its action depended on a specific displacement of the coenzyme.

In mice, Woolley and White (459) were unable to produce a nicotinic acid deficiency by feeding pyridine-3-sulfonic acid. They suggested that the lack of effect might be due to the fact that mice do not require an external source of nicotinic acid. It had been shown earlier that dogs deficient in nicotinic acid were killed by the sulfonic acid while normal dogs were apparently not harmed (456). 3-Acetylpyridine (IV) produced a similar effect. Later, Woolley (452) reported that typical signs of nicotinic acid deficiency in mice were produced by IV. The signs of the disease were prevented by an excess of nicotinic acid or nicotinamide in the rations. With several species of bacteria, no specific growth inhibition with IV was observed, confirming the results obtained by Auhagen (21).

About the time that Woods and Fildes (122, 443) described the relationship of PABA to sulfonamides, West and Coburn (430) reported that cozymase interfered with the antibacterial action of sulfapyridine, while nicotinic acid did not. Since autoclaved cozymase was used as a control, it seemed unlikely that this effect could be due to contamination with PABA. The authors called attention to a structural relationship between sulfapyridine and nicotinic acid, and suggested that the two substances might compete for the same position in the coenzyme molecule. Dorfman, Rice, Koser, and Saunders (91) studied the effect of sulfapyridine on the respiration of dysentery bacilli. When glucose was used as substrate, it was found that respiration stimulated by nicotinamide was inhibited by sulfapyridine. The order of addition of these substances was important, a greater inhibition resulting from the addition of sulfapyridine first, followed by a period of incubation before the nicotinamide was added. Similar results were observed with cozymase, and the authors suggested that the action of sulfapyridine on microorganisms might be related to the rôle of nicotinamide in their metabolism.

Numerous investigators (90, 183, 298, 371, 390, 404, 411, 442) have examined these relationships to bacterial growth and respiration with variable results. Anderson, Pilgrim, and Elvehjem (10), working with enzyme systems in which coenzyme I was necessary for maximum activity, found no inhibition with sulfapyridine or sulfathiazole. They concluded that these sulfonamides did not interfere directly with the functioning of cozymase in yeast fermentation or in several systems in normal rat liver, and suggested that the available evidence indicated the possibility that the sulfonamides might inhibit the synthesis of coenzymes.

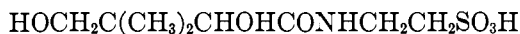
G. Pantothenic acid

Although pantothenic acid is widely distributed in nature, its physiological functions are largely unknown at present. Available evidence suggests the

possibility that it may be associated with carbohydrate metabolism (436). It seems likely that the vitamin is necessary for the normal functioning of practically all living matter (351). β -Alanine and α -hydroxy- β,β -dimethyl- γ -butyrolactone (pantolactone) or the corresponding acid (pantoic acid) are generally regarded as the precursors in the biological synthesis of pantothenic acid. Many closely related derivatives have been tested for vitamin activity, but at most they have very slight physiological action or are incomplete substitutes for natural $d(+)$ -pantothenic acid (436).



Pantothenic acid



Pantoyltaurine

Snell (380) and Kuhn, Wieland, and Möller (210) first described the taurine analogue of pantothenic acid, for which the name pantoyltaurine was suggested by McIlwain (254). With a strain of *Lactobacillus arabinosus* which requires preformed pantothenate, Snell found that pantoyltaurine was bacteriostatic, and that over a wide range of concentrations, growth was proportional to the relative amounts of pantoyltaurine and pantothenic acid. A strain of yeast stimulated by pantothenic acid was inhibited by pantoyltaurine, but growth of the same yeast stimulated by β -alanine was not reduced by the antagonist or by taurine. A number of other organisms requiring preformed pantothenic acid were susceptible to pantoyltaurine in varying degrees, and growth could be restored by additional pantothenic acid. In these cases the antibacterial action of the antagonist appeared to be produced entirely by its effect in screening the essential vitamin away from its site of action. However, organisms which synthesized their own pantothenic acid were not susceptible to pantoyltaurine. It was suggested that differences in cellular permeability or pantothenic acid metabolism might explain the lack of effect on these organisms. Since only $d(+)$ -pantothenic acid acts as a growth factor for *L. arabinosus*, Snell investigated the optical isomers of pantoyltaurine. The $d(-)$ - and $l(+)$ - α -hydroxy- β,β -dimethyl- γ -butyrolactones were condensed with the sodium salt of taurine by heating for 5 hr. at 120°C. The pantoyltaurine from the $d(-)$ -lactone proved to be about ten times as active as a growth inhibitor, and because of the racemization which undoubtedly occurred at the high temperature, all the antibacterial action was considered to be due to this isomer.

Under milder conditions (60 hr. standing at room temperature, followed by chromatographing and conversion to the quinine salt) Kuhn, Wieland, and Möller (210) obtained a $d(+)$ -pantoyltaurine which was 32 times as active as the corresponding $l(-)$ -isomer. Since their lactone intermediates were not optically pure, these investigators also concluded that all the bacteriostatic activity was probably due to the $d(+)$ -form of pantoyltaurine. With *Streptobacterium plantarum* Kuhn *et al.* found that growth inhibited by pantoyltaurine was completely restored by pantothenate and partially by β -alanine, or better, β -alanine plus pantolactone, although neither of the latter could replace pantothenic acid

as a growth factor for the organism. The bacteriostatic action decreased with time, and it was suggested that either the antagonist was gradually split or the presence of pantoyltaurine might stimulate the organisms to synthesize pantothenate. No other vitamins or growth factors tested were found to reverse the action of pantoyltaurine, thus demonstrating the specific nature of the growth inhibition. Assuming that metabolite and antagonist compete for the same apoenzyme in the cell, the inhibition constant at 50 per cent growth inhibition and from this the dissociation constant of the apoenzyme-pantothenate and apoenzyme-pantoyltaurine complexes were calculated as indicated:

$$K_p = \frac{[P] \times [Ap]}{[PAp]} = 2 \times 10^{-8}$$

$$K_t = \frac{[T] \times [Ap]}{[TAp]} = 7.6 \times 10^{-6}$$

where [P] = concentration of pantothenate, [Ap] = concentration of apoenzyme, and [T] = concentration of pantoyltaurine. These values were of the same order of magnitude as the dissociation constants for previously determined cell-free enzyme systems such as the old yellow enzyme (3.3×10^{-5}) and α -amino acid oxidase (2.5×10^{-7}).

Teague and Williams (410) reported that the fermentation by deficient yeast cells, which was accelerated by pantothenate, was not affected by pantoyltaurine.

McIlwain (254, 255) found that racemic pantoyltaurine inhibited the growth of streptococci, pneumococci, and some strains of diphtheria bacilli, but not coli, staphylococci, or *Proteus morganii*, even though the last-named organism required preformed pantothenic acid. With streptococci and pneumococci neither β -alanine nor pantoic acid interfered with the antibacterial action of pantoyltaurine, but in most cases β -alanine as well as pantothenate acted as reversing agents with susceptible strains of diphtheria bacilli. Blood serum and other natural fluids also reversed the bacteriostatic effect of pantoyltaurine, and it was shown that this action was directly related to the pantothenic acid content of these fluids. From the quantitative relationships between pantothenate and pantoyltaurine which he obtained, McIlwain considered that the results were typical of the competitive inhibition of an enzyme. He found that for streptococci the inhibition ratio was approximately 500:1, while for pneumococci it was about 1000:1. Allowing for the probable inactivity of the *l*(-)-isomer (210, 380), the ratios were 250:1 and 500:1, respectively. The ratios for several other organisms were determined to be considerably higher. On the basis of the blood pantothenate levels and the inhibition ratios, McIlwain concluded that it should be possible to obtain an antistreptococcal level of pantoyltaurine in experimental animals.

A study of the therapeutic effects of pantoyltaurine in experimental streptococcal infections was carried out by McIlwain and Hawking (264). They found that this substance was rapidly excreted even when large doses were injected. In mice pantoyltaurine was ineffective as a chemotherapeutic agent. This

result was shown to be due to the amount of pantothenic acid present in mouse blood. Rats, in which the blood pantothenate is lower, were completely protected by large frequent doses of pantoyltaurine. The simultaneous administration of pantothenic acid reversed the action of the antagonist, as it did *in vitro*. As the authors pointed out, the importance of these studies rests on the demonstration that, provided allowance is made for the concentration of the natural reversing agents present in blood and tissues, antagonists designed to inhibit specific essential metabolites can function *in vivo* as well as *in vitro*.

Attempts have been made to produce a pantothenic acid deficiency in animals by means of pantoyltaurine. Snell, Chan, Spiridanoff, Way, and Leake (383) reported obtaining symptoms of such a deficiency in mice by long-continued oral administration of pantoyltaurine. However, Woolley and White (459) and Unna (415) were unable to confirm these results.

In addition to pantoyltaurine, the following analogues of pantothenic acid were prepared by Barnett and Robinson (27):



I

Bisnordeoxypantothenic acid



II

Isonordeoxypantothenic acid



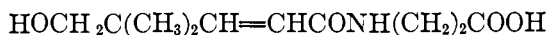
III

Homopantothenic acid



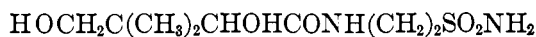
IV

Deoxypantothenic acid



V

Dehydrohomopantothenic acid



VI

Pantoyltauramide



VII

Homopantoyltaurine

These and several other derivatives synthesized by condensing pantolactone with various amino acids had very little growth factor activity.

The compounds prepared by Barnett and Robinson were studied as panto-

thenate antagonists by McIlwain (255). He found that the other taurine derivatives (VI and VII) behaved like pantoyltaurine, although they were in general less inhibitory to growth. The antistreptococcal index was 2000 for the tauramide derivative (VI) and 20,000 for VII, as compared with 500 for pantoyltaurine. With other susceptible organisms the same order of effectiveness was observed. Compounds I and II were slightly bacteriostatic for streptococci, and more so for coli and staphylococci, but pantothenic acid did not reverse the effect of these two substances. The other derivatives were inactive as antagonists. To account for the observation that the taurine derivatives in general only inhibited the growth of organisms requiring preformed pantothenic acid, McIlwain proposed the following possible explanations: (a) Destruction of the inhibitor during growth; (b) production of large amounts of pantothenic acid; or (c) other inherent differences in enzyme make-up or permeability. It was possible to rule out both (a) and (b) in the case of *E. coli*, but the factors in (c) were more difficult to assess. In relation to (b) McIlwain demonstrated that pantoyltaurine did not stimulate coli to produce more pantothenate. He also suggested that pantothenic acid produced *in situ* might be a more effective reversing agent than when supplied in the culture medium.

By successive subculturing in increasing concentrations of pantoyltaurine, McIlwain (259) obtained resistant strains of streptococci and diphtheria bacilli. Fast strains of streptococci were found to be no more resistant to sulfanilamides than the parent organisms and, conversely, sulfanilamide-fast bacteria were just as susceptible to pantoyltaurine as the parent strains. The pantoyltaurine-resistant streptococci retained a need for pantothenic acid, whereas the requirements of diphtheria bacilli changed, so that strains originally requiring pantothenate were able to grow with β -alanine and produced pantothenate when they became fast to pantoyltaurine. The resistant streptococci apparently neither produced more pantothenate nor destroyed the pantoyltaurine. Resistant diphtheria bacilli were also obtained by "training" without the use of pantoyltaurine. Organisms "trained" to utilize β -alanine efficiently were more resistant to pantoyltaurine, while those which became dependent on pantothenic acid were more susceptible.

McIlwain (259) also found that the growth of *Proteus morganii*, which was resistant to pantoyltaurine, was inhibited by a combination of this antagonist and salicylate [*cf.* Ivanovics (186)]. Concentrations of these substances which alone were without effect produced complete bacteriostasis in combination. A similar synergistic action was observed with both the normal and resistant streptococci. Pantothenate reversed the combined bacteriostatic action in all cases.

In a later investigation McIlwain (260) reported that two strains of *Proteus morganii* were susceptible to pantoyltaurine, and visible growth was prevented by ratios of antagonist to pantothenate of 2×10^5 . He also studied the mode of action of pantoyltaurine by determining its effect on the gas production of streptococci under anaerobic conditions. Although the effects of the antagonist could be reversed by pantothenate, they appeared to be too small to account for

the marked effect of pantooyltaurine on the growth of the organism. McIlwain concluded "that pantothenate is of importance to the organism because of the products to which it normally leads, whose presence in adequate concentrations is necessary for normal growth. When pantooyltaurine affects growth, it is believed to lower the rate of formation of these products to such an extent that they limit the rate of growth of the organism."

McIlwain and Hughes (265) reported that pantooyltaurine prevented the disappearance of pantothenate from streptococcal cultures, which normally occurred in its absence. This metabolism of pantothenate was associated with carbohydrate metabolism but was independent of growth and oxygen supply. Other substances which prevented glycolysis reduced pantothenate metabolism, but pantooyltaurine stopped this metabolism without interfering with glycolysis. Streptococci were shown by McIlwain (262) to inactivate pantothenic acid (i.e., destroy its growth-promoting properties) at the rate of about 2 millimicromoles per milligram of dry weight per hour. Pantooyltaurine was also inactivated by these organisms, although at a slower rate. Of various bacterial species studied, five which synthesized pantothenic acid were insensitive to pantooyltaurine, while six requiring the preformed metabolite for growth were sensitive in varying degrees to the antagonist. However, there did not appear to be any direct relationship between the rate at which the organisms destroyed pantothenic acid and their sensitivity to pantooyltaurine.

After determining the inhibition ratios of a group of pantothenic acid antagonists and related compounds (25, 26, 274), McIlwain and Hughes (266) showed that these ratios could be correlated with the effectiveness of the antagonists in preventing pantothenate destruction. Similarly, the relative antibacterial activity of pantooyltaurine against various microorganisms was directly related to its protective action. Analogous compounds which were not reversed or were inactive as growth inhibitors did not protect pantothenic acid from inactivation by the organisms. These results suggested that the two phenomena, metabolism and growth, were closely related, and that the effect of antagonists on growth might be associated with their inhibition of pantothenate inactivation. McIlwain (263) found that, while there was a latent period in the growth-inhibitory action of pantooyltaurine, pantothenate inactivation was affected immediately. However, he did not consider that this observation was inconsistent with a causal relation between the two inhibitions, because the latter process was probably carried out by the organisms in considerable excess of their minimal needs.

McIlwain (263a) also attempted to demonstrate a displacement of pantothenate in bacterial cells by pantooyltaurine. No liberation of firmly bound pantothenate was observed in the presence of excess pantooyltaurine. Larger quantities of the metabolite which were loosely associated with the organism (β -hemolytic streptococci) were released into saline solutions, but pantooyltaurine did not increase the quantity of loosely bound pantothenate released. These results were taken as additional evidence that the antagonist acts by preventing the binding of pantothenate in functioning form by susceptible organisms, rather than by the displacement of firmly bound metabolite.

While studying the biological action of α -methylpantothenic acid, Pollack (324) found that high concentrations of the compound repressed the growth-



VIII

 α -Methylpantothenic acid

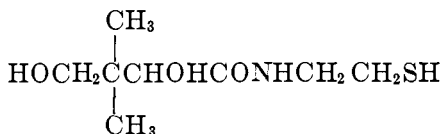
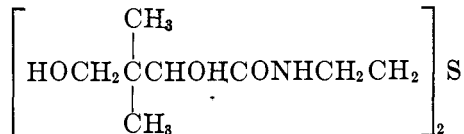
IX

 β -Methylpantothenic acid

promoting action of pantothenic acid on *Lactobacillus casei*. This repression increased with concentration only up to a point, beyond which additional amounts led to slightly increased growth. When it occurred, the growth-inhibiting action of VIII could be reversed by the addition of excess pantothenic acid. With a yeast strain the α -methyl derivative produced some growth stimulation, but this effect could not be attributed to hydrolysis of the compound followed by utilization of the pantoic acid. Under certain conditions Pollack found that α -methyl- β -alanine also appeared to inhibit the growth of yeast to a slight degree.

Nielsen, Hartelius, and Johansen (308) found that β -methylpantothenic acid (426) inhibited the growth of *Streptobacterium plantarum*, the ratio of antagonist to pantothenic acid being about 5000:1. With ratios as low as 200:1, growth was inhibited approximately 50 per cent. The effect of IX on the growth of yeast cells was apparent only in the presence of higher ratios (20,000-50,000:1), although the respiration of these cells was inhibited at somewhat lower levels. Shive and Snell (375) also examined the effect of crude VIII and IX on several microorganisms. In general, racemic IX appeared to be a somewhat more effective antagonist, and with some organisms (e.g., *Leuconostoc mesenteroides* P-60) VIII did not produce complete inhibition of growth. In confirmation of Pollack's work (324), it was found that VIII inhibited growth only to the level corresponding to its own stimulatory effect.

Barnett (25) prepared several analogues of pantoyltaurine from β -mercaptoethylamine, β, β' -diaminodiethyl sulfide, and the corresponding disulfide. From the sulfide, the sulfoxide and sulfone were obtained. The *N*-pantoyl derivatives of these compounds, e.g.,

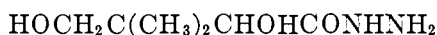
*N*-Pantoyl- β -mercaptoethylamine β, β' -Di(*N*-pantoylaminoethyl) sulfide

inhibited the growth of *Lactobacillus arabinosus*. The mercapto and disulfide compounds were reported to be as active as pantoyltaurine, while the sulfide, sulfoxide, and sulfone were less effective. All of the pantoyl derivatives were

reversed by pantothenic acid. *In vivo* tests in rats infected with streptococci apparently did not indicate that these analogues were as effective as pantoyltaurine, although no experimental details were reported.

Substitution of phenyl for methyl groups, or the introduction of a phenyl substituent alpha to the sulfonic acid group in pantoyltaurine was reported by Barnett, Dupré, Holloway, and Robinson (26) to yield inactive compounds. An attempt was also made to decrease the rate of excretion by esterification of the α -hydroxyl with *p*-toluenesulfonyl chloride, in the hope that this derivative might gradually break down *in vivo* to pantoyltaurine. However, the compound was inactive when tested against experimental streptococci infections in rats.

Madinaveitia, Martin, Rose, and Swain (274) prepared and studied a series of pantamide derivatives including the amide itself, the hydrazide (X), and dialkylamino-alkylamino, phenylalanine, hexahydroanthranilic acid and other derivatives as well as several β -aminoethyl phenyl sulfones such as XI. A majority of these compounds acted as pantothenic acid antagonists when *L. casei* ϵ and streptococci were the test organisms. X was the most potent



X

dl-Panthydrazide



XI

dl- β -(*N*-Pantoylaminoethyl) *p*-aminophenyl sulfone

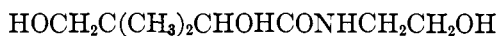
antagonist in the presence of low concentrations of pantothenic acid. At higher concentrations of the metabolite, however, this derivative and others, including XI and the corresponding *p*-methyl and *p*-methoxy derivatives, were similar to pantoyltaurine in activity. The antibacterial action of compound XI was reversed by pantothenic acid (2.5 γ /cc.) but not by PABA (1/1000), indicating that antagonism of the latter metabolite was not contributing to the antibacterial action. *In vivo* XI showed a slight chemotherapeutic effect in experimental streptococcal infections in rats.

Several alcohols and related compounds were investigated by Snell and Shive (375, 386), who showed that pantothenyl alcohol (386) (XII) and other alcohols



XII

Pantothenyl alcohol



XIII

dl-*N*-Pantoylethanolamine



XIV

N-Pantoyl-4-amino-2-butanol

XV

N-Pantoylisoserine

(XIII, XIV, and XV) inhibited the growth of several microorganisms. These derivatives were not obtained in pure form, but it was found (386) that XII prepared from *d*(-)-pantolactone was twice as active as the product from *dl*-lactone and about ten times more effective than XII from *l*(+)-pantolactone. With *Leuconostoc mesenteroides* P-60 growth stimulated by pantothenic acid was inhibited by XII over a wide range of concentrations. The competitive nature

TABLE 12

Comparative susceptibility of various organisms to inhibition by pantothenyl alcohol and pantoyltaurine (386)

ORGANISM	ANTIBACTERIAL INDEX*	
	Pantothenyl alcohol	Pantoyltaurine
<i>Leuconostoc mesenteroides</i> P60.....	300	200,000
<i>Lactobacillus acidophilus</i> VT.....	5,000	5,000
<i>Lactobacillus arabinosus</i>	5,000	1,000
<i>Lactobacillus casei</i>	10,000	10,000
<i>Lactobacillus fermentum</i>	100,000	200,000
<i>Streptococcus fecalis</i> R.....	50,000	30,000
<i>Streptococcus lactis</i> 125.....	1,000	
<i>Saccharomyces cerevisiae</i> FB.....	30,000	10,000
<i>Staphylococcus aureus</i>	Not inhibited†	
<i>Escherichia coli</i>	Not inhibited†	Not inhibited

* Ratio antagonist: metabolite for complete inhibition of growth.

† Maximum concentration tested, 3 mg./cc.

of this action was demonstrated by the constant ratio (300:1). A physical mixture of 3-amino-1-propanol and *d*(-)-pantolactone did not show any antagonistic activity. No bacteriostatic effect was observed with organisms not requiring pantothenate, such as *Staph. aureus* and *E. coli*. When XII and pantoyltaurine were compared, a marked variation in the susceptibility of different organisms to these two antagonists was observed (table 12).

Woolley and Collyer (454) synthesized and studied phenyl pantothenone, a



Phenyl pantothenone

ketone analogue of pantothenic acid. This compound inhibited the growth of a number of microorganisms. As indicated in table 13, higher concentrations of pantothenic acid reversed the growth inhibition in a number of instances. How-

ever, in two cases¹² the antagonism of organisms not requiring preformed pantothenate was not prevented by the metabolite. With *Staph. aureus*, pantothenic acid was only stimulatory, and the antibacterial effect of phenyl pantothenone was reversible. A sample of what appeared to be impure methyl pantothenone was also studied by Woolley and Collyer. The growth-inhibitory effect of this product was not prevented by pantothenic acid when either *L. casei* or *S. cerevisiae* was the test organism.

Shive and Snell (375a) prepared a group of *dl-N*-pantoylalkylamines as well as the corresponding derivatives of several substituted alkylamines. All of these compounds inhibited the growth of several microorganisms which required preformed pantothenic acid. The different antagonists varied considerably in their inhibitory effect on the same organism, and the relative activity of different

TABLE 13

Amounts of phenylpantothenone required to reduce growth of various microorganisms (454)*

ORGANISM	ANTAGONIST γ /cc.	PANTOTHENIC ACID REVERSAL	PANTOTHENIC ACID REQUIREMENT
<i>Lactobacillus casei</i>	54	+	Required
<i>Lactobacillus arabinosus</i>	180	+	Required
<i>Streptococcus hemolyticus</i> (Group B).....	60	+	Required
<i>Escherichia coli</i> †.....	2000	-	Not required
<i>Staphylococcus aureus</i>	140	+	Not required but slightly stimula- tory
<i>Saccharomyces cerevisiae</i>	33	-	Required but re- placeable by β - alanine
<i>Endomyces vernalis</i>	39	-	Not required

* Half-maximum growth in the presence of 0.04 γ /cc. of pantothenic acid.

† Complete inhibition of growth not obtained with this organism.

compounds, even in a homologous series, changed markedly with different organisms. In general, the inhibition ratios were of the same order of magnitude as those for pantothenyl alcohol.

A large series of pantoyltauramides was prepared by Winterbottom, Clapp, Miller, English, and Roblin (437). Included in this series were aliphatic aromatic and heterocyclic amine derivatives, many of which possessed a high degree



Pantoyltauranilide

of antibacterial activity. White, Lee, Jackson, Himes, and Alverson (433) found that the inhibition ratio for the most active members, e.g., pantoyltaura-

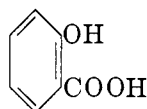
¹²The effect on *E. coli*, because of the relatively high concentrations required, may not be specific. In general, specific antagonism in the absence of reversal can only be deduced by analogy.

mido-4-chlorobenzene and pantoyltauramido-3,5-dibromobenzene, was approximately 25:1 for the *d*(+)-isomers. Several strains of streptococci and pneumococci were susceptible to these derivatives *in vitro*, but a number of other organisms tested were resistant to high concentrations (128 mg./100 cc.). Most of the resistant organisms presumably did not require preformed pantothenic acid. With a group A streptococcus in a complex medium containing 0.02 mg./100 cc. of pantothenic acid, White *et al.* obtained the following results for *d*(+)-pantoyltauranilide:

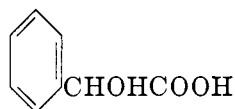
PANTOTHENIC ACID		M.E.C.	INHIBITION RATIO
Added	Total		
mg./100 cc.	mg./100 cc.	mg./100 cc.	
	0.02	1	50
0.3	0.32	16	50
1.5	1.52	64	42
6.3	6.32	128	20

The M.E.C. represented the minimum effective concentration required to inhibit growth completely for 48 hr. *In vivo*, mice were protected from experimental streptococcal infections by single oral doses of about 0.5 g./kg. of the halogen-substituted anilides. These results contrasted sharply with the activity of pantoyltaurine, which was ineffective orally and only showed chemotherapeutic activity in rats when large frequent doses were administered by injection (264).

While a majority of the antagonists probably interfere with the utilization of pantothenic acid, substances which may interfere with its synthesis have also been described. Ivanovics (185, 186) found that, in addition to a non-specific protein-denaturing action at high concentrations (0.1–0.25 *M*), small amounts of salicylic acid appeared to prevent the synthesis of pantothenate. Acetyl and



Salicylic acid



Mandelic acid

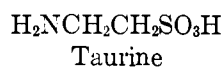
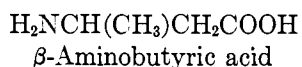
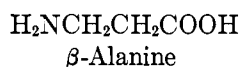
phenyl salicylates were also effective, but thiosalicylic acid and salicylamide were not. As in the case described by Fildes (124), no constant ratio between salicylate and pantothenate was observed. With staphylococci, pantolactone reversed the growth inhibition of salicylate but it was much less efficient in this respect than pantothenic acid, while β -alanine had no reversing action. *Proteus morgani*, requiring preformed pantothenate, was unaffected except by high concentrations of salicylic acid. These observations led Ivanovics to conclude that the antagonism occurred at the synthesis stage, probably in the production of pantoic acid or its conversion to pantothenic acid. In a simple medium the growth of *E. coli* was inhibited by 0.00002 *M* salicylate. The presence of protein hydrolysates

almost completely eliminated this antibacterial action. Ivanovics showed that a mixture of amino acids produced the same result. Valine alone was the most efficient but this and other amino acids, including methionine, lysine, leucine, and isoleucine in combination, were still more active in reversing salicylate. *Coli* produced eight times as much pantothenate in a casein hydrolysate medium, and it was concluded that some of the amino acids might be involved in the synthesis of pantolactone.

Stansly, Schlosser, and Alverson (394, 395) confirmed Ivanovics' observations with *E. coli* and demonstrated that pantoate ion is approximately nine times more active than pantolactone in reversing the antibacterial action of salicylate. Pantoyltaurine, presumably because it is partially hydrolyzed to pantoic acid, was also shown to prevent the bacteriostatic effect of salicylic acid (394). The formation of pantoic acid or of pantolactone was suggested as a possible explanation for the ineffectiveness of pantoyltaurine as an antibacterial agent for microorganisms which can utilize these intermediates for the synthesis of pantothenic acid.

Péroult and Greib (318) reported that, like salicylic acid, the antibacterial action of mandelic acid in concentrations of 1 per cent or less was reversed by pantothenic acid ($M/10^6$). The activity of higher concentrations of mandelic acid was only slightly affected by pantothenic acid ($M/1000$), while PABA had no effect on the bacteriostatic activity. However, with three strains of *E. coli* which were susceptible to mandelic acid in concentrations of less than 1 per cent, White (432) was unable to demonstrate any reversal with pantothenic acid.

Nielsen (306) observed that the growth-stimulating action of β -alanine



on yeast was antagonized by β -aminobutyric acid. In the absence of β -alanine this acid had neither growth-promoting nor growth-inhibiting action. Taurine, β -hydroxypropionic acid, and β -alanylglycine were inert. The following results were obtained by Nielsen and Johansen (309):

β -AMINOBUTYRIC ACID	MILLIGRAMS OF DRY YEAST PER 100 CC.		
	No β -alanine	β -Alanine (2 mg./100 cc.)	<i>dl</i> -Sodium pantothenate (1 mg./100 cc.)
mg./100 cc.			
20	11.4	12.2	26.6
10	11.6	15.6	26.2
6	11.2	17.6	26.8
2	11.8	25.8	26.8
0	11.8	34.8	26.0

These investigators concluded that β -aminobutyric acid interfered with the synthesis of pantothenic acid from β -alanine by yeast cells. They found isoserine, but not *N*-methyl- β -alanine, to have a similar action and suggested that the

antagonists might actually be converted to inert compounds containing β -aminobutyric acid or isoserine instead of β -alanine.

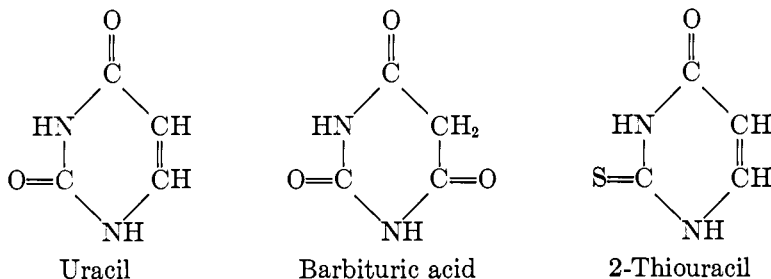
The effect of β -aminobutyric acid on the respiration of yeast was studied by Hartelius (163). His results were essentially the same as those obtained by Nielsen and Johansen (306, 309) in their growth studies. The inhibitory effect of β -aminobutyric acid on oxygen consumption was prevented by β -alanine or sodium pantothenate. Respiration stimulated by β -alanine was completely repressed by a ratio of approximately 1000:1, but the minimum effective concentration of pantothenate prevented the action of varying amounts of the antagonist. These observations also suggested that β -aminobutyric acid interfered with the synthesis rather than with the utilization of pantothenic acid.

Sarett and Cheldelin (361) investigated the effect of taurine and pantoyltaurine on 17 strains of yeast. Growth was stimulated with either β -alanine or pantothenic acid. When β -alanine (0.2–0.5 γ) stimulated growth, only one strain was inhibited 85–90 per cent with 1 mg. of taurine or 2 mg. of pantoyltaurine, all the other strains being unaffected by either antagonist. With pantothenic acid as the growth stimulant, taurine had no effect on any of the strains, but the growth of all of them was inhibited in varying degrees by pantoyltaurine.

H. Purines and pyrimidines

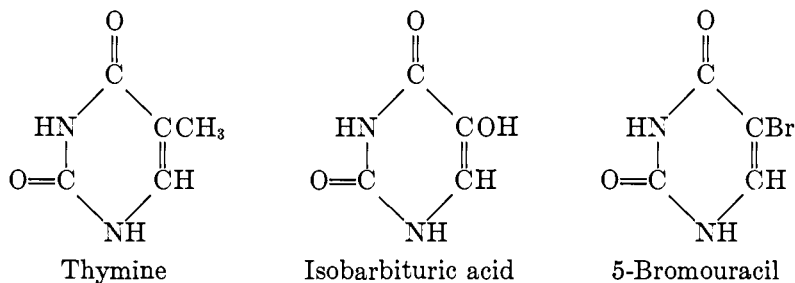
Cell nuclei are composed largely of nucleoproteins, in which are incorporated the pyrimidine- and purine-containing nucleic acids. In addition, some of these substances in the form of nucleotides function as coenzymes or are an important part of other coenzyme molecules. The pyrimidines and purines are now recognized as growth stimulants for many microorganisms (243).

Woods (444, 445) reported that $M/100$ – $M/250$ barbituric acid delayed the growth of *Staph. aureus* in a synthetic medium. The addition of uracil ($M/5000$ or less) abolished this effect and restored the normal growth rate. These observations have been confirmed by Roepke and Jones (347) with *E. coli*. They also found that 2-thiouracil produced an even greater growth inhibition than



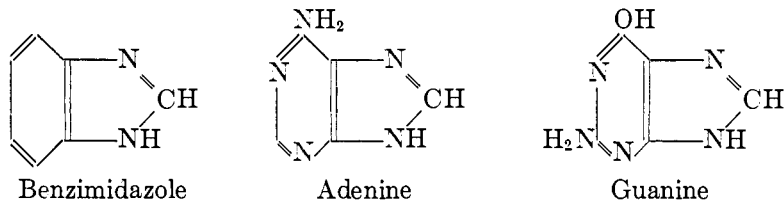
barbituric acid. In both cases uracil prevented the bacteriostatic action. The effect of 2-thiouracil was apparent only for relatively short periods of time, and high concentrations were somewhat less effective, suggesting that this substance might be gradually converted to uracil or otherwise degraded by the organisms.

Relatively large amounts of thymine will replace folic acid as a growth stimulant for certain organisms such as *L. casei* and *Strep. fecalis* R (402). Hitchings, Falco, and Sherwood (176) noted that several 5-substituted pyrimidines related to thymine, such as uramil and isobarbituric acid, inhibited the growth of *L. casei* stimulated by either thymine or folic acid. Growth could be restored by



increasing the concentration of either metabolite. Other 5-substituted derivatives, e.g., 5-bromouracil, completely inhibited thymine-stimulated growth, but had no effect, or produced a slight stimulation, when folic acid was used as the nutrient. To some extent, the reverse situation was observed with 5-nitouracil. The authors concluded that the latter results did not fit the concept of thymine as a product of an enzyme system involving folic acid as a prosthetic group or coenzyme (402). They suggested that the two metabolites might act in some fashion as alternatives rather than as two components of an anabolic system.

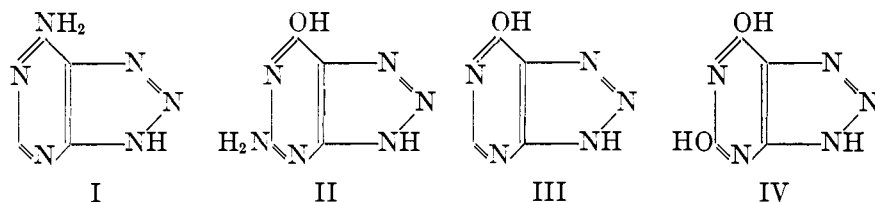
In a study of the biological effects of benzimidazole, Woolley (447) observed that the bacteriostatic action of this substance could be reversed by adenine and guanine. The latter was the more active in overcoming the effect of benzimidazole, while xanthine, hypoxanthine, and adenylic acid were ineffective in this



respect. When *S. cerevisiae* was the test organism uracil also produced no reversal, but with *Strep. fecalis* R a partial reversal was observed with relatively small concentrations of this pyrimidine. All of the organisms tested, both yeasts and bacteria, were susceptible to benzimidazole. Woolley prepared 4-aminobenzimidazole, the benzene analogue of adenine, by reduction of the 4-nitro derivative. Both of these compounds inhibited growth to about the same extent as benzimidazole. 5-Aminobenzimidazole was less effective, and the 2-hydroxy derivative showed no bacteriostatic action.

Roblin, Lampen, English, Cole, and Vaughan (341) synthesized analogues of adenine, guanine, hypoxanthine, and xanthine, in the triazolopyrimidine series,

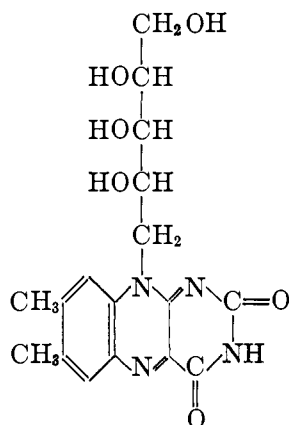
in which a nitrogen atom replaces a carbon atom of the corresponding purine. Of these, the adenine and guanine analogues (I and II) in particular exhibited



a specific antibacterial action. The results obtained with the triazolopyrimidines are summarized in table 14.

When several M.E.C.'s of these compounds were employed, the growth inhibition of *E. coli* by compound I was reversed only by adenine and hypoxanthine, guanine, xanthine, uracil, cytosine, or thymine being ineffective. Similarly with II, guanine and xanthine were the only substances tested which prevented its bacteriostatic action. When barely effective amounts of the analogues were tested, the results were less specific in that all four of the purines were more or less equivalent as reversing agents. The action of the hypoxanthine analogue (III) was apparent for short periods of time only, and again all four purines prevented it. No specific antibacterial action could be demonstrated for compound IV. In combination with sulfonamides, the guanine analogue produced a synergistic effect, so that much less of each was required to produce the same degree of bacteriostasis. None of the other triazolopyrimidines displayed this effect. Sulfonamide-fast organisms were no more resistant to II than the parent strains.

I. Riboflavin



6,7-Dimethyl-9-(*d*-1'-ribose)isoalloxazine

Riboflavin in the form of coenzymes takes part in a number of enzyme systems involved in hydrogen transfer for many important metabolic processes, including

carbohydrate and amino acid metabolism. Two such coenzymes have been identified,—a riboflavin phosphate and a riboflavin-adenine-dinucleotide. A great many flavin compounds have been synthesized and tested for vitamin activity. The substitution of other monosaccharides for *d*-ribose, in general, yielded inactive compounds. Elimination of a methyl group or substitution of one methyl by an ethyl group resulted in reduced activity, while absence of both methyl groups was accompanied by high toxicity (351).

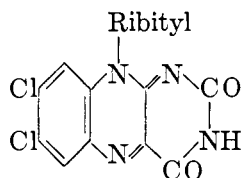
On the basis of mixed-crystal formation between methyl- and chloro-substituted compounds in the benzene series, Kuhn, Weygand, and Möller (209) prepared 6,7-dichloro-9-(*d*-1'-ribityl)isoalloxazine (dichloroflavin). Although

TABLE 14

Bacteriostatic activity and molecular inhibition ratios of triazolopyrimidines (341)

	COMPOUND	ANALOGUE OF	M.E.C.		INHIBITION*	
			<i>E. coli</i> mg./100 cc.	<i>Staph. aureus</i> mg./100 cc.	By	Ratio
I	7-Amino-1- <i>v</i> -triazolo- <i>[d]</i> -pyrimidine	Adenine	8	128	A H	640 640
II	5-Amino-7-hydroxy-1- <i>v</i> -triazolo- <i>[d]</i> pyrimidine	Guanine	64	4	G X	128 35
III	7-Hydroxy-1- <i>v</i> -triazolo- <i>[d]</i> -pyrimidine	Hypoxanthine	64	16	A H	6400 6400
IV	5,7-Dihydroxy-1- <i>v</i> -triazolo- <i>[d]</i> pyrimidine	Xanthine	128	128		

* With *E. coli*: A, adenine; H, hypoxanthine; G, guanine; X, xanthine. Ratio indicates number of moles of antagonist required to prevent visible growth in the presence of one mole of purine.



6,7-Dichloro-9-(*d*-1'-ribityl)isoalloxazine

this analogue possessed no vitamin activity, it was found to be a potent riboflavin antagonist. With the exception of yeast, the growth of all the organisms tested was inhibited by approximately 0.01 mg./cc. The antibacterial action was observed both with organisms requiring preformed riboflavin and with bacteria not dependent on an external source of the vitamin. A number of other analogues were tested for antiriboflavin action, but none of these possessed more than slight activity. Kuhn *et al.* observed that the reversing action of riboflavin on dichloroflavin varied with time. The following ratios were obtained for 50 per cent of normal growth:

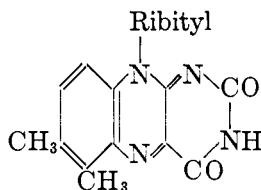
TIME	RATIO DICHLOROFLAVIN:RIBOFLAVIN	
	<i>Staphylococcus aureus</i>	<i>Streptobacterium plantarum</i>
days		
2		25:1
3	50:1	60:1
4	70:1	130:1
6	280:1	165:1

When the redox potentials of the vitamin and antivitamin were compared, it was found that the dihydro derivative of dichloroflavin was a considerably weaker reducing agent. At pH 7 the values were:

6,7-Dichloro-9-(*d*-1'-ribityl)isoalloxazine $E_0 = 0.095$ volt

6,7-Dimethyl-9-(*d*-1'-ribityl)isoalloxazine $E_0 = 0.185$ volt

Emerson and Tishler (105) reported that isoriboflavin (5,6-dimethyl-9-(*d*-1'-ribityl)isoalloxazine) suppressed growth both in riboflavin-deficient rats and in animals receiving a suboptimal intake of the vitamin. The growth-

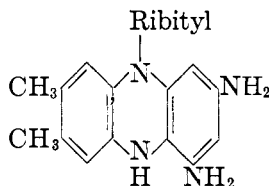


Isoriboflavin

depressing effect of 2 mg. per day of isoriboflavin was almost completely overcome by the daily administration of 40 γ of riboflavin.

With *L. casei* Foster (129) was unable to demonstrate any growth inhibition with isoriboflavin, even when it was tested at 100,000 times the concentration of riboflavin. In growth experiments with the same organism the isomer showed negligible (less than 0.5 per cent) riboflavin activity. Similar results were obtained by Sarett (359), who found that 1-ribitylamino-2-amino-4,5-dimethylbenzene also possessed only very slight riboflavin activity for the same organism.

Woolley (448) prepared 2,4-diamino-7,8-dimethyl-10-*d*-ribityl-5,10-dihydrophenazine, which is also structurally related to riboflavin. This compound in-

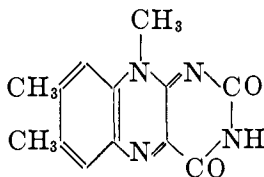


2,4-Diamino-7,8-dimethyl-10-*d*-ribityl-5,10-dihydrophenazine

hibited the growth of *L. casei*, while the corresponding dinitro derivative did not. The growth inhibition, measured by determining the pH of the culture medium,

was prevented by appropriate amounts of riboflavin. Because of the instability of the diamino compound, the dinitro derivative was frequently reduced *in situ* with finely divided iron. With one exception, Woolley found that organisms not requiring preformed riboflavin for growth were not inhibited by the diamino-phenazine. Signs of a riboflavin deficiency (reduced rate of growth, unkempt fur, and sometimes hyperirritability) were produced by both the dinitro and the diamino derivatives. Sufficient amounts of riboflavin prevented the appearance of these deficiency signs.

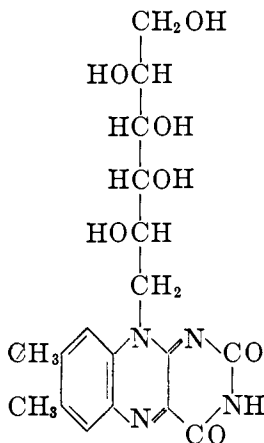
Sarett (359) reported that lumiflavin had both an inhibitory and a stimulatory action on the use of riboflavin for growth by *L. casei*. In the presence of minimal



Lumiflavin

amounts of riboflavin, lumiflavin (10–300 γ /10 cc.) inhibited growth. As the concentration of riboflavin was increased, however, a zone was reached where lumiflavin exerted no effect, and finally with still more metabolite lumiflavin showed an augmentative action on growth.

The effect of galactoflavin (6,7-dimethyl-9-(*d*-1'-dulcetyl)isoalloxazine) on the growth and survival of young rats was investigated by Emerson, Wurtz, and Johnson (105a). The antagonist was found to suppress the growth and increase

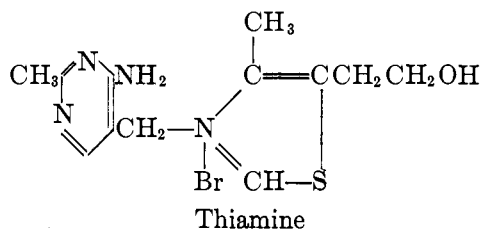


Galactoflavin

the mortality rate of rats on a riboflavin-free diet. Furthermore, the growth of animals receiving a normal supplement of the vitamin (40 γ) was markedly suppressed by the simultaneous administration of 2.16 mg. of galactoflavin. Larger quantities of riboflavin almost completely counteracted this effect.

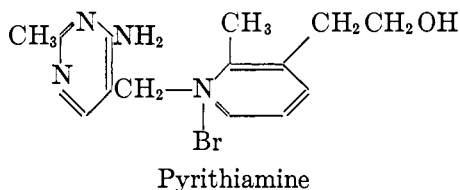
J. Thiamine; cocarboxylase

Like nicotinamide and riboflavin, thiamine is an integral part of a coenzyme



of vital importance in cell metabolism (400). Cocarboxylase (the pyrophosphoric acid ester of thiamine), together with its apoenzyme¹³, catalyzes the oxidation of pyruvic acid to acetic acid and carbon dioxide. A number of microorganisms require preformed thiamine and, of course, it is well known as a vitamin for higher animals. Although many substances chemically related to thiamine have been tested for vitamin activity, only a few have been investigated as antagonists.

Robbins (339) studied the effect on fungi of a thiamine isoster, 2-methyl-4-amino-5-pyrimidylmethyl(2-methyl-3-β-hydroxyethyl)pyridinium bromide, for which Woolley and White (457) proposed the name pyrithiamine. This compound was first synthesized by Tracy and Elderfield (413). Three species of



fungi with varying synthetic abilities were employed by Robbins (339). The growth of *Phytophthora cinnamomi*, which required preformed thiamine, was inhibited by pyrithiamine. Thiamine prevented the growth inhibition but the pyrimidine and thiazole portions of the thiamine molecule were ineffective in this respect. *Phthiomorpha gonapodioides*, unable to synthesize the pyrimidine portion of thiamine, grew well in the presence of small concentrations of pyrithiamine, but higher concentrations were inhibitory. Either thiamine or its pyrimidine moiety reversed the growth inhibition, the former being the more effective. With *Phycomyces Blakesleeanus* (able to combine the pyrimidine and thiazole portions but unable to synthesize either), no improvement in growth was observed with small concentrations of pyrithiamine and an inhibitory effect was obtained with larger amounts. Small quantities of pyrithiamine, which did not stimulate growth, produced good growth when combined with the thiazole portion. Robbins concluded that both of the latter fungi might obtain the pyrimidine moiety of thiamine by splitting pyrithiamine.

¹³The combination of coenzyme and apoenzyme (specific protein) is frequently referred to as the holoenzyme. In this case the holoenzyme is called carboxylase.

The relationship between thiamine and pyrithiamine was investigated further by Woolley and White (457, 458). In mice, they observed that pyrithiamine produced characteristic signs of thiamine deficiency. As little as 20 γ of pyrithiamine per day eventually produced deficiency symptoms and the death of the animals. All of the symptoms could be prevented or cured with appropriate amounts of thiamine.

Woolley and White (458) also studied the effect of pyrithiamine on the growth of a number of species of bacteria, yeasts, and fungi. These investigators observed that organisms requiring preformed thiamine for maximal growth were unable to grow in the presence of small amounts of pyrithiamine. Thiamine prevented this growth inhibition, one part of the vitamin reversing ten parts of pyrithiamine. Strains which required only the pyrimidine or thiazole portions

TABLE 15
Inhibition ratio of pyrithiamine for various microorganisms (458)

ORGANISM	INHIBITION* RATIO	THIAMINE REQUIREMENT
<i>Ceratostomella fimbriata</i>	7	Intact thiamine
<i>Ceratostomella pennicillata</i>	10	Intact thiamine
<i>Phytophthora cinnamomi</i>	12	Intact thiamine
<i>Endomyces vernalis</i>	130	Pyrimidine†
<i>Mucor ramannianus</i>	800	Thiazole‡
<i>Saccharomyces cerevisiae</i>	800	Pyrimidine and thiazole
<i>Staphylococcus aureus</i>	2000	Pyrimidine and thiazole
<i>Salmonella gallinarum</i>	1000	Pyrimidine and thiazole
<i>Escherichia coli</i>	2,000,000	None
<i>Lactobacillus casei</i>	5,000,000	None
<i>Streptococcus fecalis</i> R.....	5,000,000	None

* Half-maximum inhibition.

† 2-Methyl-4-amino-5-ethoxymethylpyrimidine.

‡ 4-Methyl-5-hydroxyethylthiazole.

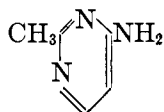
of the thiamine molecule were less susceptible. In these cases the inhibition ratio was about 1000:1. With organisms such as *E. coli*, for which thiamine was not a growth stimulant, pyrithiamine appeared to be ineffective even in concentrations 500,000 times as great as the amounts which inhibited the growth of sensitive organisms (table 15). The resistance of insusceptible bacteria could not be explained on the basis of the amount of thiamine synthesized or the production of some other reversing agent by the resistant organisms.

In a similar investigation with *Staph. aureus*, Wyss (462) obtained an inhibition ratio of pyrithiamine to thiamine of approximately 700:1 for half-maximum growth. He also found that his strain of *E. coli* was susceptible to pyrithiamine, although in this case the ratio was about 20,000:1. Wyss reported that non-injurious amounts of pyrithiamine failed to produce antibacterial concentrations in the blood of mice.

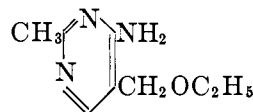
With a pyrithiamine-fast strain of yeast, Woolley (449) demonstrated that the resistance could be explained, at least in part, by a breakdown of pyrithiamine

by the yeast cells to yield the pyrimidine portion (2-methyl-4-amino-5-hydroxymethylpyrimidine) of thiamine. Both the parent yeast and the resistant strain could utilize the pyrimidine portion as well as intact thiamine. Since the fast strain grew more slowly in the absence of pyrithiamine, Woolley suggested that indirectly this antagonist served as a growth factor for the resistant yeast. His strain of *E. coli* also degraded the pyrithiamine molecule, an observation which might explain the variation in the susceptibility of different strains of this organism.

Sarett and Cheldelin (360) found that less pyrithiamine was required to inhibit the growth of *Lactobacillus fermentum* and *Penicillium digitatum* when cocarboxylase was used as a growth stimulant than when thiamine was employed. Large quantities of 2-methyl-4-aminopyrimidine and 2-methyl-4-amino-5-ethoxymethylpyrimidine also inhibited growth, and again less of either compound was required when cocarboxylase was the growth stimulant. The authors suggested



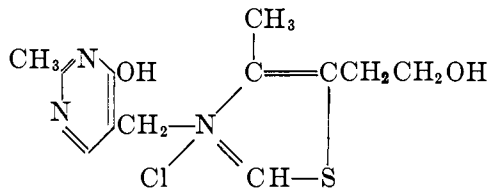
2-Methyl-4-aminopyrimidine



2-Methyl-4-amino-5-ethoxymethylpyrimidine

that these results indicate that thiamine is attached to its apoenzyme before being phosphorylated, and that cocarboxylase formed from thiamine is more firmly bound than that formed from added diphosphothiamine.

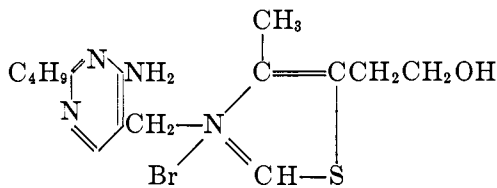
Oxythiamine (33), in which the amino group is replaced by hydroxyl, has been reported by Soodak and Cerecedo (388) to be toxic to mice on a low-



Oxythiamine

thiamine diet. It was also found to inhibit the fish principle (enzyme) which causes Chastek paralysis by destruction of thiamine.

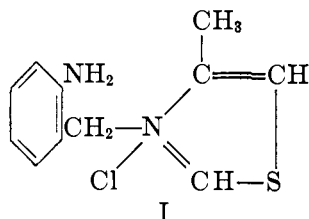
The *n*-butyl homologue of thiamine, 2-*n*-butyl-4-amino-5-pyrimidylmethyl-(2-methyl-3- β -hydroxyethyl)thiazolium bromide, was shown to possess anti-thiamine activity for rats by Emerson and Southwick (104a). Deficiency symp-



"Butylthiamine"

type compounds could be explained most readily on the basis of their effect on the respiratory enzymes. They pointed out that in their experiments PABA behaved like an inhibitor of catalysis and not like a catalyst.

During a study of the enzymatic nature of the fish principle which destroys thiamine, Sealock and Goodland (365) found that 3-(*o*-aminobenzyl)-4-methylthiazolium chloride (70) inhibited the breakdown of thiamine *in vitro*. Several

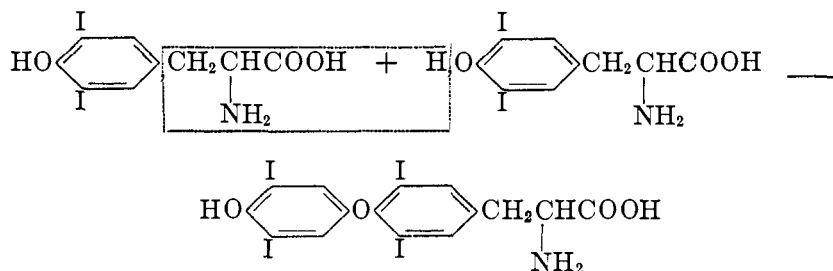


I
3-(*o*-Aminobenzyl)-4-methylthiazolium chloride

other thiazolium chloride derivatives less closely related structurally to thiamine were found to be only partly inhibitory or inactive, whereas I in 0.0005 *M* concentration completely prevented the destruction of an equal amount of thiamine¹⁴. Sealock and Goodland demonstrated that the extent of the inhibition of thiamine destruction was dependent on the ratio of the vitamin to the inhibitor. By use of the Lineweaver-Burk method, they showed that the benzylthiazolium compounds specifically inhibited thiamine destruction by competing with the vitamin for the enzyme. Calculation of the Michaelis constants indicated that the inhibitor compound combined somewhat more firmly with the fish principle than did thiamine.

K. Thyroxine

The active principle of the thyroid gland controls the metabolic rate of higher animals, and probably affects all tissue cells. Under the stimulus of the thyrotropic hormone of the anterior pituitary, thyroid hormone may be formed directly by the iodination of a polypeptide. Another possibility is the production of thyroxine through the iodination of tyrosine followed by a combination of two molecules of 3,5-diiodotyrosine as indicated (157, 158):



Thyroxine

¹⁴In this case, of course, the benzylthiazolium derivative (I) cannot be regarded as an antagonist of thiamine function, although future studies may show it to have such an action.

Overactivity of the thyroid gland in man results in hyperthyroidism, and much of the interest in thyroxine antagonists has been due to their application to the treatment of this condition (14, 174, 435). Numerous substances, mostly of biological origin, have been reported to inactivate the thyroid hormone or to suppress its formation in the thyroid gland (322). However, with the exception of those which are neutralized by iodine, no clear evidence regarding their action in relation to thyroxine has been advanced. Consequently, only substances of known structure which appear to inhibit the synthesis of thyroxine are covered in this review.

During a nutritional study with sulfaguanidine, Mackenzie, Mackenzie, and McCollum (271) observed a marked enlargement of the thyroid glands of rats receiving 1 or 2 per cent of sulfaguanidine in a purified diet. Striking histological changes in the glands were also found. The effects on the thyroid were not prevented by *p*-aminobenzoic acid or yeast. Richter and Clisby (337) noted a similar hyperplasia of the thyroid in rats fed phenylthiourea. They suggested that the hyperactive picture of the thyroid gland might result from the efforts of the animals to compensate for the reduction of metabolism produced by this substance. An attempt to isolate the goitrogenic substance present in rape seed led Kennedy (194) to study allylthiourea. He found that small (20 mg.) daily doses of this compound for eight weeks caused a three- to four-fold increase in the weight of the thyroid gland of rats.

Following up their earlier observations, Mackenzie and Mackenzie (269, 270) reported that, in addition to sulfaguanidine, a number of other sulfonamides as well as thiourea and several of its derivatives produced an enlargement of the thyroid glands of rats, mice, and dogs. The basal metabolic rate of rats receiving sulfaguanidine was reduced to -20 per cent. Sodium iodide was ineffective in preventing these effects, but small amounts of thyroxine or desiccated thyroid entirely prevented the thyroid enlargement and histologic changes. After removal of the thyroid gland, sulfaguanidine did not further depress the basal metabolic rates nor inhibit the response to small doses of thyroxine. Hypophysectomy likewise eliminated the thyroid alteration produced by the drug. On the basis of this evidence, the Mackenzies (270) concluded that the sulfonamides and thioureas probably exerted a depressing influence primarily on the functional activity (thyroxine formation) of the thyroid, and that thyroid enlargement was a reflection of increased pituitary activity resulting from this depression. Table 16 summarizes some of the results obtained with active compounds.

A number of other substances, most of which were inactive, were also investigated for antithyroid action (270). *N*⁴-Benzylsulfanilamide, in which the aromatic amino group is substituted, was practically inactive. Similarly, desamino-sulfamethyldiazine was inert, although the parent compound with the amino group present showed activity. *p*-Aminobenzoic acid also showed a slight effect. In contrast to thiourea and its diethyl and allyl derivatives, acetylthiourea and thioacetamide were reported to be inactive, as were urea and

phenylurea. Negative results were also obtained with cystine, cysteine, guanidine carbonate, sulfanilic acid, sulfonal, thiamine, and sodium and ammonium thiocyanates.

Astwood, Sullivan, Bissell, and Tyslowitz (19), after a very similar independent study, reached the same conclusions as Mackenzie and Mackenzie (270) regarding the mode of action of several sulfonamides and thiourea. They listed the compounds investigated in the following order of decreasing anti-thyroid activity: thiourea, sulfadiazine, sulfapyridine, sulfathiazole, sulfaguanidine, sulfanilylurea, sulfanilamide, and *N*⁴-succinylsulfathiazole. Not only was the goitrogenic action of sulfaguanidine completely prevented by desiccated thyroid powder, but the drug also had no influence on the toxic or calorogenic

TABLE 16
Effect of various compounds on the thyroid of young rats (270)

COMPOUND	PER CENT IN DIET	DAYS ON DIET	THYROID WEIGHT
			mg./100 g.
Sulfanilamide.....	1	58	14.0
Sulfathiazole.....	1.5	14	12.5
Sulfapyridine.....	1	14	12.0
Sulfapyridine.....	1	21	29.0
Sulfadiazine.....	0.75	45	47.0
Sulfamethyldiazine.....	0.75	45	25.0
Thiourea.....	0.05	14	13.0
Thiourea.....	0.5	14	37.4
Diethylthiourea.....	1	28	26.4
Allylthiourea.....	0.5	14	16.4
Controls.....		50	8*

* Approximate value.

action of thyroid hormone in normal or hypophysectomized rats. Astwood and coworkers concluded that the compounds under investigation acted by interfering with the synthesis of thyroid hormone. The sequence of events was considered to be as follows:

"Shortly after the drug is administered, the organism becomes unable to synthesize thyroid hormone at a normal rate, and the quantity of circulating hormone tends to fall. In response to this deficit an excess of thyrotropin is produced by the pituitary which stimulates the thyroid to hyperplasia and to the release of the normal thyroid hormone stored therein. Within 48 hours of the first administration of the drug these compensatory changes are histologically visible, and for a number of days this mechanism is adequate to maintain the metabolic rate at a normal level. Eventually, however, the store of normal thyroid hormone is exhausted, as evidenced by a complete loss of demonstrable colloid at the end of 7 to 10 days, and as new hormone can be made only at a reduced rate, the metabolic rate falls even though the thyroid hyperplasia is still advancing. The variable period observed to be required before the metabolic rate falls may be related to environmental or other factors which alter the thyroid hormone requirement and thus the time

necessary to deplete the normal stores. The actual level of hypometabolism reached under the influence of the drug may depend upon the degree to which the process of thyroid hormone synthesis is hindered, and may depend upon the dose and upon the efficiency of the compound in question."

A large number of compounds were investigated for antithyroid activity by Astwood (15), who found that the active substances fell into two general classes, compounds with the thioureyne grouping ($-\text{NH}-\overset{\text{S}}{\underset{\parallel}{\text{C}}}-\text{NH}-$) and certain

derivatives of aniline. The most potent compounds belonged to the former class. Of these, 2-thiouracil and 5,5-diethyl-2-thiobarbituric acid¹⁵ were the most active. Some of the data on a selected group of the thiourea derivatives tested are recorded in table 17. The antithyroid activity was graded as + to ++++ on the basis of the microscopic appearance of the thyroid glands after administration of the substances for 10 days in the food or drinking water.

In general, alkyl and aryl derivatives were not more active than thiourea itself, and most of them were more toxic, although *sym*-diethylthiourea was an exception. Substituted 2-thiohydantoins were more potent than thiourea but also considerably more toxic. 2-Aminothiazole also possessed some activity. Several other 5-substituted barbituric acids tested were less effective than the 5,5-diethyl derivative. In addition to the sulfonamides, a group of other aniline derivatives was studied. The *o*, *m*, and *p*-aminobenzoic acids, *p*-aminoacetanilide, and *p*-aminophenylacetic acid showed varying degrees of effectiveness, but with the exception of the sulfonamides none of the other aniline derivatives tested was active. Inorganic thiocyanates produced enlargement of the thyroid glands on prolonged administration; however, this effect was completely prevented by iodide. A large group of inactive compounds related to the active types was also reported by Astwood (15). These included uracil and several other pyrimidines, benzoic and *p*-nitrobenzoic acids, tyrosine, benzene and *p*-toluenesulfonamide, 3,5-diiodo-4-hydroxybenzoic acid, and a number of organic sulfur compounds. As a working hypothesis it was suggested that the active aniline derivatives acted through a competitive mechanism with the enzyme system responsible for the conversion of diiodotyrosine to thyroxine. The thiourea derivatives were considered as possible specific inhibitors of the same system.

Chapman (62) compared the antithyroid activity of 6-methyl-2-thiouracil with that of the parent compound and reported that the former was at least as active as thiouracil. Higgins (172) investigated the action of 2-sulfanilyl-5-aminothiazole (promizole), which he found to resemble thiourea and some of the sulfanilamides in its effect on thyroid activity. The decrease in metabolic rate

¹⁵This compound was originally reported as 2-thiobarbituric acid but a subsequent investigation by Astwood, Bissell, and Hughes (Endocrinology 36, 72 (1945)) indicated an error in labeling, and 2-thiobarbituric acid was found to be inactive.

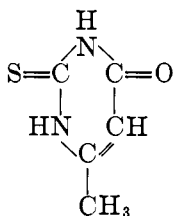
TABLE 17
Antithyroid activity of derivatives of thiourea (15)

COMPOUND	FORMULA	AVERAGE DOSE*	ACTIVITY
Thiourea.....	$\begin{array}{c} \text{S} \\ \parallel \\ \text{H}_2\text{NCNH}_2 \end{array}$	2.1 13.1	+ ++++
Methylthiourea.....	$\begin{array}{c} \text{S} \\ \parallel \\ \text{H}_2\text{NCNHCH}_3 \end{array}$	4.6 44.4	++ ++++
Thiosemicarbazide.....	$\begin{array}{c} \text{S} \\ \parallel \\ \text{H}_2\text{NCNHNH}_2 \end{array}$	5.1	+
Acetylthiourea.....	$\begin{array}{c} \text{S} \quad \text{O} \\ \parallel \quad \parallel \\ \text{H}_2\text{NCNHCCH}_3 \end{array}$	4.0 13.0	+ +++
Allylthiourea.....	$\begin{array}{c} \text{S} \\ \parallel \\ \text{H}_2\text{NCNHC}_3\text{H}_5 \end{array}$	2.1 4.9	+ +++
Guanylthiourea.....	$\begin{array}{c} \text{S} \quad \text{NH} \\ \parallel \quad \parallel \\ \text{H}_2\text{NCNHCNH}_2 \end{array}$	5.1	-
<i>sym</i> -Diethylthiourea.....	$\begin{array}{c} \text{S} \\ \parallel \\ \text{C}_2\text{H}_5\text{HNCNHC}_2\text{H}_5 \end{array}$	0.6 2.5	+ +++
Phenylthiourea.....	$\begin{array}{c} \text{S} \\ \parallel \\ \text{H}_2\text{NCNHC}_6\text{H}_5 \end{array}$	2.5	-
<i>sym</i> -Diphenylthiourea.....	$\begin{array}{c} \text{S} \\ \parallel \\ \text{C}_6\text{H}_5\text{NHCNHC}_6\text{H}_5 \end{array}$	530	++
Methylisothiourea sulfate.....	$(\text{HN}=\text{C}(\text{SCH}_3)\text{NH}_2)_2 \cdot \text{H}_2\text{SO}_4$	11.1	++
1-Acetyl-2-thiohydantoin.....	$\text{N}(\text{COCH}_3)\begin{array}{c} \text{S} \quad \text{O} \\ \parallel \quad \parallel \\ \text{CNHCCH}_2 \end{array}$	3.0 9.7	+ +++

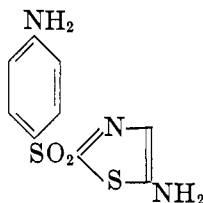
TABLE 17—Continued

COMPOUND	FORMULA	AVERAGE DOSE*	ACTIVITY
5-Benzal-2-thiohydantoin	$\begin{array}{c} \text{S} \quad \text{O} \\ \parallel \quad \parallel \\ \text{HNCNHCC}=\text{CHC}_6\text{H}_5 \end{array}$	1.3 16.5	+ ++++
2-Thiocyanuric acid	$\begin{array}{c} \text{S} \quad \text{O} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{HNCNHCNHC} \end{array}$	30.0	-
2-Thiouracil.....	$\begin{array}{c} \text{S} \quad \text{O} \\ \parallel \quad \parallel \\ \text{HNCNHCCH}=\text{CH} \end{array}$	0.6 2.3 3.6	+ ++ ++++
5,5-Diethyl-2-thiobarbituric acid.	$\begin{array}{c} \text{S} \quad \text{O} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{HNCNHCC}(\text{C}_2\text{H}_5)_2\text{C} \end{array}$	0.6 3.6 17.2	+ ++ ++++

* Milligrams per 100 g. of body weight per day in food or drinking water.



6-Methyl-2-thiouracil



2-Sulfanilyl-5-aminothiazole

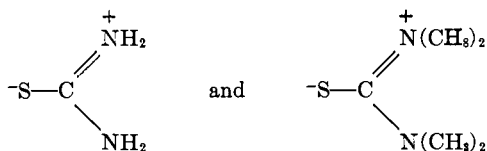
and other changes induced by promizole could be prevented by thyroxine and desiccated thyroid but not by sodium iodide.

In a subsequent study, Astwood, Bissell, and Hughes (16, 18) examined an additional group of over two hundred compounds. An improved method of assay (17), based on the weight and total iodine content of the thyroid glands of rats, was employed. Although not entirely satisfactory, this method permitted a somewhat more accurate comparison of the compounds. Thiouracil was assigned an arbitrary antithyroid activity of 1.0, and the relative activities of a representative group of organic thio compounds were listed as follows (16):

COMPOUND	RELATIVE ACTIVITY (THIOURACIL = 1.0)	COMPOUND	RELATIVE ACTIVITY (THIOURACIL = 1.0)
$\begin{array}{c} \text{NH}_2 \\ \\ \text{S}=\text{C} \\ \\ \text{NH}_2 \end{array}$ Thiourea.....	0.1	$\begin{array}{c} \text{N}=\text{N} \\ // \quad // \\ \text{HS}-\text{C} \quad \text{NH}-\text{CH} \end{array}$ 3-Mercapto-1,2,4-triazole...	0.3
$\begin{array}{c} \text{N}(\text{CH}_3)_2 \\ \\ \text{S}=\text{C} \\ \\ \text{N}(\text{CH}_3)_2 \end{array}$ Tetramethylthiourea.....	0.3	$\begin{array}{c} \text{N}=\text{N} \\ // \quad // \\ \text{HS}-\text{C} \quad \text{S}-\text{CNH}_2 \end{array}$ 2-Mercapto-5-amino-1,3,4-thiadiazole.....	1.6
$\begin{array}{c} \text{N}-\text{CH} \\ // \quad // \\ \text{HS}=\text{C} \quad \text{NH}-\text{CH} \end{array}$ 2-Mercaptoimidazole.....	1.5	$\begin{array}{c} \text{N}-\text{CH}_2 \\ // \quad // \\ \text{HS}-\text{C} \quad \text{CH}_2 \\ \\ \text{S}-\text{CH}_2 \end{array}$ 2-Mercapto-5,6-dihydro-1,3,4-thiazine.....	0.1
$\begin{array}{c} \text{N}-\text{CH} \\ // \quad // \\ \text{H}_2\text{N}-\text{C} \quad \text{S}-\text{CH} \end{array}$ 2-Aminothiazole.....	0.1	$\begin{array}{c} \text{NH}_2 \\ \\ \text{S}=\text{C} \\ \\ \text{C}_2\text{H}_5 \end{array}$ Thiopropionamide.....	<0.01
$\begin{array}{c} \text{N}-\text{CH}_2 \\ // \quad \\ \text{HS}-\text{C} \quad \text{S}-\text{CH}_2 \end{array}$ 2-Mercaptothiazoline.....	1.3	$\begin{array}{c} \text{N}(\text{CH}_3)_2 \\ \\ \text{S}=\text{C} \\ \\ \text{SCH}_3 \end{array}$ Methyl <i>N,N</i> -dimethyl-dithiocarbamate.....	<0.01
$\begin{array}{c} \text{N}-\text{CH}_2 \\ // \quad \\ \text{HS}-\text{C} \quad \text{O}-\text{CH}_2 \end{array}$ 2-Mercaptooxazoline.....	0.2	$\begin{array}{c} \text{NH}-\text{CO} \\ // \quad // \\ \text{S}=\text{C} \quad \text{CH}_2 \\ \\ \text{S}-\text{CH}_2 \end{array}$ Propiorhodanine.....	<0.01

Substitution of all four hydrogens in thiourea increased activity, although a tautomeric equilibrium between the thio and mercapto forms of this compound is impossible¹⁶. Isothioureas were ineffective, but incorporation of the sulfur

¹⁶Presumably tetramethylthiourea can still exist in resonance forms similar to thiourea: e.g.,

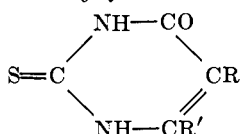


and both compounds react readily with iodine (295).

atom in a heterocyclic ring (e.g., 2-aminothiazole) did not abolish activity. The large number of inactive thio compounds investigated suggested that this grouping alone could not account for antithyroid activity. In the 2-thiobarbituric acid series it was found that active compounds were obtained only when both hydrogens in the 5-position were replaced. The dimethyl derivative was about one-tenth as active as thiouracil, while 5,5-diethyl-2-thiobarbituric acid was the most potent member of this series (antithyroid activity = 1.7).

Of all the compounds tested by Astwood *et al.* (18), the most effective ones were found among the alkyl derivatives of thiouracil synthesized by Anderson, Halverstadt, Miller, and Roblin (11). In this series, also, a great variation in

TABLE 18
The relative antithyroid activity of various substituted thiouracils (18)

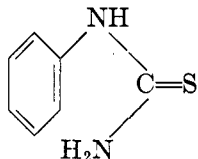


R	R'	ACTIVITY
-H	-H	1.0
-C ₂ H ₅	-H	3.5
-C ₃ H ₇ (<i>n</i>)	-H	2.0
-C ₄ H ₉ (<i>n</i>)	-H	0.6
-H	-C ₂ H ₅	8.0
-H	-C ₃ H ₇ (<i>n</i>)	11.0
-H	-C ₄ H ₉ (<i>n</i>)	3.0
-H	-CH ₂ C ₆ H ₅	10.0
-H	-C ₆ H ₅	1.0
-C ₂ H ₅	-C ₂ H ₅	2.0
-CN	-H	<0.001
-COOC ₂ H ₅	-H	<0.001
-H	-CF ₃	<0.01
-H	-NH ₂	<0.001

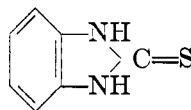
activity with changes in structure was observed, the introduction of highly polar groups reducing the antithyroid action practically to zero. Some of the results obtained with this group of compounds are recorded in table 18.

A group of 56 compounds was tested for antithyroid activity by McGinty and Bywater (248). A number of these were also tested by Astwood *et al.* (18), and in general the results of the two studies were in good agreement. Thiourea and substituted thioureas were less active than thiouracil. Of the substituted thiouracils, the 4-methyl derivative appeared to be slightly more active than the parent compound, while the *N*-phenyl derivative showed only slight activity. Using a somewhat different method of assay and a different base line (thiouracil = 100), McGinty and Bywater found the antithyroid activity of 2-mercaptothiazoline to be 131, 2-mercaptobenzimidazole 116, and 2-mercaptopyridine

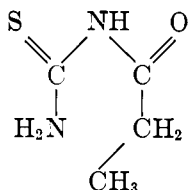
23. They pointed out an interesting analogy between open-chain and ring compounds, as follows:



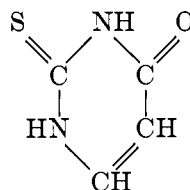
Phenylthiourea (14)



2-Mercaptobenzimidazole (116)



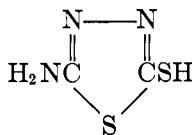
Propionylthiourea (14)



Thiouracil (100)

It was suggested (248) that the higher antithyroid activity (values given in parentheses) of the cyclic compounds in each case might be due to their tendency to exist primarily in the mercapto form.

Bywater, McGinty, and Jenesel (56) prepared and studied a series of 2-mercaptobenzimidazoles and 2-mercaptoimidazolines. None of the derivatives in either group was as active as the parent compound. The substitution of halogens, alkoxy groups, or alkyl groups reduced the antithyroid action of the former compound. Similarly, replacing the mercapto group by an amino lowered the activity from 116 to 6.



2-Mercapto-5-amino-1,3,4-thiadiazole

McGinty and Bywater (248a) also examined the antithyroid action of a number of sulfones and related compounds. Alkyl and unsubstituted phenyl sulfones were devoid of activity, whereas *p*-aminophenyl sulfones possessed some degree of action. Promizole was the most active of the sulfones, although the corresponding sulfide was slightly more effective. Most active of the compounds studied was the unsubstituted mercapto compound, 2-mercapto-5-amino-1,3,4-thiadiazole, with an antithyroid activity of 156.

Several series of thio compounds were investigated by Williams and Frame (435a). These investigators reported that no goitrogenic effect was exerted by a number of miscellaneous non-thiourea sulfur compounds, isothiureas, and thiureas in which the sulfur atom was incorporated in ring structures. *N*-Substitution of thiourea also reduced or abolished activity in most cases. The

most active compounds studied were listed in the following tentative order of decreasing effectiveness: 6-methylthiouracil, thiouracil, *o*-phenylenethiourea, 5-methylthiouracil, *p*-benzylthiouracil, 6-phenylthiouracil, tetramethylthiourea, diethylthiourea, and thiourea.

Numerous studies attempting to define more exactly the mechanism of action of these types of antithyroid compounds have been made. In reviewing this aspect of the work, Astwood (16) indicated that, beyond an interference with the synthesis of thyroxine, a more precise explanation involved considerable speculation. The lack of understanding of the natural mechanism of thyroxine formation has been a limiting factor in these studies. The oxidation of iodide to iodine, which is presumably a prerequisite to the formation of organic iodine, requires an oxidizing system of relatively high potential, such as a peroxidase and hydrogen peroxide (16). Dempsey (81) presented histochemical evidence indicating that fine granules in the thyroid cells contain a peroxidase which is inhibited by dilute solutions of thiouracil. However, Glock (143) was unable to isolate a peroxidase from thyroid tissue by chemical methods. Employing redox indicators, De Robertis and Gonçalves (83) reported that the oxidation-reduction potential of the normal thyroid cell was above +0.050 volt, while that of the colloid was about -0.20 volt. In activated cells the colloid potential rose to the same level as that of the cells (+0.050 volt). Thiourea ($M/200$) lowered the potential of both the cells and the colloid of the activated gland to -0.20 volt. On the other hand, sulfanilamide did not affect the normal oxidation-reduction potential of the thyroid cells.

Franklin and Chaikoff (131, 132) investigated the effects of various sulfonamides on the *in vitro* conversion of inorganic iodide to diiodotyrosine and thyroxine by surviving thyroid tissue slices with the aid of radioactive iodide. They found that the sulfonamides ($10^{-3}M$) inhibited the conversion, and that the degree of inhibition was related to the concentration of sulfonamide. On the other hand, sulfanilamide did not appreciably depress the iodine-concentrating capacity of surviving thyroid slices. Similar *in vitro* results using radioactive iodide were obtained with thiourea and thiouracil (133), although several groups of investigators (77, 134, 195, 334) reported that these antithyroid compounds markedly inhibit the accumulation of radioactive iodide by the thyroid gland *in vivo*. Miller, Anderson, Madison, and Salley (294) have sounded a word of warning concerning the interpretation of studies with radioactive iodide. These investigators observed a rapid exchange, under certain conditions, between radioactive iodide or iodine and diiodotyrosine.

Both thiourea and thiouracil have been reported to decrease greatly the iodine content of the thyroid (17, 30), but McGinty (247) found that the thyroid glands of animals receiving thiouracil were still capable of concentrating some iodine from large doses of potassium iodide. In the presence of the drug, however, the accumulated iodine was water soluble and did not appear to be converted to thyroid hormone.

Campbell, Landgrebe, and Morgan (57) suggested that the antithyroid activity of thiourea might be due to its well-known reactivity toward iodine. On this

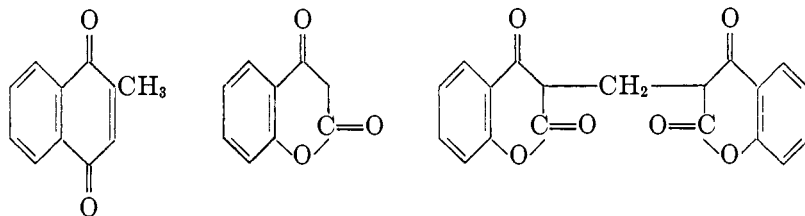
basis, the iodine formed in the thyroid would react so rapidly with thiourea that it would not be available for conversion to diiodotyrosine and thyroxine. The same concept was examined experimentally by Miller, Roblin, and Astwood (295) with respect to thiouracil and other thio compounds. It was found that in neutral buffered solutions thiouracil reacted almost instantaneously with one equivalent of iodine to form a disulfide which could be isolated in almost quantitative yields as the disodium salt. Tyrosine and casein could not be iodinated in the presence of thiouracil, but sulfonamides reacted with iodine more slowly than tyrosine and the action of these compounds could not be accounted for by this mechanism.

L. Vitamin K; prothrombin

A number of 1,4-naphthoquinones, the simplest of which is the 2-methyl derivative, restore normal prothrombin levels in animals on a vitamin K-deficient diet. Since the prothrombin concentration (which affects blood coagulation) is reduced in this deficiency, it is generally assumed that the K vitamins are involved in the formation of prothrombin (351).

When Stahmann, Huebner, and Link (392) found that 3,3'-methylenebis(4-hydroxycoumarin), dicumarol, was the hemorrhagic agent in spoiled sweet clover hay which increased prothrombin time, the action of this anticoagulant was investigated from the standpoint of vitamin K antagonism. Link (234) and Quick (329) have reviewed the present evidence on this subject. While the results are not unequivocal, the effect of small single doses of dicumarol appears to be prevented by 2-methyl-1,4-naphthoquinone (50, 79, 228, 314, 374), or vitamin K₁ oxide (80, 244). The mechanism of action of dicumarol is not entirely clear at present, and it is quite possible that the majority of known anticoagulants might better be classified primarily as prothrombin antagonists. Available evidence suggests that dicumarol inhibits the synthesis of prothrombin perhaps by interfering with the utilization of the K vitamins, rather than by any direct action on prothrombin (329).

Witts (438) pointed to a basic similarity between the structures of 2-methyl-1,4-naphthoquinone and 4-hydroxycoumarin and suggested that dicumarol might act by interfering with the utilization of vitamin K by the liver.

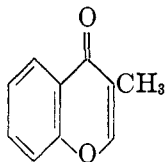


2-Methyl-1,4- 4-Hydroxycoumarin
naphthoquinone (keto form)

Dicumarol (keto form)

Meunier and Mentzer (289) also called attention to these structural relationships and reported that they were able to prevent completely the effects of

dicumarol by repeated daily doses of 2-methyl-1,4-naphthoquinone. They also reported that 4-hydroxycoumarin and 3-methylchromone possessed a slight antihemorrhagic and vitamin K-like action. The methylxanthines—theophyl-

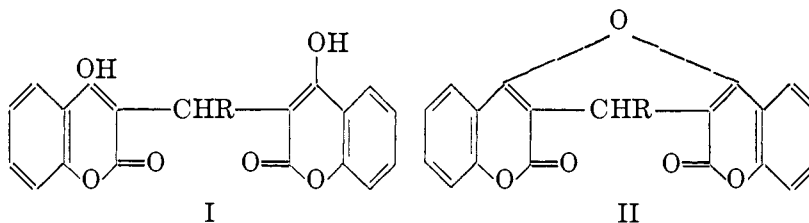


3-Methylchromone

line, theobromine, and caffeine—have been found by Field, Larsen, Spero, and Link (120) to decrease prothrombin time and counteract the hypoprothrombinemic action of dicumarol. However, Quick (329a) was unable to confirm these observations in rabbits and dogs.

In vitamin K-deficient chicks Glavind and Jansen (142) showed that there was a quantitative relationship between the metabolite and dicumarol. The proportions of the two substances could be adjusted so that the rate of prothrombin formation was unaffected by either. With the smallest active doses of the vitamin the ratio was 200–500:1, but the lack of constancy of this ratio precluded the possibility of a simple stoichiometric relationship. It was suggested that dicumarol might exert a specific toxic effect on prothrombin formation in the liver, which was compensated by the stimulating effect of vitamin K.

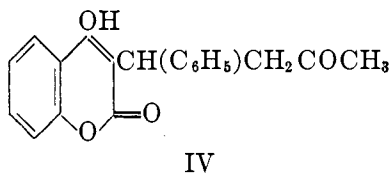
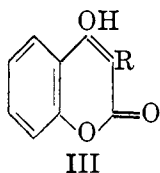
Overman, Stahmann, Huebner, Sullivan, Spero, Doherty, Ikawa, Graf, Roseman, and Link (313) have summarized their extensive studies on the relation of structure to anticoagulant activity among compounds of the 4-hydroxycoumarin type. On the basis of a single oral dose assay, they concluded that 3,3'-methylenebis(4-hydroxycoumarin) was the most potent member of this class of substances. Replacement of a hydrogen of the methylene group by an alkyl or aryl group as indicated in formula I lowered activity more or less in relation to the size of the group.



Jensen and Jensen (189), Lehmann (227), and Fantl (116) reported the ethylidene derivative ($R = CH_3$) equal to or less active than dicumarol. Overman *et al.* (313) investigated a large series of compounds of type I. The first two members of the series, the ethylidene and propylidene derivatives, were about one-third as active as dicumarol, while longer chains, phenyl or carboxyl groups, decreased the activity still more. Replacement of the methylene group by a sulfur atom reduced the activity to $\frac{1}{10}$ that of dicumarol. Monoesters and

glucosides immediately formed the inactive anhydrides (II) on neutralization. Substitution of the methylene carbon atom facilitated anhydride formation, and might have accounted for the decrease in anticoagulant activity of type I compounds.

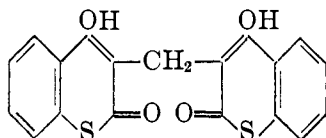
Another group of substances studied by Overman and his associates (313) was that of the 3-substituted-4-hydroxycoumarins (III). The methyl derivative of this type has been described as inactive or slightly anticoagulant by several investigators (116, 189, 227, 313), although Meunier and Mentzer (289) reported the compound to have slight vitamin K activity. In general, Overman *et al.*



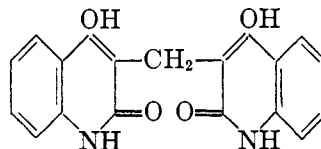
(313) found that compounds of type III, when R was alkyl, possessed very low anticoagulant action. When R was cyano, bromo, nitro, or oximino, the derivatives were inactive. However, the presence of a benzene ring and a ketone in the R group increased the effectiveness, so that 3-(α -phenyl- β -acetyethyl)-4-hydroxycoumarin (IV) was $\frac{1}{4}$ as active as dicumarol.

The diesters, ethers, and acetals of 3,3'-methylenebis(4-hydroxycoumarin) investigated by Overman *et al.* (313) showed a lower degree of activity than the parent substance. It was suggested that these compounds owed their action to an *in vivo* breakdown to dicumarol. Substitution of the benzene ring with methyl, bromo, or hydroxyl groups destroyed the activity in practically every case.

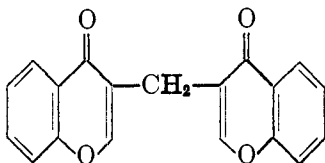
Several compounds structurally related to dicumarol were investigated by Mentzer, Meunier, Buu Høi, and Cagniant (288, 290). The sulfur analogue (V) was reported to be $\frac{1}{17}$ as effective, while the corresponding derivative in



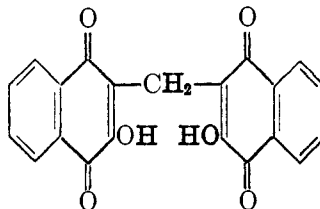
3,3'-Methylenebis(4-hydroxy-1-thiocoumarin)



3,3'-Methylenebis(4-hydroxy-2-quinolone)



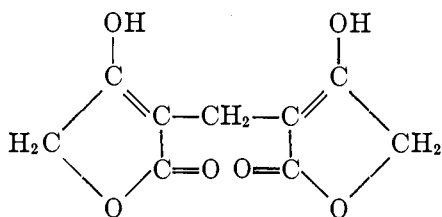
VII



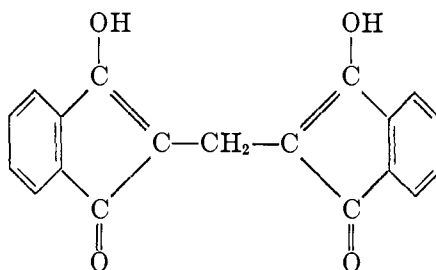
VIII

which the oxygen atoms were replaced by NH's to form a quinolone derivative (VI) was about $\frac{1}{50}$ as active as dicumarol. In view of the vitamin K activity of 3-methylchromone and phthiocol, these investigators also prepared 3,3'-methylenebischromone (VII) and 3,3'-methylenebis(2-hydroxy-1,4-naphthoquinone) (VIII). The former possessed about $\frac{1}{50}$ the activity of the parent compound; the latter $\frac{1}{25}$, but the action was of short duration.

Jansen and Jensen (189) and Overman *et al.* (313) studied other compounds related to dicumarol, such as methylenebis(tetronic acid) (IX), 3,3'-methylenebis(4-hydroxy-6-methyl- α -pyrone), and 2,2'-methylenebis(indanedione) (X).



IX



X

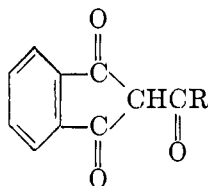
None of these compounds was an anticoagulant. Similarly, 3,3'-methylenebis(coumarin) was inactive, although coumarin itself showed a trace of activity (313). Degradation products of 3,3'-methylenebis(4-hydroxycoumarin) were also without anticoagulant action (189, 313). Overman *et al.* (313) concluded that the minimum structural requirements in this series are an intact 4-hydroxycoumarin residue with the 3-position substituted by a hydrogen atom or a carbon residue. Every compound fulfilling these requirements appeared to have some activity.

When added to blood or plasma *in vitro*, dicumarol does not affect clotting power (58), and its action in experimental animals is detectable only after a lapse of 12 to 24 hr. (315). These facts led Link, Overman, Sullivan, Huebner, and Scheel (235) to suggest that 3,3'-methylenebis(4-hydroxycoumarin) might undergo a chemical change in the animal body before it becomes active. Since salicylic acid can be obtained from dicumarol *in vitro* in practically quantitative yields (392), and of all the compounds studied by Overman *et al.* (313) only those which could yield salicylic acid had anticoagulant action, Link and his associates (235) studied the effect of this acid on prothrombin time. They found that single doses of salicylic acid produced an increase in the prothrombin time of rats maintained on a basal artificial ration low in vitamin K. This anticoagulant action, like that produced by dicumarol, could be prevented by K vitamins and 2-methyl-1,4-naphthoquinone. The activity of salicylic acid was less marked than that of the same amount of dicumarol, and the effect of the K vitamins on the action of the former was much more pronounced.

Rapport, Wing, and Guest (332) observed that methyl salicylate produced a severe degree of hypoprothrombinemia in rabbits but not in dogs. In rheumatic fever patients receiving salicylic acid or its acetyl derivative, they observed a marked prolongation of the prothrombin time.

Lester (229) was unable to detect salicylates in the urine of rats receiving dicumarol. He also pointed out that this anticoagulant was 25–100 times as effective as salicylic acid, and much more vitamin K was required to prevent its action. Link (234) has suggested that the failure to find the degradation product might not be conclusive, since when salicylic acid was administered to animals only part of it could be recovered in the urine.

A series of indanedione derivatives of type XI was studied by Kabat, Stohman, and Smith (192). They found that when R was isobutyl or α -naphthyl,



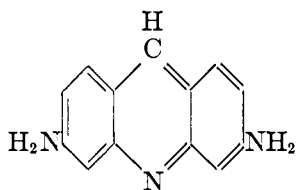
XI

the compounds exerted a marked effect on the prothrombin time of rabbits. Large doses of vitamin K had at best only a very slight effect on the hypoprothrombinemia produced by the isobutyl derivative. When R was ethyl the activity of the product was lower, but the *tert*-butyl derivative was the most active of the group studied—approximately equivalent to dicumarol. Kabat *et al.* considered it unlikely that these indandiones could be degraded to salicylic acid *in vivo*. They also reported that dibenzoylmethane produced a slight hypoprothrombinemia, although it also probably could not be metabolized to salicylic acid in the body.

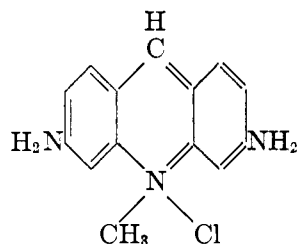
M. Miscellaneous antagonists

Numerous other cases of metabolite antagonists have been reported in addition to those reviewed in the preceding sections. In some instances the number of these studies is too limited to be set apart in separate sections. Other cases are of questionable significance, with respect to either the specificity of the antagonism or the identification of the reversing substance as an essential metabolite. Some of these investigations, which fall more or less within the scope of this review, have been grouped together in this section. It is obviously improbable, however, that all of the substances reported to prevent the biological activity of the diamidines or antimalarials such as quinine and quinacrine are directly related to their mode of action (*cf.* Section IV).

McIlwain (253) found that the growth of bacteria such as *E. coli* and *Strep. hemolyticus*, inhibited by acriflavine or proflavine, could be restored by two types of substances not normally required preformed by the organisms. The first of these was yeast nucleic acid and related substances, such as adenylic acid and cozymase. Yeast nucleic acid was the most effective reversing agent and showed a direct relationship between concentration and effect. The formation of insoluble complexes or salts between proflavine and yeast nucleic acid or adenylic acid was demonstrated. As in the reversal experiments, an excess of



2,8-Diaminoacridine
(proflavine)

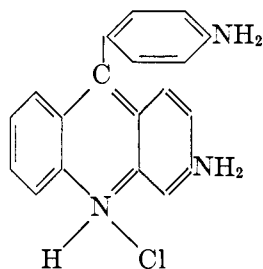


2,8-Diamino-10-methylacridinium
chloride (acriflavine)

nucleic acid was required, suggesting partial dissociation of the salt. Substances of the second type included a mixture of amino acids which was active only over a limited range of acriflavine concentrations. In the presence of this group of substances, but not in their absence, synthetic hydrogen carriers such as methylene blue and riboflavin were effective in further restoring growth. McIlwain considered that the acridine derivatives deprived the organism of the use of certain enzymes or metabolites so that their requirements became more exacting than normal. He suggested that the antibacterial action was due to the combination with and inactivation of enzyme systems of which the nucleotides were essential parts. The amino acids were regarded as substrates or products some of which could be replaced by hydrogen carriers.

Similar results were obtained by Martin and Fisher (281), who showed that the bacteriostatic action of proflavine toward *Staph. aureus* was prevented by adenylic acid and cozymase as well as yeast nucleic acid. No insoluble complexes were formed between proflavine and adenylic acid in saturated aqueous solutions. A number of metabolic intermediates associated with these substances, such as succinic, lactic, pyruvic, and acetic acids, were also reported to interfere with the action of proflavine.

Fitzgerald, Babbitt, and Lee (127, 128) studied the effect of various substances upon the lytic action of bacteriophage on *E. coli*. Of the many types of compounds investigated, 2-amino-5-(*p*-aminophenyl)acridinium chloride (Phosphine GRN), acriflavine, and other acridines were the most active in this respect. Al-



2-Amino-5-(*p*-aminophenyl)acridinium chloride

though these derivatives inhibited bacterial growth as well, it was possible to demonstrate marked inhibition of phage multiplication at concentrations of Phosphine GRN which were only slightly bacteriostatic. Moreover, other types

of substances at equally bacteriostatic concentrations failed to prevent the lysis of *E. coli*. It was concluded that the acridines might interfere with some mechanism essential for the reproduction of bacteriophage. Further investigation (128) established the fact that the antiphage effect could be counteracted by yeast nucleic acid. Numerous other substances tested (including vitamins, amino acids, and purines) failed to affect the action of the acridines. The specific nature of the effect of nucleic acid was suggested by studies with acridine-resistant *E. coli*. Strains of this organism made resistant to several of the acridines were still equally susceptible to lysis by bacteriophage. However, the Phosphine GRN-resistant organism was not lysed under the same conditions (i.e., absence of acridine derivative) except in the presence of 10,000 times as large an infecting dose of phage. The presence of nucleic acid again rendered this resistant strain as susceptible to lysis as the original parent strain. Loss of

TABLE 19
Competition between hydrogen and acridine ions* (9)

ACRIDINE	INHIBITORY CONCENTRATION OF ACRIDINE IONS $M \times 10^4$				RATIO OF ACRIDINE TO HYDROGEN IONS			
	pH = 5.7	pH = 6.8	pH = 7.5	pH = 8.3	pH = 5.7	pH = 6.8	pH = 7.5	pH = 8.3
5-Amino.....	1000	130	32	8	510	810	1000	1600
3-Amino.....	740†	57	20		190†	285	641	
2,7-Diamino.....	950	111	25	6.5	500	690	810	1300
3,7-Diamino.....	830†	115	18		210†	580	590	
Average values..	880	103	24	7	352	591	760	1450

* *E. coli* in synthetic medium.

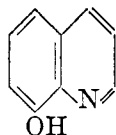
† pH 5.4.

resistance to Phosphine GRN, brought about by many serial passages in a drug-free medium, resulted in the return of normal phage susceptibility.

By a comprehensive investigation of over 100 acridine derivatives, Albert, Rubbo, Goldacre, Davey, and Stone (9) demonstrated that the compounds which were 75 per cent or more dissociated at pH 7.3 showed a much greater bacteriostatic activity at that pH than the weak bases. Over a range of hydrogen-ion concentrations in which the degree of ionization of the strong bases did not vary appreciably there was as much as a 100,000-fold change in their minimum effective concentrations. On the other hand, the antibacterial action of the weakly basic acridines did not change greatly with pH. At low pH values (5.4), where all the compounds were practically completely dissociated, both strong and weak bases were essentially equally active. But under conditions of unequal ionization (i.e., at pH 7.3) the activity appeared to be determined by the concentration of cations. Moreover, from pH 5.7 to 8.3, where the hydrogen-ion concentration varies over 400-fold, the ratio of acridine to hydrogen ions was relatively constant (table 19). The authors concluded that their results suggested a competition between acridine ions and hydrogen ions for the same

position on a bacterial enzyme surface. They assumed that this position was probably an ionized acidic group, the formation of weakly ionized complexes with the acridine ions inactivating the acidic group in the enzyme.

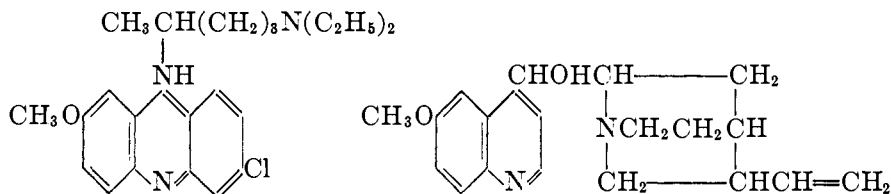
Combining the knowledge that microorganisms require traces of several metals



8-Hydroxyquinoline

for growth and that 8-hydroxyquinoline forms undissociated metal complexes, with the known fungistatic action of this compound, Zentmyer (471) reasoned that its effect might be due to a combination with essential metallic ions. Since complex formation does not occur much below pH 3.5, he first demonstrated that the fungistatic action was a function of pH, no effect being observed at low pH values. Zinc was found to be the most important trace element for several fungi, and with these organisms it was shown that the fungistatic action of 8-hydroxyquinoline could be prevented by 1–2 molar equivalents of zinc. With smaller ratios partial growth was obtained, while increasing concentrations of the fungicide were required to inhibit growth in the presence of increasing concentrations of zinc. The author suggested that a zinc–protein enzyme system might be involved, and demonstrated that other substances which form chelate inner complex salts with metals, such as ammonium nitrosophenyl hydroxylamine (Cupferron), also possess fungistatic activity.

Silverman and Evans (376, 377) observed that various substances, including spermidine, spermine, trimethylenetetramine, and pantothenic acid, prevented



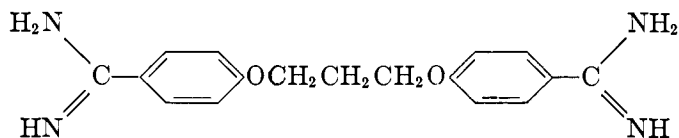
Quinacrine

Quinine

the antibacterial action of quinacrine against *E. coli*. The lesser bacteriostatic action of quinine was also relieved by spermidine. Of the polyamines, spermine was active at a somewhat higher dilution, but spermidine was less toxic to the bacteria. With several soil organisms, it was found that spermidine failed to reverse the action of quinacrine against highly susceptible organisms with exacting growth requirements. However, where the degree of susceptibility to quinacrine and the growth requirements were similar to those of *E. coli*, spermidine prevented the bacteriostatic effect. The authors suggested that spermidine might be an essential metabolite for certain bacteria and that the antibacterial action of quinacrine was due to a competitive inhibition. They were unable to

isolate spermine or spermidine from *E. coli*, a result attributed to the minute amounts which might be present. The ability of thiamine, riboflavin, pantothenic acid, glutathione, and nicotinic acid to reverse the action of quinacrine was not considered to be due to specific metabolic effects, since the relative amounts required were much greater.

Snell (382) studied the effect of several polyamines (spermidine, trimethylenetetramine, and tetramethylenepentamine) on the antibacterial action of propami-



Propamidine

dine for *L. casei* and *Strep. fecalis* R. With the former organism only trimethylenetetramine was effective in preventing bacteriostasis at less than growth-inhibitory concentrations. For the latter, spermidine was the most efficient in restoring growth inhibited by propamidine. This effect of the polyamines was evident only over a limited range, and with other bacteria such as *L. arabinosus*, which was susceptible to propamidine only at high concentration, no amount of any of the polyamines was capable of reversing the antibacterial action.

A detailed study of the reversing action of spermine and spermidine was carried out by Miller and Peters (291). They found that the growth-inhibitory action of $M/10,000$ quinacrine on *E. coli* in a synthetic medium was never completely reversed by these substances. Growth could be restored to 50 per cent of normal with 2 molar equivalents of spermine or 5 molar equivalents of spermidine. Putrescine was ineffective as a reversing agent. Although the polyamines produced a slight growth stimulation, it appeared to be too small to account for the results. In a complex medium less spermine was required to produce the same degree of reversal. The quinacrine side chain (1-diethylamino-4-aminopentane) showed no effect in either medium. With propamidine ($M/10,000$) in a synthetic medium spermine was able to restore growth completely in a ratio of 1:10, while at 1:1 growth was 50 per cent of normal. The authors concluded that spermine and spermidine were probably not acting as essential metabolites because of the high ratios involved.

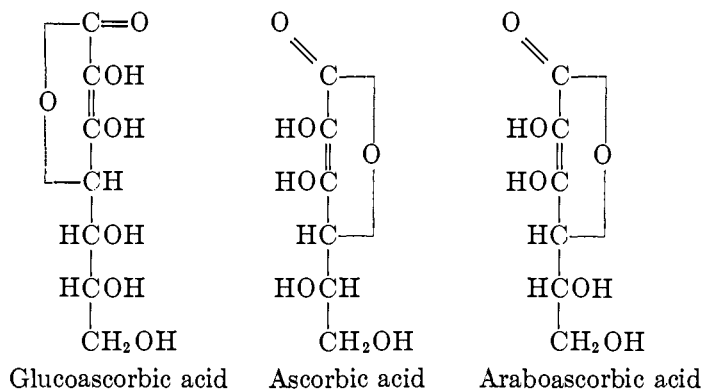
Other substances have also been reported to prevent the antibacterial action of various diamidines and antimalarials. Thus, Elson (102) found that the bacteriostatic action of propamidine on staphylococci and coli could be prevented by lecithin. Baker, Harrison, and Miller (22) had previously demonstrated a similar effect of lecithins toward various cationic and anionic detergents. Bichowsky (37) noted that the growth-inhibitory effect of 4,4'-diamidinostilbene and 4,4'-diamidinodiphenoxypentane on several microorganisms could be abolished by relatively high concentrations of yeast or animal sodium nucleate. A number of simple substances tested, including adenylic acid, purines, and amino acids, were without effect. It was suggested that the diamidines might act on living cells by combining with the nucleoproteins.

Elson (103) suggested that many of the preceding results might be interpreted

as a competitive adsorption between propamidine or other basic antibacterial agents and relatively non-toxic cations, such as the phospholipids and naturally occurring polyamines as well as hydrogen ions, for critical anionic positions on the bacterial cell. Similar considerations were applied to basic dyes by the Stearns (401) and later by Albert and coworkers (6, 7, 9) and Valko and DuBois (416) to other cationic antibacterial agents. It was pointed out by Elson that the protective effect of phosphate ions and nucleic acid might result from the formation of weakly dissociated salts which, in effect, reduced the concentration of toxic cations.

The relation between quinacrine, quinine, propamidine, methylene blue, and riboflavin was examined by Madinaveitia (273), who found that riboflavin prevented the bacteriostatic action of all the other substances when *L. casei* was the test organism. Johnson and Lewin (190) also found that the growth-inhibitory action of quinine on *E. coli*, exhibited over a narrow range of concentration, was reversed by riboflavin as well as by crude cozymase. The latter was the more effective reversing agent, and the authors pointed out that the results were consistent with the view that the effects of quinine on growth and viability might result in part from either a competition or loose combination with cozymase. *In vivo*, Seeler (366) reported that pyridoxine almost completely prevented the antimalarial activity of minimal doses of quinine and quinacrine in blood-induced *P. lophurae* infections in ducks. He suggested the possibility that a competition between the antimalarials and a pyridoxine-like compound for a position in some enzyme system of the parasite might explain this phenomenon.

The production of a scurvy-like condition in mice and cotton rats fed glucoascorbic acid (2,3-enediol-*d*-glucoheptono-1,4-lactone) in a purified diet was

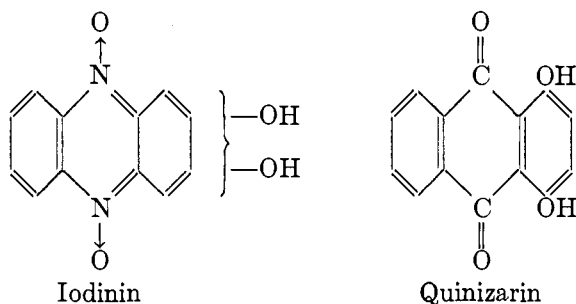


described by Woolley and Krampitz (455). The harmful action of this substance was not prevented by large amounts of ascorbic acid. However, fresh cabbage or dried grass was effective in counteracting the condition. When 10 per cent of glucoascorbic acid was incorporated in the diet of mice, the resulting symptoms (failure to grow, diarrhea, and hemorrhage) were fatal within two weeks. A majority of the animals survived a 5 per cent diet and eventually showed a gradual return to normal. Closely related substances, such as sodium araboascorbate, did not produce the scurvy-like condition. Unlike mice and cotton rats, which synthesize ascorbic acid, guinea pigs require the preformed vitamin.

In these animals, Woolley (450) found that glucoascorbic acid produced a scurvy-like condition which could be prevented by suitable quantities of ascorbic acid. He concluded that the results in guinea pigs suggested that the condition could be regarded as an ascorbic acid deficiency in all the animals.

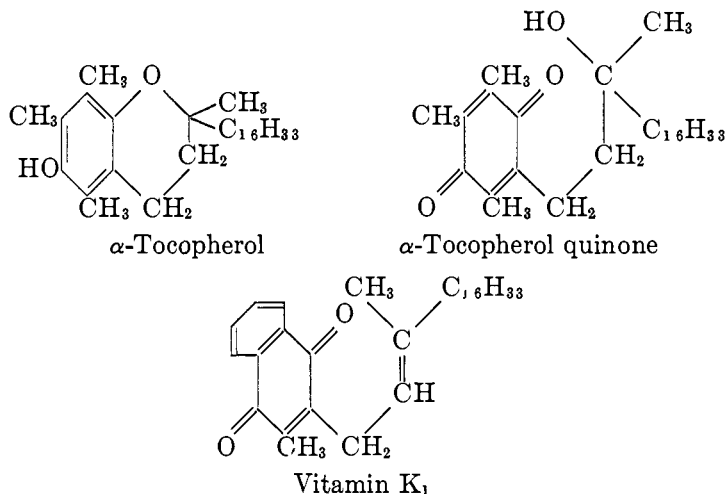
These results were not confirmed by Banerjee and Elvehjem (24), employing rats, chicks, or guinea pigs. Ascorbic acid itself was found to produce similar but less severe symptoms in rats. No hemorrhages were observed, and it was suggested that the failure to grow might be due to the diarrhea. Vitamin C-free liver powder, 2 per cent, with 10 per cent of either glucoascorbic or ascorbic acid allowed normal growth, although the rats showed moderate diarrhea. Similar results were obtained with both chicks and guinea pigs. The authors suggested that the unfavorable effects of glucoascorbic acid might be due, in part, to a modification of the intestinal flora resulting in a deficiency in some factor other than vitamin C which is present in liver powder.

McIlwain (258) investigated the interference of various naphthoquinones and anthraquinones with the bacteriostatic action of iodinin and phenazine di-*N*-oxide. He found that the growth-inhibitory action of the latter substances on



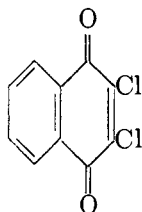
streptococci was prevented by relatively small amounts of quinizarin or 2-methyl-1,4-naphthoquinone. The interaction between this latter substance and iodinin appeared to be competitive. A destruction of the *N*-oxides occurred, probably by reduction. As no interaction between iodinin and the quinones occurred in the absence of the organisms, McIlwain concluded that the quinones must have participated to some degree in their metabolism. Although this result could be explained equally well as a function of hydrogen transport, he felt that the structural similarity between the *N*-oxides and quinones suggested that the two classes of compounds functioned at common sites in the organism and that the action of iodinin might be due to its inhibition of systems normally concerned with such quinones.

Woolley (453) reported that the administration of α -tocopherol quinone to pregnant mice produced symptoms resembling in some respects an α -tocopherol (vitamin E) deficiency. However, α -tocopherol acetate failed to prevent these symptoms. In addition to the death and resorption of the embryos, excessive vaginal bleeding was observed in the pregnant mice receiving α -tocopherol quinone. The administration of vitamin K_1 overcame both the hemorrhage and the resorption. Equal doses of α -tocopherol quinone were without toxic effects on non-pregnant mice, and the compound did not appear to affect the



prothrombin time of any of the animals. Since other vitamin K antagonists (*q.v.*) such as dicumarol failed to produce fetal resorption, Woolley concluded that α -tocopherol quinone bore an antagonistic relationship to both vitamin E and vitamin K.

The growth-inhibitory action of 2,3-dichloronaphthoquinone on various



2,3-Dichloro-1,4-naphthoquinone

microorganisms was examined by Woolley (453a). With organisms such as *Saccharomyces cerevisiae* and *Endomyces vernalis*, 2-methyl-1,4-naphthoquinone or vitamin K₁ prevented the growth inhibition. Over a limited range of concentrations a competitive inhibition appeared to be involved. With other organisms 2-methyl-1,4-naphthoquinone was found to inhibit growth in the same range of concentrations as the dichloro derivative. Consequently, in these cases no reversal could be demonstrated. No vitamin K deficiency could be demonstrated in mice fed purified diets containing 2,3-dichloronaphthoquinone.

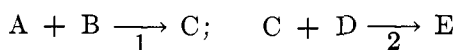
IV. GENERAL CONSIDERATIONS

This review covers a diversified group of antagonists whose physiological actions vary from an effect on the growth of microorganisms to the prevention of a muscular contraction induced by histamine or an influence on the clotting time of blood. The following discussion represents an effort to correlate these apparently unrelated effects on the basis of an underlying similarity in the antagonistic mechanisms involved.

tion in the presence of a normally adequate amount of β -alanine. But the minimum effective concentration of pantothenic acid completely prevents the action of any amount of the antagonist. These generalizations hold, provided amounts which act specifically are not exceeded, for almost any substance will have a non-specific poisoning action on cells if present in sufficiently high concentrations. The differentiation in types of antagonism has been expressed diagrammatically (137) in relation to bacterial growth, as illustrated in figure 3.

In the isobol *ABC* there is no indication of direct antagonism. Here, increasing the concentration of antagonist results in undiminished growth until at *B*, growth is inhibited. However, increasing the concentration of metabolite at this point does not restore growth. *D* represents competitive inhibition of the utilization of a metabolite, where a graded response occurs over a wide range of concentrations. This isobol is drawn through points giving the same degree of growth and shows the more or less constant ratio involved in this type of antagonism. *EFG* indicates that the antagonist affects the synthesis of the metabolite, so that at point *F* increasing the concentration of antagonist no longer affects growth.

Because of the complex and intimate relationships among metabolites in living cells, the distinction between interference with synthesis and interference with utilization can only be applied at a particular stage in any metabolic reaction. A substance which interferes with the utilization of *p*-aminobenzoic acid, for example, probably in turn blocks the synthesis of a more complex metabolite from it. Consequently, more than one metabolite may frequently be found to reverse the biological activity of an antagonist, but only one should show a constant ratio with the antagonist. The sequence in the biochemical synthesis of a complex metabolite *E* may be represented as follows:



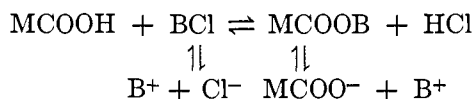
Under certain conditions, an antagonist blocking reaction 1 by a competitive inhibition of the utilization of *A* to form *C* could be reversed not only by additional *A* but also to some extent by *B*, which would displace reaction 1 toward *C*, and by *D*, tending to make up for a deficiency of *C* in reaction 2. Finally, the presence of *E* should eliminate the requirement for *C* altogether, unless this substance also served other metabolic functions or the cell was unable to utilize preformed *E*. A combination of two antagonists, one of which blocked reaction 1 and the other reaction 2, should be very efficient in preventing the formation of *E*. There is also the possibility of the existence of another metabolite *E*¹, able to take over the function of *E*. This metabolite could then supply an alternative independent route to the same metabolic end point, and so overcome the action of an antagonist which blocked reaction 1 or 2. Under these circumstances, however, there would be no direct relationship between the antagonist and *E*. It is evident that unless the biochemical or physiological relationships are known, it is frequently difficult to establish direct antagonisms in the absence of a competitive inhibition.

B. Mechanisms of action

Direct antagonism involving either the synthesis or the utilization of a metabolite may be due to any one of the following mechanisms: (1) salt or complex formation; (2) chemical reaction, forming an inactive product; (3) oxidation of a metabolite required in the reduced form, or *vice versa*; (4) competitive inhibition of an enzyme or tissue receptor associated with a metabolite by a structurally related antagonist.

It is interesting to compare the classification of enzyme inhibitors (153) with those drawn from the fields of pharmacology (69) and chemotherapy (122) which form the basis for the present scheme (*cf.* Section II). As indicated in the Introduction, not all direct antagonists are structurally related to the affected metabolite. However, it is also apparent that the greatest degree of specificity is probably associated with this type of antagonism. In fact, the preceding classification falls more or less in the order of increasing specificity, which is of primary importance to antagonistic mechanisms in living cells.

Salt or complex formation is likely to be relatively non-specific, except in certain instances where an antagonist forms a specific insoluble or undissociated complex with a metabolite such as one of the essential trace metals. More frequently, the antagonists which act by this mechanism are organic acids or bases or heavy metals which form salts with metabolites of opposite charge. For example, the antibacterial action of the organic bases may be represented as a simple double decomposition reaction as indicated:



In this equation M represents the residue of an acidic metabolite and B⁺ the cation of an organic base, the action of which depends on the dissociation constants of BCl and MCOOB. Since there are numerous acidic and basic metabolites, including proteins, the problem of determining the specific metabolite involved is frequently gratuitous. Several different metabolites may be found to reverse the same antagonist (*cf.* Section III, M), and relatively non-toxic ions of the same or opposite charge which are not metabolites have been shown to act in the same manner (416). Many instances of the so-called "interference phenomenon" (460) or chemotherapeutic interference (125) can probably be explained by a mechanism similar to that demonstrated for the protective action of relatively non-toxic ions (416). Consequently, in most cases of antagonisms involving salt formation, it can only be concluded that a competition for hydrogen or hydroxyl ions (ion exchange) is involved.

Antagonists which combine chemically with metabolites to form inactive products, and those which affect oxidation-reduction systems, form an intermediate group with respect to their specificity of action. In the former category, the effects may sometimes be limited to a particular chemical grouping such as the mercapto group (*cf.* Section III, D). Since there are numerous —SH-con-

taining essential metabolites, however, it is usually not possible to demonstrate complete specificity. Nevertheless, the action of trivalent arsenicals and some of the antibiotics like clavacin can be explained most readily through their inactivation of vital mercapto groups. On the other hand, any antagonist which reacts with mercapto compounds may be reversed by metabolites containing this grouping, whether or not this mechanism explains its biological activity, and regardless of whether the —SH compounds are actually metabolites. As a result, special care should be taken in interpreting the observations with highly reactive substances such as penicillin. All substances which inactivate this antibiotic obviously cannot form the basis for an explanation of its mode of action. To a considerable extent this type of difficulty is inherent in all mechanisms involving a chemical reaction, although this approach may be of value in certain cases when the metabolite contains unique chemical groupings. Otherwise, the effects are frequently too non-specific to be of general interest in living cells.

Many oxidizing agents have antibacterial activity which is relatively non-specific with respect to the affected metabolite. Within narrow limits of potential, it is possible that more specific effects involving an oxidation or reduction mechanism may be found. For example, although the mechanism of action of thiouracil is not well understood at present, it is evident that an oxidation of iodide ion to organic iodine is essential for the synthesis of thyroxine. By reducing any free iodine as rapidly as it is formed, thiouracil may effectively prevent the formation of organic iodine (295). It is also possible, of course, that thiouracil inhibits an enzyme system associated with the conversion of iodide ion to organic iodine by some other mechanism. In any event, it is evident that in the absence of any apparent structural relationship, a highly specific action on the synthesis of thyroxine is found in the thiouracil-type compounds.

Under different circumstances, the same type of antagonist, e.g., heavy metals, may act through divergent mechanisms. For instance, auric ions can form insoluble salts with acidic metabolites or essential —SH groups, but they can also oxidize the latter to inactive disulfides. Consequently, depending on the conditions, the mechanism of action of gold salts may involve salt formation or oxidation.

The action of all of the foregoing types of antagonists appears to involve a competitive mechanism. Thus, the formation of undissociated salts may be regarded as a competition for hydrogen or hydroxyl ions; the arsenicals may compete for vital mercapto groups; and possibly thiouracil competes with a metabolite for iodine. In all cases a definite ratio can usually be demonstrated, although the range of concentrations for thiouracil is rather narrow (16), owing possibly to the limited oxidative capacity of the thyroid gland.

The distinguishing feature of the other class of competitive antagonists—namely, the close structural relationship between antagonist and metabolite—probably accounts for the high degree of specificity associated with their biological activity. This specificity allows the isolation of a single metabolic

process which might otherwise be impossible under the complex conditions occurring in living cells.

The mechanism underlying the action of antagonists structurally related to metabolites is based on studies with isolated enzyme systems (*cf.* Section II,A). Similar phenomena are found in the cross-reactions and the effect of haptens in the field of immunology (217, 276). The metabolite and antagonist are assumed to compete for an active center or receptor in which there are polar groups oriented in such a manner as to exert a strong, specific attractive force on the metabolite. These forces, involving both primary and secondary valencies, are probably similar to those postulated for immunological reactions (317). Because of the similarity in the nature and steric configuration of the active groupings of the metabolite and the antagonist, both are presumably attracted to the same active center, but the antagonist is unable to carry out the normal physiological function of the metabolite because of slight differences in one of the essential groupings.

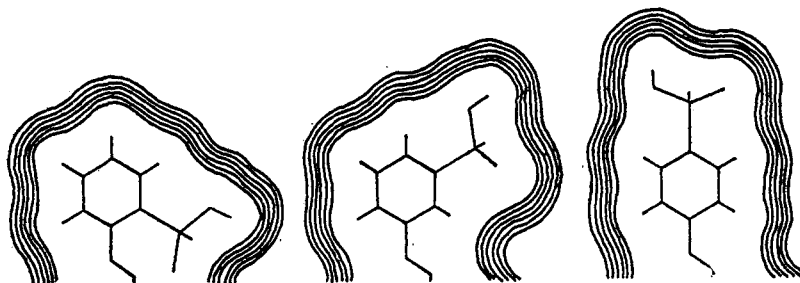


FIG. 4. Antibody cavities specific to *o*-, *m*-, and *p*-azophenylarsonic acid haptenic groups, respectively. (Scale drawings with circumferential contours corresponding to close fit; radial dilatation in increments of 0.2 Å.) Pauling and Pressman (317).

While the exact nature of the active centers or receptors is unknown, they may be pictured as cavities in a complex molecule similar to those suggested for hapten groupings in antibody molecules (figure 4). If one assumes that superimposed on steric configuration there are polar groups at appropriate points which bind the metabolite to the surface of the cavity, the high degree of specificity of both metabolite and antagonist can be accounted for.

The relative attractive forces operating between the metabolite and the active centers, compared with the attraction of the antagonist to the active centers, will determine the efficiency of the antagonist. This efficiency is reflected in the molar inhibition ratio between antagonist and metabolite. With few exceptions, these ratios are greater than unity, because the firmness of the binding of the metabolite to the receptor is usually greater than the binding between the antagonist and the receptor.

From pharmacological studies with acetylcholine and epinephrine Gaddum (136) has shown that the quantitative data for many competitive antagonisms can be interpreted by a simple equation. If M is the concentration of metabo-

lite, and A the concentration of antagonist competing for the same receptors, and m and a the corresponding proportions of the receptors occupied by each, so that $(1 - m - a)$ is the proportion of free receptors, then for equilibrium:

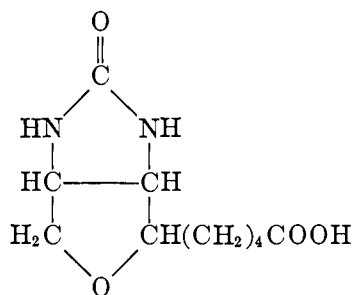
$$K_1M(1 - m - a) = m, \text{ and } K_2A(1 - m - a) = a$$

Eliminating a gives:

$$K_1M = (1 + K_2A) \frac{m}{1 - m} \quad (1)$$

When $A = 0$, this expression becomes the simple mass action equilibrium. Equation 1 is applicable so long as the reaction is of the first order. Actually, in certain cases the reaction does not appear to be of the first order, although this observation may be related to the complexity of these phenomena in living cells (137).

Table 20 illustrates a number of examples of structurally related competitive metabolite antagonists which act as growth inhibitors. It is doubtful, however, that one should conclude from such results that substituting the same groupings in other metabolites will lead to competitive antagonists in all cases. For instance, although replacing the sulfur atom in methionine by oxygen produces a competitive antagonist (341), a corresponding change in the biotin molecule results in a compound, oxybiotin, with the same type of biological activity as biotin



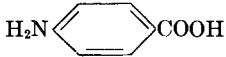
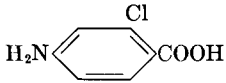
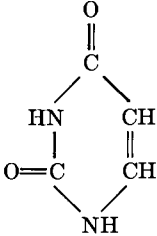
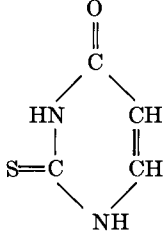

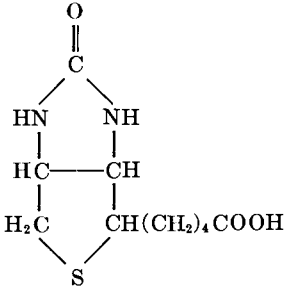
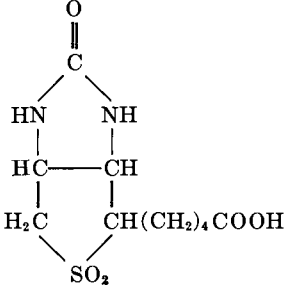
Oxybiotin

itself (95, 178, 178a, 323a, 357, 358). Similarly, the replacement of the carboxyl group in anthranilic acid by a sulfonic acid group leads to an inert compound with respect to the antagonism of anthranilic acid (381). The evidence drawn from more extensive studies with isolated enzymes also suggests that each system may exhibit its own peculiarities, and structural changes which produce effective antagonists for one system may not produce the same results in another case.

C. Applications and conclusions

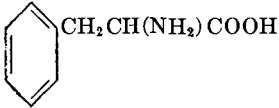
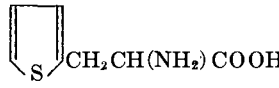
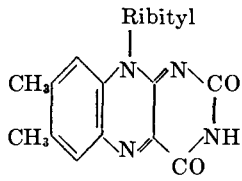
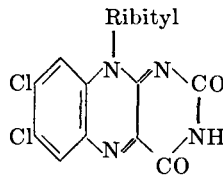
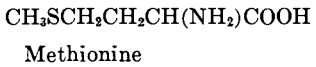
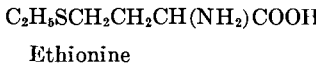
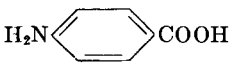
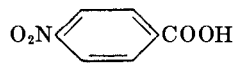
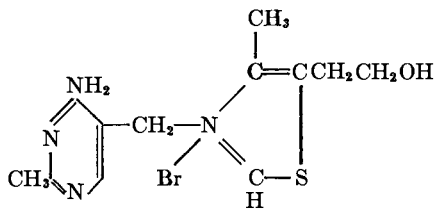
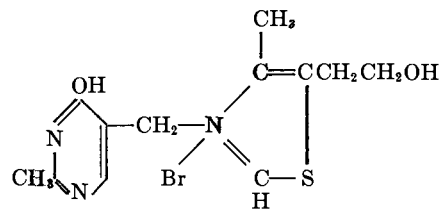
The intimate relation of enzymes to life processes makes them the logical starting point for a discussion of the application of antagonists in living cells. Wherever possible, the study of antagonists with isolated enzyme systems should simplify many of the problems encountered under more complex conditions. It



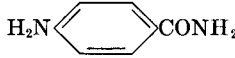

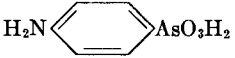
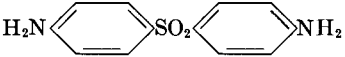
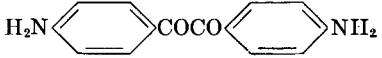
TABLE 20
Structurally related competitive metabolite antagonists

ATOM OR GROUP REPLACED	REPLACING ATOM OR GROUP	EXAMPLE		REFERENCE
		Metabolite	Antagonist	
-H	-Cl	 <p><i>p</i>-Aminobenzoic acid</p>	 4-Amino-2-chlorobenzoic acid	Wyss <i>et al.</i> (464)
-H	-CH ₃	$\text{H}_2\text{NCH}_2\text{CH}_2\text{COOH}$ β -Alanine	$\text{H}_2\text{NCH}(\text{CH}_3)\text{CH}_2\text{COOH}$ β -Aminobutyric acid	Nielson (306)
=O	=S	 Uracil	 2-Thiouracil	Roepke and Jones (347)
-S-	-O-	$\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ Methionine	$\text{CH}_3\text{OCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ Methoxinine	Roblin <i>et al.</i> (341)
-S-		 Biotin sulfone	 Biotin sulfone	Dittmer and du Vigneaud (85)

		<p>Adenine</p>	<p>Benzimidazole</p>	Woolley (447)
		<p>Guanine</p>	<p>5-Amino-7-hydroxy-1-<i>v</i>-triazolo[<i>d</i>]pyrimidine</p>	Roblin <i>et al.</i> (341)
		<p>Thiannine</p>	<p>Pyrithiamine</p>	Robbins (339) Woolley and White (457, 458)
		<p>Biotin</p>	<p>δ-(2,3-Ureylencyclohexyl)valeric acid</p>	English <i>et al.</i> (108)

TABLE 20—Continued
Structurally related competitive metabolite antagonists

ATOM OR GROUP REPLACED	REPLACING ATOM OR GROUP	EXAMPLE		REFERENCE
		Metabolite	Antagonist	
—CH=CH—	—S—	 <p>Phenylalanine</p>	 <p>β-2-Thienylalanine</p>	du Vigneaud <i>et al.</i> (420)
—CH ₃	—Cl	 <p>Riboflavin</p>	 <p>Chloroflavin</p>	Kuhn <i>et al.</i> (209)
—CH ₃	—C ₂ H ₅	 <p>Methionine</p>	 <p>Ethionine</p>	Dyer (96) Harris and Kohn (159)
—NH ₂	—NO ₂	 <p><i>p</i>-Aminobenzoic acid</p>	 <p><i>p</i>-Nitrobenzoic acid</p>	Miller (292)
—NH ₂	—OH	 <p>Thiamine</p>	 <p>Oxythiamine</p>	Soodak and Cerecedo (388)

—COOH	—CH ₂ OH	HOCH ₂ C(CH ₃) ₂ CHOHCONHCII ₂ CH ₂ COOH Pantothenic acid	HOCH ₂ C(CH ₃) ₂ CHOHCONHCII ₂ CH ₂ CH ₂ OH Pantothenyl alcohol	Snell and Shive (386)
—COOH	—COC ₆ H ₅	Pantothenic acid	HOCH ₂ C(CH ₃) ₂ CHOHCONHCII ₂ CH ₂ COC ₆ H ₅ Phenyl pantothenone	Woolley and Collyer (454)
—COOH	—SO ₃ H	(CH ₃) ₂ CHCH(NH ₂)COOH Valine	(CH ₃) ₂ CHCH(NH ₂)SO ₃ H α -Aminoisobutanesulfonic acid	McIlwain (252)
—COOH	—SO ₂ NH ₂	 <i>p</i> -Aminobenzoic acid	 Sulfanilamide	Woods (443)
—COOH	—CONH ₂	<i>p</i> -Aminobenzoic acid	 <i>p</i> -Aminobenzamide	Hirsch (175)
—COOH	—PO ₃ H ₂	<i>p</i> -Aminobenzoic acid	 Phosphanilic acid	Kuhn <i>et al.</i> (206)
—COOH	—AsO ₃ H ₂	<i>p</i> -Aminobenzoic acid	 Arsanilic acid	Peters (319)
—COOH	—SO ₂ —	<i>p</i> -Aminobenzoic acid	 <i>p, p'</i> -Diaminodiphenyl sulfone	Levaditi and Perault (231)
—COOH	—COCO—	<i>p</i> -Aminobenzoic acid	 <i>p, p'</i> -Diaminobenzil	Kuhn <i>et al.</i> (206)

cannot be arbitrarily concluded, however, that because substances inhibit certain enzymes they function by the same mechanisms in living cells.

The examples in table 1 (Section II) indicate a few of the numerous inhibitors which have been studied with isolated enzymes. Unfortunately, many of the enzymes involved in the utilization of simple metabolites are unknown, particularly those associated with anabolic reactions. This situation led Kuhn *et al.* (210) to reverse the usual procedure and calculate the dissociation constant of the hypothetical enzyme associated with the utilization of pantothenic acid from data obtained with living cells. Such an application may be somewhat speculative in nature, but it illustrates the versatility of the metabolite antagonist approach.

A number of respiratory enzymes have been studied extensively, and the relation of the simple metabolites to the holoenzymes established. These systems are frequently vital to all cells so that, from the chemotherapeutic point of view, antagonists affecting respiratory processes appear to offer fewer possibilities because of their potential toxicity. The rapidly fatal effect of pyriethamine for higher animals (457) demonstrates the toxic action of this antagonist in mammalian cells dependent on an external source of thiamine. On the other hand, much valuable information in the fields of animal and bacterial nutrition can be obtained with this type of antagonist.

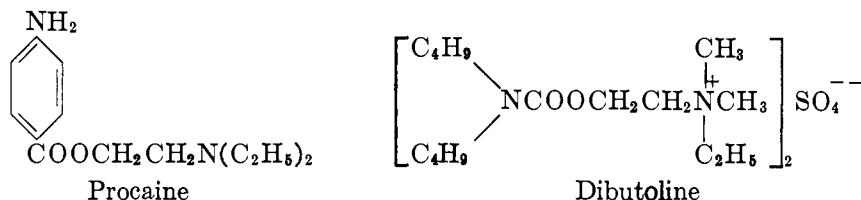
Because the rate of growth and of multiplication of host cells is probably much slower than that of pathogenic organisms, antagonists which affect anabolic reactions seem to offer greater promise as chemotherapeutic agents. The sulfanilamides are, in all likelihood, an outstanding example of this type of antagonism. The arsenicals, on the other hand, probably owe their lower toxicity to the host to the fact that they are concentrated in the invading parasites (166, 467).

One of the complicating factors in the application of metabolite antagonists to chemotherapy is the presence of too high a concentration of some metabolites in the body fluids and tissues of higher animals and man. This difficulty was encountered by McIlwain and Hawking (264), who were able to protect rats but not mice from experimental streptococcal infections with pantooyltaurine because of the lower concentration of pantothenic acid in the body fluids of rats. In any case, the pharmacological properties of pantooyltaurine would probably limit its usefulness as a chemotherapeutic agent. However, other pantothenic acid antagonists have been found which are highly effective in mice (433, 437) and may prove to be of value in chemotherapy. As Welch (428) pointed out, the study of antagonists of metabolites which occur only in minute amounts in body fluids is most likely to lead to practical applications in chemotherapy.

There is also another phenomenon which is of theoretical interest and at the same time limits the application of competitive inhibitions of bacterial growth to chemotherapy; that is, the susceptibility of various organisms to antagonists of metabolites required preformed, compared with those which they are capable of synthesizing. In several instances, such as *p*-aminobenzoic acid and purine

antagonists, the growth of practically all microorganisms appears to be inhibited regardless of their requirements. Antagonists of other metabolites, e.g., biotin, pantothenic acid, and thiamine, seem to inhibit the growth of only those organisms which require the preformed metabolite. Analogous observations have been made with isolated enzyme systems (*cf.* table 1), and the explanation may rest on a difference in the manner in which various organisms utilize the particular metabolite. A simpler explanation can be based on the observation that certain closely related analogues may actually serve as intermediates or be degraded to yield intermediates which can be utilized by the organisms for the synthesis of the metabolite. Under these circumstances, only organisms lacking the synthetic ability can be expected to be sensitive to this type of antagonist. Evidence for this explanation has been presented for biotin sulfone (86) and desthiobiotin (87, 408a), *p*-nitrobenzoic acid (292), pantoyltaurine (394), and pyriothiamine (339, 449).

The pharmacological action of several drugs has been accounted for on the basis of enzyme inhibitions (Section II), and antagonists have been found which block the action of acetylcholine, epinephrine, and histamine in isolated tissues or intact animals. Clark (69) explained the action of direct antagonists on the basis of a union with specific receptors. Similarly, Thimann (412) suggested that the action of many local anesthetics such as procaine and cocaine might be due to their structural resemblance to acetylcholine. He assumed that nerve stimulation is brought about by a combination of acetylcholine with receptors, which may resemble the active centers of enzymes. By competing with the metabolite for the receptors, the local anesthetics are assumed to block the nerve stimulation and so produce anesthesia. Both procaine (165) and dibutoline (320) have been shown to prevent acetylcholine-induced contractions in certain types of muscle tissue.¹⁷



The relation of structure to pharmacological action is sometimes complicated by the observation that different members of a closely related series produce opposite pharmacological effects. This enigma may be resolved in certain of those cases which involve the antagonism of a metabolite normally destroyed by an enzyme system. If the drugs act both as direct antagonists and as enzyme inhibitors, the net effect of any member of the group will depend on its predominant action. Thus, the primary effect of one substance, A₁, may be to

¹⁷Using leech muscle, Hazard, Corteggiani, and Pelou (167a) did not observe any antagonism of acetylcholine with procaine. In fact, the local anesthetic appeared to sensitize the muscle to acetylcholine.

inhibit the enzyme system which destroys the metabolite. Because it protects the metabolite from destruction, its action will be like that of the metabolite. But the predominant effect of another member of the series, A_2 , may be a direct antagonism of the metabolite so that its pharmacological action will be the opposite of A_1 .

It should be emphasized again that antagonisms in isolated tissues or intact animals may be exceedingly complex. For instance, ephedrine in certain cases enhances the action of epinephrine, presumably by decreasing the rate of hormone destruction (138), but under the same conditions higher concentrations of ephedrine are antagonistic (362). The antagonistic effect of ergotoxine toward the action of epinephrine on the rabbit's ileum is very pronounced, but it does not noticeably affect the latter's action on the rabbit's colon (69). Atropine, an acetylcholine antagonist, also counteracts some of the actions of epinephrine. Moreover, epinephrine and acetylcholine themselves can produce opposite effects in the same tissue, and so neutralize each other. Finally, the antagonists are usually not without some pharmacological activity of their own. Thus, diverse tissues may respond differently to the same stimulus, and one may assume that variations in the pattern of receptors determine both the type and intensity of pharmacological response and antagonistic action (69).

The mechanisms of action of all drugs obviously cannot be explained on the basis of a direct antagonism. Nevertheless, it is apparent that simple enzymatic reactions, the growth of microorganisms, and the function or response of various tissues may be antagonized by mechanisms which are strikingly similar. Consequently, it may not be unreasonable to assume that this resemblance is more than superficial and that, for example, the inhibitory action of malonic acid on succinic dehydrogenase, the antibacterial action of pantooyltaurine, and the antagonistic effect of trimethyloctylammonium chloride toward the action of acetylcholine on smooth muscle are closely related phenomena. In each case the antagonist appears to compete with the metabolite for an active center or receptor and in so doing block the normal reaction, whether it be the oxidation of succinic acid, the growth of a microorganism dependent on pantothenic acid, or the contraction of a muscle under the stimulus of acetylcholine.

Since in many respects it is a relatively new and rapidly developing field, it is not possible to assess all the implications inherent in the broad concept of metabolite antagonists. However, as an approach to the mechanism of action of a number of drugs, as a guide in the synthesis of new therapeutic agents, and as a means of evaluating the normal mode of synthesis and function of metabolites in living cells, the concept appears to offer many possibilities as yet unexplored.

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V. REFERENCES

- (1) ACKERMANN, D.: *Naturwissenschaften* **27**, 515 (1939).
- (2) ACKERMANN, D., AND MAUER, H.: *Arch. ges. Physiol. (Pflüger's)* **247**, 450 (1944); *Chem. Abstracts* **39**, 3803 (1945).
- (3) ACKERMANN, D., AND WASMUTH, W.: *Z. physiol. Chem.* **259**, 28 (1939).
- (4) ACKERMANN, D., AND WASMUTH, W.: *Z. physiol. Chem.* **260**, 155 (1939).
- (5) AESCHLIMANN, J. A., AND REINERT, M.: *J. Pharmacol.* **43**, 413 (1931).
- (6) ALBERT, A.: *Lancet* **1942**, **II**, 633.
- (7) ALBERT, A.: *Australian J. Sci.* **6**, 137 (1944).
- (8) ALBERT, A., FALK, J. E., AND RUBBO, S. D.: *Nature* **153**, 712 (1944).
- (9) ALBERT, A., RUBBO, S. D., GOLDACRE, R. J., DAVEY, M. E., AND STONE, J. D.: *Brit. J. Exptl. Path.* **26**, 160 (1945).
- (10) ANDERSON, E. G., PILGRIM, F. J., AND ELVEHJEM, C. A.: *Proc. Soc. Exptl. Biol. Med.* **55**, 39 (1944).
- (11) ANDERSON, G. W., HALVERSTADT, I. F., MILLER, W. H., AND ROBLIN, R. O., JR.: *J. Am. Chem. Soc.* **67**, 2197 (1945).
- (12) ANDERSON, T. F.: *Science* **101**, 565 (1945).
- (13) ARMSTRONG, E. F.: *Proc. Roy. Soc. (London)* **73**, 516 (1904).
- (14) ASTWOOD, E. B.: *J. Am. Med. Assoc.* **122**, 78 (1943).
- (15) ASTWOOD, E. B.: *J. Pharmacol.* **78**, 79 (1943).
- (16) ASTWOOD, E. B.: *Harvey Lectures*. Science Press Printing Company, Lancaster, Pennsylvania (1945).
- (17) ASTWOOD, E. B., AND BISSELL, A.: *Endocrinology* **34**, 282 (1944).
- (18) ASTWOOD, E. B., BISSELL, A., AND HUGHES, A. M.: *Endocrinology*, in press.
- (19) ASTWOOD, E. B., SULLIVAN, J., BISSELL, A., AND TYSLOWITZ, R.: *Endocrinology* **32**, 210 (1943).
- (20) ATKINSON, N., AND STANLEY, N. F.: *Australian J. Exptl. Biol. Med. Sci.* **21**, 249, 255 (1943).
- (21) AUHAGEN, E.: *Z. physiol. Chem.* **274**, 48 (1942).
- (22) BAKER, Z., HARRISON, R. W., AND MILLER, B. F.: *J. Exptl. Med.* **74**, 621 (1941).
- (23) BALLS, A. K., AND KÖHLER, F.: *Ber.* **64**, 34 (1931).
- (24) BANERJEE, S., AND ELVEHJEM, C. A.: *Proc. Soc. Exptl. Biol. Med.* **60**, 4 (1945).
- (25) BARNETT, J.: *J. Chem. Soc.* **1944**, 5.
- (26) BARNETT, J., DUPRÉ, D. J., HOLLOWAY, B. J., AND ROBINSON, F. A.: *J. Chem. Soc.* **1944**, 94.
- (27) BARNETT, J. W., AND ROBINSON, F. A.: *Biochem. J.* **36**, 357, 364 (1942).
- (28) BARRON, E. S. G., AND SINGER, T. P.: *Science* **97**, 356 (1943).
- (29) BARRON, E. S. G., AND SINGER, T. P.: *J. Biol. Chem.* **157**, 221 (1945).
- (30) BAUMANN, E. J., METZGER, N., AND MARINE, D.: *Endocrinology* **34**, 44 (1944).
- (31) BEHRENS, O. K.: *J. Biol. Chem.* **141**, 503 (1941).
- (32) BELL, P. H., AND ROBLIN, R. O., JR.: *J. Am. Chem. Soc.* **64**, 2905 (1942).
- (33) BERGEL, F., AND TODD, A. R.: *J. Chem. Soc.* **1937**, 1504.
- (34) BERNHEIM, F.: *The Interaction of Drugs and Cell Catalysts*. Burgess Publishing Company, Minneapolis, Minnesota (1942).
- (35) BERNHEIM, F., AND BERNHEIM, M. L. C.: *Cold Spring Harbor Symposia Quant. Biol.* **7**, 174 (1939).
- (36) BEST, C. H., AND MCHENRY, E. W.: *Physiol. Rev.* **11**, 371 (1931).
- (37) BICHOWSKY, L.: *Proc. Soc. Exptl. Biol. Med.* **57**, 163 (1944).
- (38) BLANCHARD, K. C.: *J. Biol. Chem.* **140**, 919 (1941).
- (39) BLANCHARD, K. C.: Personal communication.
- (40) BLASCHKO, H., RICHTER, D., AND SCHLOSSMANN, H.: *Biochem. J.* **31**, 2187 (1937).
- (41) BLISS, E. A., AND LONG, P. H.: *Bull. Johns Hopkins Hosp.* **69**, 14 (1941).
- (42) BLOCH, H., AND ERLÉNMEYER, H.: *Helv. Chim. Acta* **25**, 694, 1062 (1942).

- (43) BONNER, D., TATUM, E. L., AND BEADLE, G. W.: Arch. Biochem. **3**, 71 (1943).
- (44) BOURGUE, J. E., AND LOEW, E. R.: Am. J. Physiol. **133**, 341 (1943).
- (45) BOVET, D., HORCLOIS, R., AND FOURNEL, J.: Compt. rend. soc. biol. **138**, 165 (1944).
- (46) BOVET, D., HORCLOIS, R., AND WALTHERT, F.: Compt. rend. soc. biol. **138**, 99 (1944).
- (47) BOVET, D., AND STAUB, A. M.: Compt. rend. soc. biol. **124**, 547 (1937).
- (48) BRACKETT, S., WALETZKY, M., AND BAKER, M.: To be published.
- (49) BRAUN, H., AND SCHAEFFER, H.: Z. Hyg. Infektionskrank. **89**, 339 (1919).
- (50) BRODIE, D. C., HIESTAND, W. A., AND JENKINS, G. L.: J. Am. Pharm. Assoc. **34**, 73 (1945).
- (51) BRUECKNER, A. H.: Yale J. Biol. Med. **15**, 813 (1943).
- (52) BUCHMAN, E. R., HEEGAARD, E., AND BONNER, J.: Proc. Natl. Acad. Sci. U. S. **26**, 561 (1940).
- (53) BURCHELL, H. B., AND VARCO, R. L.: J. Pharmacol. **75**, 1 (1942).
- (54) BURTNER, R. R., AND CUSIC, J. W.: J. Am. Chem. Soc. **65**, 262 (1943).
- (55) BURTNER, R. R., AND CUSIC, J. W.: J. Am. Chem. Soc. **65**, 1582 (1943).
- (56) BYWATER, W. G., MCGINTY, D. A., AND JENESEL, N. D.: J. Pharmacol. **85**, 14 (1945).
- (57) CAMPBELL, D., LANDGREBE, F. W., AND MORGAN, T. N.: Lancet **1944**, **II**, 630.
- (58) CAMPBELL, H. A., AND LINK, K. P.: J. Biol. Chem. **133**, 21 (1941).
- (59) CAVALLITO, C. J., AND BAILEY, J. M.: Science **100**, 390 (1944).
- (60) CAVALLITO, C. J., BAILEY, J. M., HASKELL, T. H., MCCORMICK, J. R., AND WARNER, W. F.: J. Bact. **50**, 61 (1945).
- (61) CHAMBON, M., LAVAUD, M. T., AND MARTIN, P.: Compt. rend. soc. biol. **137**, 469 (1943).
- (62) CHAPMAN, C. J.: Quart. J. Pharm. Pharmacol. **17**, 314 (1944).
- (63) CHAUCHARD, B., AND CHAUCHARD, P.: Compt. rend. soc. biol. **137**, 708 (1943).
- (64) CHEN, G., GEILING, E. M. K., AND MACHATTON, R. M.: J. Infectious Diseases **76**, 152 (1945).
- (65) CHICK, H.: J. Hyg. **8**, 92 (1908).
- (66) CHOW, B. F., AND MCKEE, C. M.: Proc. Soc. Exptl. Biol. Med. **58**, 175 (1945).
- (67) CLARK, A. J.: J. Physiol. **61**, 547 (1926).
- (68) CLARK, A. J.: *The Mode of Action of Drugs on Cells*. Williams & Wilkins Company, Baltimore (1933).
- (69) CLARK, A. J.: Heffter's *Handbuch der experimentellen Pharmakologie*, Ergänzt. 4. J. Springer, Berlin (1937).
- (70) CLARKE, H. T.: J. Am. Chem. Soc. **66**, 652 (1944).
- (71) CLIMENKO, D. R., HOMBURGER, E., AND MESSER, F. H.: J. Lab. Clin. Med. **27**, 289 (1941).
- (72) COHEN, A., KING, H., AND STRANGWAYS, W. I.: J. Chem. Soc. **1931**, 3043.
- (73) COHEN, A., KING, H., AND STRANGWAYS, W. I.: J. Chem. Soc. **1932**, 2505.
- (74) COLWELL, C. A., AND MCCALL, M.: Science **101**, 592 (1945).
- (75) CONTROULIS, J., AND BANKS, C. K.: J. Am. Chem. Soc. **67**, 1946 (1945).
- (76) COOMBS, H. I.: Biochem. J. **21**, 1259 (1927).
- (77) COUCEIRO, A., VIEIRA, L. G., AND MORAES, J. DE: Rev. brasil. biol. **4**, 173 (1944); Chem. Abstracts **39**, 350 (1945).
- (78) COWLES, P. B.: Yale J. Biol. Med. **14**, 599 (1942).
- (79) CROMER, H. E., JR., AND BARKER, N. W.: Proc. Staff Meetings Mayo Clinic **19**, 217 (1944).
- (80) DAVIDSON, C. S., AND MACDONALD, H.: New Engl. J. Med. **229**, 353 (1943).
- (81) DEMPSEY, E. W.: Endocrinology **34**, 27 (1944).
- (82) DENKEWALTER, R., COOK, M. A., AND TISHLER, M.: Science **102**, 12 (1945).
- (83) DEROBERTIS, E., AND GONÇALVES, J. M.: Endocrinology **36**, 245 (1945).
- (84) DIMOND, N. S.: Science **94**, 420 (1941).
- (85) DITTMER, K., AND DU VIGNEAUD, V.: Science **100**, 129 (1944).

- (86) DITTMER, K., DU VIGNEAUD, V., GYÖRGY, P., AND ROSE, C. S.: Arch. Biochem. **4**, 229 (1944).
- (87) DITTMER, K., MELVILLE, D. B., AND DU VIGNEAUD, V.: Science **99**, 203 (1944).
- (88) DIXON, T. F.: Nature **153**, 289 (1944).
- (89) DOERMANN, A. H.: Arch. Biochem. **5**, 373 (1944).
- (90) DORFMAN, A., AND KOSER, S. A.: J. Infectious Diseases **71**, 241 (1942).
- (91) DORFMAN, A., RICE, L., KOSER, S. A., AND SAUNDERS, F.: Proc. Soc. Exptl. Biol. Med. **45**, 750 (1940).
- (92) DOUDOROFF, M.: Proc. Soc. Exptl. Biol. Med. **53**, 73 (1943).
- (93) DOUGLAS, C. G., HALDANE, J. S., AND HALDANE, J. B. S.: J. Physiol. **44**, 275 (1912).
- (94) DUBOS, R. J.: J. Am. Med. Assoc. **124**, 633 (1944).
- (95) DUSCHINSKY, R., DOLAN, L. A., FLOWER, D., AND RUBIN, S. H.: Arch. Biochem **6**, 480 (1945).
- (96) DYER, H. M.: J. Biol. Chem. **124**, 519 (1938).
- (97) EAGLE, H.: J. Pharmacol. **66**, 436 (1939).
- (98) EAKIN, R. E., SNELL, E. E., AND WILLIAMS, R. J.: J. Biol. Chem. **140**, 535 (1941).
- (99) EDLBACHER, S., BAUR, H., AND BECKER, M.: Z. physiol. Chem. **265**, 61 (1940).
- (100) EDLBACHER, S., JUCKER, P., AND BAUR, H.: Z. physiol. Chem. **247**, 63 (1937).
- (101) ELLIS, F. W.: Federation Proc. **4**, 117 (1945).
- (102) ELSON, W. O.: J. Biol. Chem. **154**, 717 (1944).
- (103) ELSON, W. O.: J. Infectious Diseases **76**, 193 (1945).
- (104) ELVEHJEM, C. A., AND TEPLEY, L. J.: Chem. Rev. **33**, 185 (1943).
- (104a) EMERSON, G. A., AND SOUGHWICK, P.: J. Biol. Chem. **160**, 169 (1945).
- (105) EMERSON, G. A., AND TISHLER, M.: Proc. Soc. Exptl. Biol. Med. **55**, 184 (1944).
- (105a) EMERSON, G. A., WURTZ, E., AND JOHNSON, O. H.: J. Biol. Chem. **160**, 165 (1945).
- (106) EMMELIN, N., KAHLSON, G. S., AND LINDSTRÖM, K.: Acta Physiol. Scand. **3**, 39 (1941).
- (107) ENGLEHARDT, E., AND LOEWI, O.: Arch. exptl. Path. Pharmacol. **150**, 1 (1930).
- (108) ENGLISH, J. P., CLAPP, R. C., COLE, Q. P., HALVERSTADT, I. H., LAMPEN, J. O., AND ROBLIN, R. O., JR.: J. Am. Chem. Soc. **67**, 295 (1945).
- (109) ENGLISH, J. P., CLARK, J. H., CLAPP, J. W., SEEGER, D., AND EBEL, R. H.: J. Am. Chem. Soc., in press.
- (110) ERLICH, P.: Ber. **42**, 17 (1909).
- (111) ERLENMEYER, H., BLOCH, H., AND KIEFER, H.: Helv. Chim. Acta **25**, 1066 (1942).
- (112) ERLENMEYER, H., AND WÜRGLER, W.: Helv. Chim. Acta **25**, 249 (1942).
- (113) EULER, H. v.: Ber. **75**, 1876 (1942).
- (114) EULER, H. v., AND JOSEPHSON, K.: Z. physiol. Chem. **157**, 122 (1926).
- (115) EULER, H. v., AND AHLSTRÖM, L.: Z. physiol. Chem. **279**, 176 (1943).
- (116) FANTL, P.: Australian J. Sci. **6**, 23 (1943).
- (117) FEENEY, R. E., AND STRONG, F. M.: J. Am. Chem. Soc. **64**, 881 (1942).
- (118) FELDBERG, W.: Ann. Rev. Physiol. **3**, 671 (1941).
- (119) FELDT, A.: Klin. Wochschr. **20**, 945 (1941).
- (120) FIELD, J. B., LARSEN, G. E., SPERO, L., AND LINK, K. P.: J. Biol. Chem. **156**, 725 (1944).
- (121) FILDES, P.: Biochem. J. **32**, 1600 (1938).
- (122) FILDES, P.: Lancet **1940**, **I**, 955.
- (123) FILDES, P.: Brit. J. Exptl. Path. **21**, 67 (1940).
- (124) FILDES, P.: Brit. J. Exptl. Path. **22**, 293 (1941).
- (125) FINDLAY, G. M.: *Recent Advances in Chemotherapy*. P. Blakiston's Son, Philadelphia (1939).
- (126) FINDLAY, G. M.: Brit. J. Exptl. Path. **21**, 356 (1940).
- (127) FITZGERALD, R. J., AND BABBITT, D.: J. Immunol., in press.
- (128) FITZGERALD, R. J., AND LEE, M. E.: J. Immunol., in press.
- (129) FOSTER, J. W.: J. Bact. **48**, 97 (1944).

- (130) FOX, C. L., JR., AND ROSE, H. M.: Proc. Soc. Exptl. Biol. Med. **50**, 142 (1942).
- (131) FRANKLIN, A. L., AND CHAIKOFF, I. L.: J. Biol. Chem. **148**, 719 (1943).
- (132) FRANKLIN, A. L., AND CHAIKOFF, I. L.: J. Biol. Chem. **152**, 295 (1944).
- (133) FRANKLIN, A. L., CHAIKOFF, I. L., AND LERNER, S. R.: J. Biol. Chem. **153**, 151 (1944).
- (134) FRANKLIN, A. L., LERNER, S. R., AND CHAIKOFF, I. L.: Endocrinology **34**, 265 (1944).
- (135) GADDUM, J. H.: J. Physiol. **61**, 141 (1926).
- (136) GADDUM, J. H.: J. Physiol. (Proceedings) **89**, 7 (1937).
- (137) GADDUM, J. H.: Trans. Faraday Soc. **39**, 323 (1943).
- (138) GADDUM, J. H., AND KWIATKOWSKI, J.: J. Physiol. **94**, 87 (1938).
- (139) GEIGER, W. B., AND CONN, J. E.: J. Am. Chem. Soc. **67**, 112 (1945).
- (140) GILBERT, A. J., AND GOLDMAN, F.: Proc. Soc. Exptl. Biol. Med. **44**, 458 (1940).
- (141) GLADSTONE, G. P.: Brit. J. Exptl. Path. **20**, 189 (1939).
- (142) GLAVIND, J., AND JANSEN, K. F.: Acta Physiol. Scand. **8**, 173 (1944); Chem. Abstracts **39**, 4976 (1945).
- (143) GLOCK, G. E.: Nature **154**, 460 (1944).
- (144) GOETCHIUS, G. R., AND LAWRENCE, C. A.: J. Bact. **49**, 575 (1945).
- (145) GORDON, W. G., AND JACKSON, R. W.: J. Biol. Chem. **110**, 151 (1935).
- (146) GOULD, R. G., KANE, L. W., AND MUELLER, J. H.: J. Bact. **47**, 287 (1944).
- (147) GREDNER, K., AND SCHUMBRICK, W.: Arch. exptl. Path. Pharmacol. **202**, 155 (1943) (from reference 283).
- (148) GREEN, H. N.: Brit. J. Exptl. Path. **21**, 38 (1940).
- (149) GREEN, H. N., AND BIELSCHOWSKI, F.: Brit. J. Exptl. Path. **23**, 13 (1942).
- (150) GROSS, R. E.: Z. physiol. Chem. **112**, 236 (1921).
- (151) GUGGENHEIM, M.: *Die biogenen Amine*. S. Karger, Basel (1940).
- (152) GYÖRGY, P., MELVILLE, D. B., BURK, D., AND DU VIGNEAUD, V.: Science **91**, 243 (1940).
- (153) HALDANE, J. B. S.: *Enzymes*. Longmans, Green and Company, New York (1930).
- (154) HALLENBECK, G. A.: Am. J. Physiol. **139**, 329 (1943).
- (155) HALPERN, B. N.: Compt. rend. soc. biol. **131**, 509 (1939).
- (156) HALPERN, B. N.: Arch. intern. pharmacodynamie **66**, 339 (1942).
- (157) HARRINGTON, C. R.: J. Chem. Soc. **1944**, 193.
- (158) HARRINGTON, C. R.: Proc. Roy. Soc. (London) **132**, 223 (1944).
- (159) HARRIS, J. S., AND KOHN, H. I.: J. Pharmacol. **73**, 383 (1941).
- (160) HARRIS, J. S., AND KOHN, H. I.: J. Biol. Chem. **141**, 989 (1941).
- (161) HARRIS, S. A., WOLF, D. E., MOZINGO, R., AND FOLKERS, K.: Science **97**, 447 (1943).
- (162) HARRIS, S. A., WOLF, D. E., MOZINGO, R., ANDERSON, R. C., ARTH, G. E., HEYL, D., WILSON, A. N., AND FOLKERS, K.: J. Am. Chem. Soc. **66**, 1756 (1944).
- (163) HARTELIUS, V.: Naturwissenschaften **31**, 440 (1943).
- (164) HARTRIDGE, H., AND ROUGHTON, F. J. W.: Proc. Roy. Soc. (London) **B94**, 336 (1923).
- (165) HARVEY, A. M.: Bull. Johns Hopkins Hosp. **65**, 223 (1939).
- (166) HAWKING, F.: J. Pharmacol. **59**, 123 (1937).
- (167) HAWKING, F.: Brit. J. Exptl. Path. **25**, 63 (1944).
- (167a) HAZARD, R., CORTEGGIANI, E., AND PELOU, A.: Compt. rend. soc. biol. **138**, 427 (1944).
- (168) HELLERMAN, L.: Physiol. Rev. **17**, 454 (1937).
- (169) HELLERMAN, L.: Cold Spring Harbor Symposia Quant. Biol. **7**, 165 (1939).
- (170) HENRY, R. J.: Bact. Rev. **7**, 175 (1943).
- (171) HENRY, R. J.: *The Mode of Action of Sulfonamides*, Publication of the Josiah Macy Jr. Foundation, Review Series, **2**, No. 1 (1944).
- (172) HIGGINS, G. M.: Am. J. Med. Sci. **210**, 347 (1945).
- (173) HILL, J. H., AND MARTIN, L.: Medicine **11**, 141 (1932).
- (174) HIMSWORTH, H. P.: Lancet **1943**, II, 465.

- (175) HIRSCH, J.: *Science* **96**, 139 (1942).
- (176) HITCHINGS, G. H., FALCO, E. A., AND SHERWOOD, M. B.: *Science* **102**, 251 (1945).
- (177) HOFMANN, K.: *J. Am. Chem. Soc.* **67**, 694 (1945).
- (178) HOFMANN, K., MCCOY, R. H., FELTON, J. R., AXELROD, A. E., AND PILGRIM, F. J.: *Arch. Biochem.* **7**, 393 (1945).
- (178a) HOFMANN, K., AND WINNICK, T.: *J. Biol. Chem.* **160**, 449 (1945).
- (179) HOUSEWRIGHT, R. D., AND KOSER, S. A.: *J. Infectious Diseases* **75**, 113 (1944).
- (180) HÜFNER, G.: *J. prakt. Chem.* [2] **30**, 68 (1884).
- (181) ISAACS, M. L.: *Science* **75**, 46 (1932).
- (182) ISBELL, H.: *J. Biol. Chem.* **144**, 567 (1942).
- (183) IVANOVICS, G.: *Z. Immunitäts.* **101**, 58 (1941).
- (184) IVANOVICS, G.: *Z. Immunitäts.* **102**, 238 (1942).
- (185) IVANOVICS, G.: *Naturwissenschaften* **30**, 104 (1942).
- (186) IVANOVICS, G.: *Z. physiol. Chem.* **276**, 33 (1942).
- (187) JADASSOHN, W., FIERZ-DAVID, H. E., AND VOLLENWEIDER, H.: *Schweiz. med. Wochschr.* **73**, 122 (1943); *Chem. Abstracts* **38**, 6362 (1944).
- (188) JADASSOHN, W., FIERZ-DAVID, H. E., AND VOLLENWEIDER, H.: *Helv. Chim. Acta* **27**, 1384 (1944); *Chem. Abstracts* **39**, 3836 (1945).
- (189) JANSSEN, K. F., AND JENSEN, K. A.: *Z. physiol. Chem.* **277**, 66 (1942).
- (190) JOHNSON, F. H., AND LEWIN, I.: *Science* **101**, 281 (1945).
- (191) JOHNSON, O. H., GREEN, D. E., AND PAULI, R.: *J. Biol. Chem.* **153**, 37 (1944).
- (192) KABAT, H., STOHLMAN, E. F., AND SMITH, M. I.: *J. Pharmacol.* **80**, 160 (1944).
- (193) KAPLAN, H., AND LEUBNER, G. W.: *J. Am. Chem. Soc.* **67**, 1076 (1945).
- (194) KENNEDY, T. H.: *Nature* **150**, 233 (1942).
- (195) KESTON, A. S., GOLDSMITH, E. D., GORDON, A. S., AND CHARIPPER, H. A.: *J. Biol. Chem.* **152**, 241 (1944).
- (196) KIMMIG, J.: *Klin. Wochschr.* **20**, 235 (1941).
- (197) KLOTZ, I. M.: *J. Am. Chem. Soc.* **66**, 459 (1944).
- (198) KLOTZ, I. M., AND GUTMANN, H. R.: *J. Am. Chem. Soc.* **67**, 558 (1945).
- (199) KNIGHT, B. C. J. G.: *Proc. Roy. Soc. Med.* **37**, 492 (1944).
- (200) KOGL, F., AND BORG, W. A. J.: *J. physiol. Chem.* **281**, 65 (1944); *Chem. Abstracts* **39**, 4664 (1945).
- (201) KOHN, H. I.: *Ann. N.Y. Acad. Sci.* **44**, 503 (1943).
- (202) KOHN, H. I., AND HARRIS, J. S.: *J. Pharmacol.* **73**, 343 (1941).
- (203) KOHN, H. I., AND HARRIS, J. S.: *J. Pharmacol.* **77**, 1 (1943).
- (204) KUHN, R.: *Z. Physiol. Chem.* **129**, 57 (1923).
- (205) KUHN, R.: *Die Chemie* **55**, 1 (1942).
- (206) KUHN, R., MÖLLER, E. F., AND WENDT, G.: *Ber.* **76**, 405 (1943).
- (207) KUHN, R., MÖLLER, E. F., WENDT, G., AND BEINERT, H.: *Ber.* **75**, 711 (1942).
- (208) KUHN, R., AND SCHWARZ, K.: *Ber.* **74**, 1617 (1941).
- (209) KUHN, R., WEYGAND, F., AND MÖLLER, E. F.: *Ber.* **76**, 1044 (1943).
- (210) KUHN, R., WIELAND, W., AND MÖLLER, E. F.: *Ber.* **74**, 1605 (1941).
- (211) KÜLZ, F.: *Arch. exptl. Path. Pharmacol.* **98**, 339 (1923).
- (212) KWARTLER, C., AND LUCAS, P.: *J. Am. Chem. Soc.* **65**, 1804 (1943).
- (213) LAMPEN, J. O., AND PETERSON, W. H.: *J. Am. Chem. Soc.* **63**, 2283 (1941).
- (214) LAMPEN, J. O., AND PETERSON, W. H.: *Arch. Biochem.* **2**, 443 (1943).
- (215) LAMPEN, J. O., UNDERKOFER, L. A., AND PETERSON, W. H.: *J. Biol. Chem.* **146**, 277 (1942).
- (216) LANDAU, S. W., AND GAY, L. N.: *Bull. Johns Hopkins Hosp.* **74**, 55 (1944).
- (217) LANDSTEINER, K.: *The Specificity of Serological Reactions*. C. C. Thomas, Baltimore (1936).
- (218) LANDY, M., AND DICKEN, D. M.: *J. Biol. Chem.* **146**, 109 (1943).
- (219) LANDY, M., DICKEN, D. M., BICKING, M. M., AND MITCHELL, W. R.: *Proc. Soc. Exptl. Biol. Med.* **49**, 441 (1942).
- (220) LANDY, M., LARKUM, N. W., AND OSWALD, E. J.: *Proc. Soc. Exptl. Biol. Med.* **52**, 338 (1943).

- (221) LANDY, M., LARKUM, N. W., OSWALD, E. J., AND STREIGHTOFF, F.: *Science* **97**, 265 (1943).
- (222) LANDY, M., AND WYENO, J.: *Proc. Soc. Exptl. Biol. Med.* **46**, 59 (1941).
- (223) LAWRENCE, C. A., AND GOETCHIUS, G. R.: *Proc. Soc. Exptl. Biol. Med.* **58**, 356 (1945).
- (224) LEHMANN, G., AND KNOEFEL, P. K.: *J. Pharmacol.* **74**, 274 (1942).
- (225) LEHMANN, G., AND KNOEFEL, P. K.: *J. Pharmacol.* **80**, 335 (1944).
- (226) LEHMANN, G., AND YOUNG, J. W.: *J. Pharmacol.* **83**, 90 (1945).
- (227) LEHMANN, J.: *Lancet* **1943**, **I**, 458.
- (228) LEHMANN, J.: *Lancet* **1943**, **I**, 661.
- (229) LESTER, D.: *J. Biol. Chem.* **154**, 305 (1944).
- (230) LEVADITI, C.: *Compt. rend. soc. biol.* **135**, 1109 (1941).
- (231) LEVADITI, C., AND PÉRAULT, R.: *Compt. rend. soc. biol.* **135**, 1043 (1941).
- (232) LEWIS, J. C.: *J. Biol. Chem.* **146**, 441 (1942).
- (233) LILLY, V. G., AND LEONIAN, L. H.: *Science* **99**, 205 (1944).
- (234) LINK, K. P.: *Harvey Lectures*. Science Press Printing Company, Lancaster, Pennsylvania (1944).
- (235) LINK, K. P., OVERMAN, R. S., SULLIVAN, W. R., HUEBNER, C. F., AND SCHEEL, L. D.: *J. Biol. Chem.* **147**, 463 (1943).
- (236) LINNEWEG, F.: *Monatsschr. Kinderheilk.* **85**, 215 (1940-41) (from reference 216).
- (237) LOCKWOOD, J. S.: *J. Immunol.* **35**, 155 (1938).
- (238) LOEW, E. R., AND CHICKERING, O.: *Proc. Soc. Exptl. Biol. Med.* **48**, 65 (1941).
- (239) LOEW, E. R., AND KAISER, M. E.: *Proc. Soc. Exptl. Biol. Med.* **58**, 235 (1945).
- (240) LOEW, E. R., KAISER, M. E., AND ANDERSON, M.: From reference 75.
- (241) LOEW, E. R., KAISER, M. E., AND MOORE, V.: *J. Pharmacol.* **83**, 120 (1945).
- (242) LOEWI, O., AND NAVRATIL, E.: *Arch. ges. Physiol. (Pflüger's)* **214**, 678 (1926).
- (243) LORING, H. S.: *Ann. Rev. Biochem.* **13**, 309 (1944).
- (244) LUCAS, S. P., AND AGGELER, P. M.: *Proc. Soc. Exptl. Biol. Med.* **56**, 36 (1944).
- (245) LWOFF, A., NITTI, F., TRÉFOUËL, MME. J., AND HAMON, V.: *Ann. inst. Pasteur* **67**, 9 (1941).
- (246) McCARTY, M.: *Proc. Soc. Exptl. Biol. Med.* **46**, 133 (1941).
- (247) McGINTY, D. A.: From reference 16.
- (248) McGINTY, D. A., AND BYWATER, W. G.: *J. Pharmacol.* **84**, 342 (1945).
- (248a) McGINTY, D. A., AND BYWATER, W. G.: *J. Pharmacol.* **85**, 129 (1945).
- (249) McILWAIN, H.: *Brit. J. Exptl. Path.* **21**, 136 (1940).
- (250) McILWAIN, H.: *Nature* **146**, 653 (1940).
- (251) McILWAIN, H.: *J. Chem. Soc.* **1941**, 75.
- (252) McILWAIN, H.: *Brit. J. Exptl. Path.* **22**, 148 (1941).
- (253) McILWAIN, H.: *Biochem. J.* **35**, 1311 (1941).
- (254) McILWAIN, H.: *Brit. J. Exptl. Path.* **23**, 95 (1942).
- (255) McILWAIN, H.: *Biochem. J.*, **36**, 417 (1942).
- (256) McILWAIN, H.: *Lancet* **1942**, **I**, 412.
- (257) McILWAIN, H.: *Nature* **151**, 270 (1943).
- (258) McILWAIN, H.: *Biochem. J.* **37**, 265 (1943).
- (259) McILWAIN, H.: *Brit. J. Exptl. Path.* **24**, 203, 212 (1943).
- (260) McILWAIN, H.: *Biochem. J.* **38**, 97 (1944).
- (261) McILWAIN, H.: *Biol. Rev. Cambridge Phil. Soc.* **19**, 135 (1944).
- (262) McILWAIN, H.: *Biochem. J. (Proceedings)* **38**, 28 (1944).
- (263) McILWAIN, H.: *Biochem. J.* **39**, 279 (1945).
- (263a) McILWAIN, H.: *Biochem. J.* **39**, 329 (1945).
- (264) McILWAIN, H., AND HAWKING, F.: *Lancet* **1943**, **I**, 459.
- (265) McILWAIN, H., AND HUGHES, D. E.: *Biochem. J.* **38**, 187 (1944).
- (266) McILWAIN, H., AND HUGHES, D. E.: *Biochem. J.* **39**, 133 (1945).

- (267) McINTOSH, J., AND WHITBY, L. E. H.: *Lancet* **1939**, I, 431.
- (268) MACKAY, M.: *Australian J. Exptl. Biol. Med. Sci.* **16**, 137 (1938).
- (269) MACKENZIE, J. B., AND MACKENZIE, C. G.: *Federation Proc.* **1**, 122 (1942).
- (270) MACKENZIE, C. G., AND MACKENZIE, J. B.: *Endocrinology* **32**, 185 (1943).
- (271) MACKENZIE, J. B., MACKENZIE, C. G., AND MCCOLLUM, E. V.: *Science* **94**, 518 (1941).
- (272) MACLEOD, C. M.: *J. Exptl. Med.* **41**, 215 (1940).
- (273) MADINAVEITIA, J.: *Biochem. J. (Proceedings)* **38**, 27 (1944).
- (274) MADINAVEITIA, J., MARTIN, A. R., ROSE, F. L., AND SWAIN, G.: *Biochem. J.* **39**, 85 (1945).
- (275) MAIER, J., AND RILEY, E.: *Proc. Soc. Exptl. Biol. Med.* **50**, 152 (1942).
- (276) MARRACK, J. R.: *The Chemistry of Antigens and Antibodies*. His Majesty's Stationery Office, London (1939).
- (277) MARSHALL, E. K., JR., LITCHFIELD, J. T., JR., AND WHITE, H. J.: *J. Pharmacol.* **75**, 89 (1942).
- (278) MARTIN, A. R., AND ROSE, F. L.: *Biochem. J.* **39**, 91 (1945).
- (279) MARTIN, A. R., ROSE, F. L., AND SWAIN, G.: *Nature* **154**, 639 (1944).
- (280) MARTIN, G. J., AND FISHER, C. V.: *J. Biol. Chem.* **144**, 289 (1942).
- (281) MARTIN, G. J., AND FISHER, C. V.: *J. Lab. Clin. Med.* **29**, 383 (1944).
- (282) MATTI, J., NITTI, F., MOREL, M., AND LWOFF, A.: *Ann. inst. Pasteur* **67**, 240 (1941).
- (283) MAYER, R. L.: To be published.
- (284) MAYER, R. L., HUTTRER, C. P., AND SCHOLZ, C. R.: *Federation Proc.* **4**, 129 (1945).
- (285) MAYER, R. L., HUTTRER, C. P., AND SCHOLZ, C. R.: *Science* **102**, 93 (1945).
- (286) MELVILLE, D. B., DITTMER, K., BROWN, G. B., AND DU VIGNEAUD, V.: *Science* **98**, 497 (1943).
- (287) MENDEZ, R.: *J. Pharmacol.* **32**, 451 (1928).
- (288) MENTZER, C., AND MEUNIER, P.: *Bull. soc. chim. biol.* **25**, 379 (1943).
- (289) MEUNIER, P., AND MENTZER, C.: *Bull. soc. chim. biol.* **25**, 80 (1943).
- (290) MEUNIER, P., MENTZER, C., BUU-HOÏ, AND CAGNIANT: *Bull. soc. chim. biol.* **25**, 384 (1943).
- (291) MILLER, A. K., AND PETERS, L.: *Arch. Biochem.* **6**, 281 (1944).
- (292) MILLER, J. K.: *J. Pharmacol.* **71**, 14 (1941).
- (293) MILLER, J. K.: *J. Pharmacol.*, **72**, 354 (1941).
- (294) MILLER, W. H., ANDERSON, G. W., MADISON, R. K., AND SALLEY, D. J.: *Science* **100**, 340 (1944).
- (295) MILLER, W. H., ROBLIN, R. O., JR., AND ASTWOOD, E. B.: *J. Am. Chem. Soc.* **67**, 2201 (1945).
- (296) MITCHELL, H. H.: In *Vitamins and Hormones*. Academic Press, Inc., New York (1943).
- (297) MODERN, F. S., AND THIENES, C. S.: *Arch. intern. pharmacodynamie* **53**, 413 (1936).
- (298) MÖLLER, E. F., AND BIRKOFER, L.: *Ber.* **75**, 1108, 1118 (1942).
- (299) MÖLLER, E. F., AND SCHWARZ, K.: *Ber.* **74**, 1612 (1941).
- (300) MORRIS, H. C., AND DRAGSTEDT, C. A.: *Proc. Soc. Exptl. Biol. Med.* **59**, 311 (1945).
- (301) MORRISON, R. S., AND LISSAK, K.: *Am. J. Physiol.* **123**, 404 (1938).
- (302) MUIR, R. D., SHAMLEFFER, V., AND JONES, L. R.: *J. Bact. (Proceedings)* **41**, 84 (1941).
- (303) MUIR, R. D., SHAMLEFFER, V., AND JONES, L. R.: *J. Bact.* **44**, 95 (1942).
- (304) MURRAY, D. R. P.: *Biochem. J.* **23**, 292 (1929).
- (305) MYRBÄCK, K.: *Z. physiol. Chem.* **158**, 160 (1926).
- (306) NIELSEN, N.: *Naturwissenschaften* **31**, 146 (1943).
- (307) NIELSEN, N., AND HARTELIUS, V.: *Compt. rend. trav. lab. Carlsberg* **22**, 271 (1938).
- (308) NIELSEN, N., HARTELIUS, V., AND JOHANSEN, G.: *Naturwissenschaften* **32**, 294 (1944).
- (309) NIELSEN, N., AND JOHANSEN, G.: *Naturwissenschaften* **31**, 235 (1943).

- (310) NORTHEY, E. H.: *Chem. Rev.* **27**, 85 (1940).
- (311) NORTHEY, E. H.: *Sulfonamides and Allied Compounds*. Reinhold Publishing Corporation, New York (in press).
- (312) NUNGESTER, W. J., HOOD, M. N., AND WARREN, M. K.: *Proc. Soc. Exptl. Biol. Med.* **52**, 287 (1943).
- (313) OVERMAN, R. S., STAHMANN, M. A., HUEBNER, C. F., SULLIVAN, W. R., SPERO, L., DOHERTY, D. G., IKAWA, M., GRAF, L., ROSEMAN, S., AND LINK, K. P.: *J. Biol. Chem.* **153**, 5 (1944).
- (314) OVERMAN, R. S., STAHMANN, M. A., AND LINK, K. P.: *J. Biol. Chem.* **145**, 155 (1942).
- (315) OVERMAN, R. S., STAHMANN, M. A., SULLIVAN, W. R., HUEBNER, C. F., CAMPBELL, H. A., AND LINK, K. P.: *J. Biol. Chem.* **142**, 941 (1942).
- (316) PARK, C. R., AND WOOD, W. B., JR.: *Bull. Johns Hopkins Hosp.* **70**, 19 (1942).
- (317) PAULING, L., AND PRESSMAN, D.: *J. Am. Chem. Soc.* **67**, 1003 (1945).
- (318) PÉRAULT, R., AND GREIB, E.: *Compt. rend. soc. biol.* **138**, 506 (1944).
- (319) PETERS, L.: *J. Pharmacol.* **79**, 32 (1943).
- (320) PETERSON, C. G., AND PETERSON, D. R.: *J. Pharmacol.* **84**, 236 (1945).
- (321) PETERSON, W. H.: *Biol. Symposia* **5**, 31 (1941).
- (322) PETROVA, A. N.: *Advances in Modern Biology (U.S.S.R.)* **15**, 65 (1942).
- (323) PFALTZ, H.: *Z. Vitaminforsch.* **13**, 236 (1943); *Chem. Abstracts* **38**, 6339 (1944).
- (323a) PILGRIM, F. J., AXELROD, A. E., WINNICK, T., AND HOFMANN, K.: *Science* **102**, 35 (1945).
- (324) POLLACK, M. A.: *J. Am. Chem. Soc.* **65**, 1335 (1943).
- (325) PORTER, J. R., AND MEYERS, F. P.: *Arch. Biochem.* **8**, 169 (1945).
- (326) QUASTEL, J. H.: *Physiol. Rev.* **19**, 135 (1939).
- (327) QUASTEL, J. H.: *Trans. Faraday Soc.* **39**, 348 (1943).
- (328) QUASTEL, J. H., AND WOOLDRIDGE, W. R.: *Biochem. J.* **22**, 689 (1928).
- (329) QUICK, A. J.: *Physiol. Rev.* **24**, 297 (1944).
- (329a) QUICK, A. J.: *J. Biol. Chem.* **161**, 33 (1945).
- (330) RAIZISS, G. W., SEVERAC, M., AND MOETSCH, J. C.: *J. Am. Pharm. Assoc. (Sci. Ed.)* **31**, 198 (1942).
- (331) RAMANAMANJARY, W.: *Compt. rend. soc. biol.* **138**, 480 (1944).
- (332) RAPOPORT, S., WING, M., AND GUEST, M. G.: *Proc. Soc. Exptl. Biol. Med.* **53**, 40 (1943).
- (333) RAVENTÓS, J.: *Quart. J. Exptl. Physiol.* **26**, 361 (1937).
- (334) RAWSON, R. W., TANNHEIMER, J. F., AND PEACOCK, W.: *Endocrinology* **34**, 245 (1944).
- (335) REINER, L., AND LEONARD, C. S.: *Arch. intern. pharmacodynamie* **43**, 49 (1932).
- (336) RENNICK, B., CHESSE, D., HAYS, H. W., MATHIESON, D., MAYER, R. L., AND YONKMAN, F. F.: *Federation Proc.* **4**, 133 (1945).
- (336a) RICHARDS, R. K., EVERETT, G. M., AND KUETER, K. E.: *J. Pharmacol.* **84**, 387 (1945).
- (337) RICHTER, C. P., AND CLISBY, K. H.: *Arch. Path.* **33**, 46 (1942).
- (338) RIEVESCHL, G., JR., AND HUBER, W. F.: Unpublished results.
- (339) ROBBINS, W. J.: *Proc. Natl. Acad. Sci. U.S.* **27**, 419 (1941).
- (340) ROBLIN, R. O., JR., AND BELL, P. H.: *Ann. N.Y. Acad. Sci.* **44**, 449 (1943).
- (341) ROBLIN, R. O., JR., LAMPEN, J. O., ENGLISH, J. P., COLE, Q. P., AND VAUGHAN, J. R., JR.: *J. Am. Chem. Soc.* **67**, 290 (1945).
- (342) ROBLIN, R. O., JR., WILLIAMS, J. H., WINNEK, P. S., AND ENGLISH, J. P.: *J. Am. Chem. Soc.* **62**, 2002 (1940).
- (343) ROBLIN, R. O., JR., AND WINNEK, P. S.: *J. Am. Chem. Soc.* **62**, 1999 (1940).
- (344) ROBLIN, R. O., JR., WINNEK, P. S., AND ENGLISH, J. P.: *J. Am. Chem. Soc.* **64**, 567 (1942).
- (345) ROCHA E SILVA, M.: *J. Pharmacol.* **77**, 198 (1943).
- (346) ROCHA E SILVA, M.: *J. Pharmacol.* **80**, 399 (1944).
- (347) ROEPKE, R. R., AND JONES, M. J.: Unpublished results.

- (348) ROEPKE, R. R., PERKINS, A. B., AND MERCER, F.: To be published.
- (349) RONA, P., AND PAVLOVIC, R.: *Biochem. Z.* **130**, 225 (1922).
- (350) ROSE, H. M., AND FOX, C. L., JR.: *Science* **95**, 412 (1943).
- (351) ROSENBERG, H. R.: *Chemistry and Physiology of the Vitamins*. Interscience Publishers, New York (1942).
- (352) ROSENTHAL, S. M., AND VOEGTLIN, C.: *J. Pharmacol.* **39**, 347 (1930).
- (353) ROSENTHAL, S. R., AND BROWN, M. L.: *J. Immunol.* **38**, 259 (1940).
- (354) ROSENTHAL, S. R., AND MINARD, D.: *J. Exptl. Med.* **70**, 415 (1939).
- (355) RUBBO, S. D., AND GILLESPIE, J. M.: *Nature* **146**, 838 (1940).
- (356) RUBBO, S. D., MAXWELL, M., FAIRBRIDGE, R. L., AND GILLESPIE, J. M.: *Australian J. Exptl. Biol. Med. Sci.* **19**, 185 (1941).
- (357) RUBIN, S. H., DREKTER, L., AND MOYER, E. H.: *Proc. Soc. Exptl. Biol. Med.* **58**, 352 (1945).
- (358) RUBIN, S. H., FLOWER, D., ROSEN, F., AND DREKTER, L.: *Arch. Biochem.* **8**, 79 (1945).
- (359) SARETT, H. P.: *Federation Proc.* **4**, 101 (1945).
- (360) SARETT, H. P., AND CHELDELIN, V. H.: *J. Biol. Chem.* **156**, 91 (1944).
- (361) SARETT, H. P., AND CHELDELIN, V. H.: *J. Bact.* **49**, 31 (1945).
- (362) SCHAUMANN, O.: *Arch. exptl. Path. Pharmacol.* **138**, 208 (1928).
- (363) SCHMELKES, F. C., AND RUBIN, M.: *J. Am. Chem. Soc.* **66**, 1631 (1944).
- (364) SCHMELKES, F. C., WYSS, O., MARKS, H. C., LUDWIG, B. J., AND STRANDSKOV, F. B.: *Proc. Soc. Exptl. Biol. Med.* **50**, 145 (1942).
- (365) SEALOCK, R. R., AND GOODLAND, R. L.: *J. Am. Chem. Soc.* **66**, 507 (1944).
- (366) SEELER, A. O.: *Proc. Soc. Exptl. Biol. Med.* **57**, 113 (1944).
- (367) SEELER, A. O., GRAESSLE, O., AND DUSENBERRY, E. D.: *J. Bact.* **45**, 205 (1943).
- (368) SELBIE, F. R.: *Brit. J. Exptl. Path.* **21**, 90 (1940).
- (369) SEVAG, M. G., HENRY, J., AND RICHARDSON, R. A.: *J. Bact.* **49**, 71 (1945).
- (370) SEVAG, M. G., RICHARDSON, R. A., AND HENRY, J.: *J. Bact.* **49**, 79 (1945).
- (371) SEVAG, M. G., AND SHELBURNE, M.: *J. Bact.* **43**, 447 (1942).
- (372) SEVAG, M. G., SHELBURNE, M., AND MUDD, S.: *J. Gen. Physiol.* **25**, 805 (1942).
- (373) SEVAG, M. G., SHELBURNE, M., AND MUDD, S.: *J. Bact.* **49**, 65 (1945).
- (374) SHAPIRO, S., REDISH, M. H., AND CAMPBELL, H. A.: *Proc. Soc. Exptl. Biol. Med.* **52**, 12 (1943).
- (375) SHIVE, W., AND SNELL, E. E.: *Science* **102**, 401 (1945).
- (375a) SHIVE, W., AND SNELL, E. E.: *J. Biol. Chem.* **160**, 287 (1945).
- (376) SILVERMANN, M., AND EVANS, E. A., JR.: *J. Biol. Chem.* **150**, 265 (1943).
- (377) SILVERMANN, M., AND EVANS, E. A., JR.: *J. Biol. Chem.* **154**, 521 (1944).
- (378) SIMON, C. E., AND WOOD, M. A.: *Am. J. Med. Sci.* **147**, 524 (1914).
- (379) SINGER, T. P., AND BARRON, E. S. G.: *J. Biol. Chem.* **157**, 241 (1945).
- (380) SNELL, E. E.: *J. Biol. Chem.* **139**, 975 (1941); **141**, 121 (1941).
- (381) SNELL, E. E.: *Arch. Biochem.* **2**, 389 (1943).
- (382) SNELL, E. E.: *J. Biol. Chem.* **152**, 475 (1944).
- (383) SNELL, E. E., CHAN, L., SPIRIDANOFF, S., WAY, E. L., AND LEAKE, C. D.: *Science* **97**, 168 (1943).
- (384) SNELL, E. E., AND GUIRARD, B. M.: *Proc. Nat. Acad. Sci.* **29**, 66 (1943).
- (385) SNELL, E. E., AND MITCHELL, H. K.: *Arch. Biochem.* **1**, 93 (1942).
- (386) SNELL, E. E., AND SHIVE, W.: *J. Biol. Chem.* **158**, 551 (1945).
- (387) SOBOTKA, H., AND GLICK, D.: *J. Biol. Chem.* **105**, 199 (1934).
- (388) SOODAK, M., AND CERECEDO, L. R.: *J. Am. Chem. Soc.* **66**, 1988 (1944).
- (389) SPINK, W. W., AND JERMSTA, J.: *Proc. Soc. Exptl. Biol. Med.* **47**, 395 (1941).
- (390) SPINK, W. W., VIVINO, J. J., AND MICKELSON, O.: *Proc. Soc. Exptl. Biol. Med.* **50**, 31 (1942).
- (391) SPIZIZEN, J.: *J. Infectious Diseases* **73**, 212 (1943).
- (392) STAHMANN, M. A., HUEBNER, C. F., AND LINK, K. P.: *J. Biol. Chem.* **138**, 513 (1941).

- (393) STAMP, T. C.: *Lancet* **1939**, **II**, 10.
- (394) STANSLY, P. G., AND ALVERSON, C. M.: *Science*, in press.
- (395) STANSLY, P. G., AND SCHLOSSER, M. E.: *J. Biol. Chem.* **161**, 513 (1945).
- (396) STAUB, A.-M.: *Ann. inst. Pasteur* **63**, 400 (1939).
- (397) STAUB, A.-M.: *Ann. inst. Pasteur* **63**, 485 (1939).
- (398) STAUB, A.-M., AND BOVET, D.: *Compt. rend. soc. biol.* **125**, 818 (1937).
- (399) STEDMAN, E., AND STEDMAN, E.: *Biochem. J.* **25**, 1147 (1931).
- (400) STEPHENSON, M.: *Bacterial Metabolism*. Longmans, Green and Company, London (1939).
- (401) STEARN, E. W., AND STEARN, A. E.: *J. Bact.* **9**, 491 (1924).
- (402) STOKES, J. L.: *J. Bact.* **48**, 201 (1944).
- (403) STRANGWAYS, W. I.: *Ann. Trop. Med. Parasitol.* **31**, 387 (1937).
- (404) STRAUS, E., DINGLE, J. H., AND FINLAND, M.: *J. Immunol.* **42**, 313, 331 (1941).
- (405) STRAUSS, E., LOWELL, F. C., AND FINLAND, M.: *J. Clin. Investigation* **20**, 189 (1941).
- (406) SUMNER, J. B., AND SOMERS, G. F.: *Enzymes*. Academic Press, New York (1943).
- (407) TAMMANN, G.: *Z. physiol. Chem.* **16**, 271 (1891).
- (408) TAMURA, J. T.: *J. Bact.* **47**, 529 (1944).
- (408a) TATUM, E. L.: *J. Biol. Chem.* **160**, 455 (1945).
- (409) TATUM, E. L., AND BEADLE, G. W.: *Proc. Natl. Acad. Sci. U. S.* **28**, 234 (1942).
- (410) TEAGUE, P. C., AND WILLIAMS, R. J.: *J. Gen. Physiol.* **25**, 777 (1942).
- (411) TEPLEY, L. J., AXELROD, A. E., AND ELVEHJEM, C. A.: *J. Pharmacol.* **77**, 207 (1943).
- (412) THIMANN, K. V.: *Arch. Biochem.* **2**, 87 (1943).
- (413) TRACY, A. H., AND ELDERFIELD, R. C.: *J. Org. Chem.* **6**, 54 (1941).
- (414) UNGAR, G., PARROT, J.-L., AND BOVET, D.: *Compt. rend. soc. biol.* **124**, 445 (1937).
- (415) UNNA, K.: *Proc. Soc. Exptl. Biol. Med.* **54**, 55 (1943).
- (416) VALKO, E. I., AND DuBOIS, A. S.: *J. Bact.* **47**, 15 (1944).
- (417) VALLERY-RADOT, P., BOVET, D., MAURIC, G., AND HOLTZER, A.: *Compt. rend. soc. biol.* **136**, 354 (1942).
- (418) VALLERY-RADOT, P., BOVET, D., MAURIC, G., AND HOLTZER, A.: *Compt. rend. soc. biol.* **136**, 385 (1942).
- (419) VIGNEAUD, V. DU: *Science* **96**, 455 (1942).
- (420) VIGNEAUD, V. DU, McKENNIS, H., JR., SIMMONDS, S., DITTMER, K., AND BROWN, G. B.: *J. Biol. Chem.* **159**, 385 (1945).
- (421) VIGNEAUD, V. DU, MELVILLE, D. B., FOLKERS, K., WOLF, D. E., MOZINGO, R., KERESZTESY, J. C., AND HARRIS, S. A.: *J. Biol. Chem.* **146**, 475 (1942).
- (422) VOEGTLIN, C.: *Physiol. Rev.* **5**, 63 (1925).
- (423) VOEGTLIN, C., DYER, H. A., AND LEONARD, C. S.: *U.S. Pub. Health Rep.* **38**, 1882 (1923).
- (424) VOEGTLIN, C., JOHNSON, J. M., AND DYER, H. A.: *Proc. Natl. Acad. Sci. U.S.* **11**, 344 (1925).
- (425) WAGNER-JAUREGG, TH.: *Naturwissenschaften* **31**, 335 (1943).
- (426) WEINSTOCK, H. H., JR., MAY, E. L., ARNOLD, A., AND PRICE, D.: *J. Biol. Chem.* **135**, 343 (1940).
- (427) WEIS, O., AND JONES, L. R.: *J. Bact. (Proceedings)* **41**, 82 (1941).
- (428) WELCH, A. D.: *Physiol. Rev.* **25**, 687 (1945).
- (429) WELLS, J. A., AND MORRIS, H. C.: *Federation Proc.* **4**, 140 (1945).
- (429a) WELLS, J. A., MORRIS, H. C., BULL, H. B., AND DRAGSTEDT, C. A.: *J. Pharmacol.* **85**, 122 (1945).
- (430) WEST, R., AND COBURN, A. F.: *J. Exptl. Med.* **72**, 91 (1940).
- (431) WIEDLING, S.: *Science* **94**, 389 (1941).
- (432) WHITE, H. J.: Unpublished results.
- (433) WHITE, H. J., LEE, M., JACKSON, E. R., HIMES, A., AND ALVERSON, C. M.: To be published.

- (434) WILCOX, H. B., JR., AND SEEGAL, B. C.: *J. Immunol.* **44**, 219 (1942).
- (435) WILLIAMS, R. H., AND BISSELL, G. W.: *Science* **98**, 156 (1943).
- (435a) WILLIAMS, R. H., AND FRAME, E. G.: *Bull. Johns Hopkins Hosp.* **77**, 314 (1945).
- (436) WILLIAMS, R. J.: In *Advances in Enzymology*. Interscience Publishers, New York (1943).
- (437) WINTERBOTTOM, R., CLAPP, J. W., MILLER, W. H., ENGLISH, J. P., AND ROBLIN, R. O., JR.: To be published.
- (438) WITTS, L. J.: *Glasgow Med. J.* **19**, 57 (1942).
- (439) WOHL, A., AND GLIMM, E.: *Biochem. Z.* **27**, 349 (1910).
- (440) WOOD, J. L. AND DU VIGNEAUD, V.: *J. Am. Chem. Soc.* **67**, 210 (1945).
- (441) WOOD, W. B., JR.: *J. Exptl. Med.* **75**, 369 (1942).
- (442) WOOD, W. B., JR., AND AUSTRIAN, R.: *J. Exptl. Med.* **75**, 383 (1942).
- (443) WOODS, D. D.: *Brit. J. Exptl. Path.* **21**, 74 (1940).
- (444) WOODS, D. D.: *Biochem. J.* **36**, 4 (1942).
- (445) WOODS, D. D.: Personal communication.
- (446) WOODS, D. D., AND FILDES, P.: *J. Soc. Chem. Ind.* **59**, 133 (1940).
- (447) WOOLLEY, D. W.: *J. Biol. Chem.* **152**, 225 (1944).
- (448) WOOLLEY, D. W.: *J. Biol. Chem.* **154**, 31 (1944).
- (449) WOOLLEY, D. W.: *Proc. Soc. Exptl. Biol. Med.* **55**, 179 (1944).
- (450) WOOLLEY, D. W.: *Federation Proc.* **3**, 97 (1944).
- (451) WOOLLEY, D. W.: *Science* **100**, 579 (1944).
- (452) WOOLLEY, D. W.: *J. Biol. Chem.* **157**, 455 (1945).
- (453) WOOLLEY, D. W.: *J. Biol. Chem.* **159**, 59 (1945).
- (453a) WOOLLEY, D. W.: *Proc. Soc. Exptl. Biol. Med.* **60**, 225 (1945).
- (454) WOOLLEY, D. W., AND COLLYER, M. L.: *J. Biol. Chem.* **159**, 263 (1945).
- (455) WOOLLEY, D. W., AND KRAMPITZ, L. O.: *J. Exptl. Med.* **78**, 333 (1943).
- (456) WOOLLEY, D. W., STRONG, F. M., ELVEHJEM, C. A., AND MADDEN, R. J.: *J. Biol. Chem.* **124**, 715 (1938).
- (457) WOOLLEY, D. W., AND WHITE, A. G. C.: *J. Biol. Chem.* **149**, 285 (1943).
- (458) WOOLLEY, D. W., AND WHITE, A. G. C.: *J. Exptl. Med.* **78**, 489 (1943).
- (459) WOOLLEY, D. W., AND WHITE, A. G. C.: *Proc. Soc. Exptl. Biol. Med.* **52**, 106 (1943).
- (460) WRIGHT, H. N., AND HIRSCHFELDER, A. D.: *J. Pharmacol.* **39**, 39 (1930).
- (461) WYSS, O.: *Proc. Soc. Exptl. Biol. Med.* **48**, 122 (1941).
- (462) WYSS, O.: *J. Bact.* **46**, 483 (1943).
- (463) WYSS, O., GRUBAUGH, K. K., AND SCHMELKES, F. C.: *Proc. Soc. Exptl. Biol. Med.* **49**, 618 (1942).
- (464) WYSS, O., RUBIN, M., AND STRANDSKOV, F. B.: *Proc. Soc. Exptl. Biol. Med.* **52**, 155 (1943).
- (465) YONKMAN, F. F., CHESS, D., MATHIESON, D., AND HANSEN, N.: *Federation Proc.* **4**, 143 (1945).
- (466) YONKMAN, F. F., HAYS, H. W., AND RENNICK, B.: *Federation Proc.* **4**, 144 (1945).
- (467) YORKE, W., MURGATROYD, F., AND HAWKING, F.: *Ann. Trop. Med. Parasitol.* **25**, 351 (1931).
- (468) YOUNG, R. H., AND GILBERT, R. P.: *J. Allergy* **12**, 235 (1941).
- (469) YUAN, H. C., AND LI, H. C.: *J. Chinese Chem. Soc.* **5**, 214 (1937) (from reference 420).
- (470) ZELLER, E. A.: *Helv. Chim. Acta* **21**, 1645 (1938).
- (471) ZENTMYER, G. A.: *Science* **100**, 294 (1944).