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The physiological action of thiamine analogues in thiamine dependent systems

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NOTE TO USERS

THE PHYSIOLOGICAL ACTION OF THIAMINE ANALOGUES IN THIAMINE DEPENDENT SYSTEMS

by

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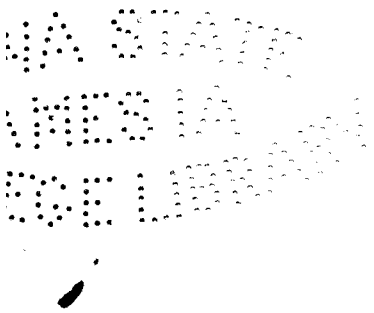

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I. INTRODUCTION

The fundamental catalytic unit of the living cell, the enzyme, has been the object of intense study since its discovery. As the numbers of enzymes and enzyme catalyzed biological reactions have evolved from the few rather ill-defined examples of one hundred years ago into the now numerous, highly specialized and integrated complexes of enzymes, the concept of the enzyme as one of the all-important units of all living processes has been firmly established.

Many of the basic contributions to the knowledge of enzymes have been made through the use of enzyme inhibitors, ions, compounds or natural substances that were found capable of inhibiting enzymatic action. As the functions of inhibitors began to be understood, their use developed into an exceptionally versatile and useful tool for the enzyme investigator.

The important discovery that structural analogues of metabolically required compounds can often interfere with or inhibit the action of specific enzymes which are dependent on the uncomplicated presence of the natural compounds, has added a new and important member to the group of enzyme inhibitors; one that has contributed immensely to both theoretical and practical knowledge in the last few years.

The allowable modifications of structure that will confer inhibitor properties to an analogue of a metabolically required compound are quite specific, and are most difficult to predict. The need for more fundamental knowledge in this respect is imperative if progress in this field is to continue. Systematic step-wise studies of structure modification

must be made, such as those that have been made with thiamine analogues for the Chastek-Paralysis enzyme; studies which have shown that certain structural configurations of benzyl-(3)-4-methylthiazolium chloride analogues of thiamine have been strikingly effective as compared to others in inhibiting the thiamine destroying powers of this enzyme.

The fact that certain of these structural modifications of thiamine characterized by benzyl-(3)-thiazolium analogues of thiamine can effectively compete with thiamine for a given enzyme makes it quite logical that these same types of analogues might also affect other enzyme systems involving thiamine.

The systematic investigation of some selected benzyl-(3)-thiazolium analogues of thiamine, in thiamine dependent systems, has been undertaken to determine their action in these systems, and where possible to determine through additional study the effect of the structural modification of the analogues on this action.

II. HISTORICAL

A. Structural Analogues in Vitamin Research

The use of structural analogues of the vitamins in biochemical research has evolved from a simple test for vitamin activity of the analogue to a tool of major importance in studies of vitamin mechanisms, vitamin enzymes, and determination of vital processes in cellular life.

As the structures of vitamins became known or were being elucidated, numerous synthetic analogues were prepared and tested for their vitamin activity, or their similarity to known degradation products of the natural vitamins. While in most instances the publication of the nature and properties of the various analogues actually used in elucidating the structure or synthesis of a new vitamin came with or after the announcement of structure or total synthesis, the work was without doubt done prior to this and the knowledge derived from the analogues contributed markedly to the successful termination of the research. Numerous fragment analogues of the components of thiamine, riboflavin and pyridoxine can be noted even in the final resumés of total synthesis of these vitamins (1).

Analogues and fragment analogues of vitamins have definitely contributed to the knowledge of vitamin activity. In addition through studies with these analogues, the necessary functional groups, their positions, and the general spatial requirements of many of the vitamins have been determined. Typical evidence accumulated through analogue studies with thiamine shows that the pyrimidine and thiazole rings, the methylene bridge between them (2), an unsubstituted amino group on the

4-position of the pyrimidine ring, a 5-hydroxy-alkyl-group (3), and a free 2-position in the thiazole ring are generally necessary for activity of the thiamine molecule.

Through the use of analogues, studies can be made on the inner workings of various cells. The ability or inability of specific organisms to utilize fragments of vitamins in effecting total synthesis of the true vitamin for its own use has been investigated. For example, Phycomyces blakesleeanus, a fungus, was shown (4) to be able to use thiamine or equimolar mixtures of the thiazole and pyrimidine moieties of thiamine.

Bonner and Buchman (5) used the Phycomyces assay to determine the fate of various analogues of the vitamin thiazole in isolated pea-roots which have the ability to synthesize thiamine from its pyrimidine and thiazole moieties and to utilize the thiamine for growth. The thiazole derivatives, all substituted in the 5-position, were able to support growth of pea-roots. They found that pea-roots possessed the ability to convert some of the thiazole analogues into the true thiamine thiazole while on the others they could not effect such a conversion. It was shown through these studies that the pea-roots possessed the necessary mechanisms to modify some analogues into the vitamin thiazole for use, and also that they could utilize certain analogues functionally even though it was not possible to change them into the natural compound.

These same authors (5) demonstrated the ability of pea-roots to synthesize the thiamine thiazole moiety of the vitamin if given the appropriate intermediates. Chloroacetopropyl alcohol and thioformamide, which give the thiamine thiazole in vitro, when supplied to the pea-roots were synthesized into the thiamine thiazole moiety. When pea-roots were supplied with the intermediates that would result in a vitamin thiazole

derivative with the hydroxyl group in the alpha instead of in the beta-position on the 5-alkyl side chain of the thiazole, they could not accomplish the synthesis. The α-hydroxy analogue if supplied to the pea-roots preformed, however, could be utilized to some extent in root growth. Apparently, therefore, pea-roots contain a relatively specific enzyme that can accomplish thiazole synthesis from appropriate precursors and another that can link thiazoles to appropriate pyrimidines to form thiamine or useful thiamine analogues.

A relatively new concept in the use of structural analogues in vitamin research is that of the inhibition of the natural physiological activity of the vitamin by a structural analogue of that vitamin. The historical approach to this phase of vitamin analogues will be presented later, but the usefulness of this tool should be mentioned at this point.

The ability to create artificial vitamin deficiency symptoms or diseases in laboratory animals, with all or portions of the characteristic deficiency manifestations, through structural analogues of the metabolite concerned has found wide application in vitamin research. In one of the more recent examples, the recognition and characterization of a new manifestation of pyridoxine deficiency has become possible through the use of a structural analogue, desoxypyridoxine, of known antagonism to pyridoxine (6). Stoerk and Eisen (7) demonstrated that dietary induced pyridoxine deficient rats showed a marked reduction in ability to form circulating antibodies to sheep erythrocytes. The condition was attributed to atrophy of the thymus in the pyridoxine deficient rats (8). When the antagonistic analogue desoxypyridoxine was fed, a disease was elicited which would be prevented by increasing pyridoxine in the ration. The feeding of this inhibitory structural analogue also caused an atrophy of the thymus of the rats with the resulting reduction in anti-bodies. The

similarity in results obtained in the case of both the natural and the artificial deficiency gave additional evidence for the relation of pyridoxine to thymus atrophy and reduced antibody formation.

The use of vitamin analogues in studies of isolated enzyme systems *in vitro* has been demonstrated by several workers (9, 10). This type of study illustrates the application of a new tool to the study of the vitamin enzymes.

B. Vitamin Enzymes

The enzymatic or "bio-catalytic" role of certain vitamins operating as key components of the enzyme molecule in vital reactions of metabolism is now firmly established. Catalytic function for vitamins had been predicted from the knowledge of the fact that relatively trace amounts of vitamins can prevent a metabolic chaos in an organism that is entirely out of proportion to the absolute amount of vitamin present.

Thiamine was established as a component of an enzyme system by the work of Lohmann and Schuster (11), who, in 1937, showed that cocarboxylase, the coenzyme of yeast carboxylase, was the pyrophosphate ester of thiamine. Since this discovery of the role of diphosphothiamine, it has been demonstrated to be the major functional form of thiamine in enzyme reactions in animal tissues (12, 13), and in bacterial (14, 15) and yeast cells (11, 16). The ability of the animal organism and its tissues, and the bacterial and yeast cells to convert thiamine to diphosphothiamine has been established (11, 15).

The components of the vitamin enzyme yeast carboxylase have been the subject of quite thorough investigation. Green and his collaborators (17) have isolated and purified a yeast carboxylase which consists of a

diphosphothiamine-metal-protein, and Kubowitz and Lüttgens (18) have also prepared a purified carboxylase from yeast which was shown to contain a ratio of one gram atom of magnesium and one mole of diphosphothiamine to 75,000 grams of protein. Green, Westerfield, Vennesland and Knox (19) have isolated a crude carboxylase from various animal tissues which consists of a diphosphothiamine, a divalent metal (Mg^{++} or Mn^{++}) and protein. Both the synthetic diphosphothiamine and the natural isolated diphosphothiamine are equally active in the restoration of activity to a resolved carboxylase enzyme system (11). The apoenzyme of carboxylase, which has been isolated and studied, seems to be protein in nature with reported molecular weights of 75,000 (18) and 150,000 (20).

At the present, it may be assumed that the entire enzymatic role of thiamine is mediated through enzyme systems including at least the following components:

diphosphothiamine	-	metal	-	protein
(coenzyme)		(divalent cation)		(apoenzyme)

However, the complete complex has not been isolated from all systems where diphosphothiamine has been shown to exert a catalytic influence. In some of these systems the need for metal ions and specific protein has not been ruled out, but on the contrary is strongly indicated (21).

In fact, the reactions catalyzed by the diphosphothiamine enzyme systems are not all clearly defined, but much of the catalytic effect centers about the reactions of the very reactive carbohydrate intermediate, pyruvic acid (14, 15), the true metabolic pathways of which are not completely understood. In the case of yeast and some microbial species the established metabolic pathway, catalyzed by the

diphosphothiamine enzyme, for pyruvic acid is its simple decarboxylation to acetaldehyde and carbon dioxide (22), whereas in animal and many bacterial cells this reaction does not seem to be of significant importance in the total metabolism. Pyruvate reactions catalyzed by diphosphothiamine in these cells are more complex, involving oxidative decarboxylation, dismutations and similar reactions (23), and often requiring in addition the presence of other enzyme systems of the flavoprotein or phosphopyridine classes (24, 25).

The present state of knowledge of thiamine-enzymes in pyruvate reactions has not advanced to the point where the whole picture can be presented without some reservations and conflicting opinions. However, evidence as a whole for the fact that normal pyruvate metabolism is dependent on the presence of a diphosphothiamine enzyme or enzymes is quite conclusive. In so far as normal adequate pyruvate metabolism is necessary to the whole of carbohydrate metabolism, and through this to the whole metabolic relationships of the intact cell, normal metabolism is related to the presence and function of the diphosphothiamine enzymes. Since carbohydrate metabolism is the net result of numerous enzymatic reactions, each mediated by a specific enzyme, the important roles of other vitamin enzymes must be considered.

Nicotinic acid or its amide appeared as an established component of an enzyme system several years prior to the discovery of its vitamin function. von Euler, Albers, and Schlenk (26, 27), in 1935, showed that coenzyme I contained one molecule of nicotinic acid amide, one molecule of adenine, two molecules of d-ribose, and two molecules of phosphoric acid, and that it was a dinucleotide. Warburg, Christian and Griese (28), in 1935, reported that the coenzyme II, was a dinucleotide consisting of one molecule of nicotinic acid amide, one molecule of adenine,

two molecules of pentose and three molecules of phosphoric acid, but it was not until 1937 that Elvehjem's group (29) announced that nicotinic acid was the anti-canine black tongue factor.

Since the discovery of the nutritional function of nicotinic acid, the bulk of experimental evidence links a major physiological role to its presence in the di- and triphosphopyridine dinucleotides (30, 31). The possibility of a significant role for free nicotinic acid has also been suggested (32) as well as a function of nicotinic acid in another and different type of respiration catalyst (33, 34).

The two known nicotinic acid or phosphopyridine nucleotide enzymes exist as either the di-or triphosphopyridine nucleotides, which are prosthetic groups associated with a specific protein carrier. Coenzyme I, the diphosphopyridine nucleotide and coenzyme II, the triphosphopyridine nucleotide, when associated with their respective specific proteins, constitute a portion of the groups of known enzymes classed as dehydrogenases (35).

The active center of these two coenzymes was elucidated soon after their discovery by Warburg and Christian (36). A reversible oxidation-reduction with resultant loss or gain of hydrogen is effected in the nicotinic acid amide portion of the coenzyme.

Specificity of these enzymes for their particular substrates, as with enzymes in general, comes from the specific protein carrier, while the removal and transfer of hydrogen involves the associated prosthetic group (30).

The role of this group of enzymes or their place in the scheme of biological oxidation is largely confined to oxidations (hydrogen removal) of some of the intermediates of carbohydrate metabolism. The oxidation of the reduced phosphopyridine nucleotides is accomplished in

most instances by the flavoproteins (35), the riboflavin enzymes.

Riboflavin became associated with enzymatic action at approximately the same time it became identified as a member of the vitamin B complex. In 1932, Banga, Szent-Györgyi and Vargha (37), and Warburg and Christian (38) isolated yellow-colored reversible oxidation-reduction systems of enzymatic character that were later shown to include riboflavin as portions of the systems. Theorell (39), in 1935, isolated the yellow pigment of Warburg and Christian's "yellow enzyme" and showed it to be riboflavin-5'-phosphoric acid.

Warburg and Christian (40), in 1938, were able to show that the prosthetic group of d-amino acid oxidase was a riboflavin-adenine dinucleotide compound consisting of one riboflavin, one adenine, one pentose and two phosphoric acid molecules. Other flavoprotein enzymes with this same prosthetic group (41, 42) as well as with prosthetic groups containing additional groups or groupings plus the riboflavin-adenine dinucleotide (43, 44), have since been isolated. Cytochrome c reductase is a flavoprotein enzyme containing as a prosthetic group an alloxazine mononucleotide (45).

Since the discovery of the nutritional significance of riboflavin, there is much evidence (46, 47) which associates major nutritional significance of riboflavin to the activity of the flavoprotein enzymes. Other roles may be considered as quite possible.

The number of flavoprotein enzymes identified is reasonably large, and in each case the riboflavin molecule is incorporated in the prosthetic group which is in turn associated with a specific protein (41) for the particular reaction catalyzed (43). The flavoprotein enzymes act in carbohydrate metabolism by virtue of the active center associated with the riboflavin molecule in the prosthetic group. A

reversible oxidation-reduction center in the isoalloxazine nucleus capable of taking up and giving off hydrogen is involved. The flavoprotein enzymes act as intermediate transfer agents, accepting hydrogen from the phosphopyridine nucleotide enzymes and transferring it to the cytochrome enzymes. For example, cytochrome c reductase (45) is exceptionally effective in removal of hydrogen from the phosphopyridine nucleotide and then transferring it to the cytochromes. In some instances the enzymes act directly not requiring intervention by the phosphopyridine nucleotide enzymes (40).

The most recent of the B vitamins shown to have significant enzymatic activity related to its nutritional function is pyridoxine. After papers appeared by Gunsalus and Bellamy (48), and Snell (49) in May and June, 1944, Gunsalus and his collaborators (50, 51), and other workers (52) started a rapid flow of publications that culminated in the identification of pyridoxal phosphate, the phosphorylated aldehyde derivative of pyridoxine, as the codecarboxylase of tyrosine decarboxylase and other amino acid decarboxylases. Schlenk and Snell (53), in 1945, reported work which linked the pyridoxine group of compounds with transamination. Another rapid flow of publications appeared from many laboratories implicating pyridoxal phosphate (54, 55) with the prosthetic group of the transaminases. More recent investigations (56) have expressed the view that a possibly more complex or different form of pyridoxal phosphate may be the true transaminase coenzyme.

Although the nutritional role of pyridoxine has never been entirely defined, association with enzymatic action may be a major function of pyridoxine in cellular metabolism. Activity of the amino acid decarboxylase and transaminase systems have been shown to be significantly

decreased in pyridoxine deficient tissues and microorganisms (57, 58).

Pyridoxal phosphate has been shown to be the codecarboxylase or prosthetic group (50) for several amino acid decarboxylases, typified by tyrosine decarboxylase. The protein carriers of these enzymes, all highly specific, have been isolated in the cell free states (50), and both natural and synthetic codecarboxylases have been shown to activate the apoenzyme. Specific apoenzymes for several transaminase systems have been isolated in water clear, cell free conditions and pyridoxal phosphate has exhibited the requisite activating effect (54).

The definite association of vitamins with enzyme systems is largely limited to thiamine, riboflavin, nicotinic acid, and pyridoxine. Suggested vitamin enzyme functions for some of the other vitamins have been made but with the exception of vitamin A (59) conclusive experimental proof has not been forthcoming.

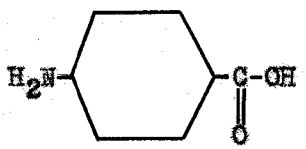
C. Vitamin Analogues as Inhibitors

Much of the present day concept of structural analogue inhibition of vitamin action came into being through the enlightening researches of Woods (60), coupled with the reasoning and interpretation he applied to his results. Woods was able to show that the growth inhibiting action of sulfanilimide against certain bacteria could be reversed by p-aminobenzoic acid.

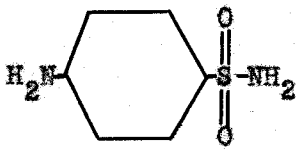
Investigation of the background of this discovery reveals that Woods himself, as well as his immediate associates, were quite aware of and receptive to a trend of thought of the time that the bacteriostatic action of the sulfonamides might be due to enzyme inhibition -- a trend of thought which was crystallized by his work. Chemical evidence of

structural similarity as well as interpretation of his preliminary results as competitive inhibition (60) led him to the actual testing of structural analogues of sulfanilimide as inhibitors of sulfonamide action.

The structural similarity of p-aminobenzoic acid to sulfanilamide is striking as may be seen in the two formulas:



p-aminobenzoic acid



p-aminobenzenesulfonamide
(sulfanilimide)

The antagonism of the two compounds was competitive, in that the mole ratios of p-aminobenzoic acid to sulfonamide present determined the occurrence or failure of the inhibition and not the absolute amounts present. Wyss (61) showed by mathematical analysis that the inhibitions observed between p-aminobenzoic acid and sulfanilimide were competitive.

At the time of Woods' work, p-aminobenzoic acid had not been associated with cellular life or function, either as an essential nutrient, a growth factor, or a constituent of protein. However, its isolation from yeast by Blanchard (62) in 1941, and the recognition of its growth promoting powers for certain bacteria (63) gave it a place in the physiological scheme, lending support to Woods' (60) contention that p-aminobenzoic acid might be considered as an essential metabolite.

Recent work culminating in the elucidation and identification (64) of the folic acid complex, places p-aminobenzoic acid squarely on a firm footing as an essential component in vital animal and bacterial processes. Pteroic acid, pteroyl glutamic acid, the tri- and hepta-

glutamates of pteroic acid all contain p-aminobenzoic acid (64) as an integral part of their structure.

While the sulfonamide-p-aminobenzoic acid inhibitions have been perhaps the most thoroughly studied (65) of all structural analogue inhibitions, the new metabolic role ascribed to p-aminobenzoic acid in the folic acid picture opens a new and fruitful field of study. Allowing the assumption that if p-aminobenzoic acid acts as a substrate in the synthesis of pteroic acid or the pteroyl glutamates in nature, then structural analogues such as sulfonamides might be expected to compete with it in this reaction.

Two researches which fall in line with this theory are Miller's (66), who demonstrated in 1944 that sulfonamides markedly inhibited the formation of folic acid by bacteria, and Lampen and Jones' (67), who have found that in the species of bacteria tested, those which required folic acid and therefore presumably could not synthesize it, were not susceptible to inhibition by sulfonamide drugs. It may be presumed that these bacterial species have no synthetic mechanism to be inhibited. Lampen and Jones (67) also noted that in some bacterial species pteroyl glutamic acid could negate the inhibition by sulfonamide drugs in the same manner as p-aminobenzoic acid itself, though in this case the antagonistic action of the folic acid was not competitive as would be expected if folic acid were the product and p-aminobenzoic acid the reactant of a reaction subject to competitive inhibition.

An attractive speculative theory for the explanation of sulfonamide action based on competitive structural inhibition can be derived (68) from these data — one which will undoubtedly be well explored in the future.

The p-aminobenzoic acid-sulfanilimide antagonism observed by Woods, supported by the establishment of p-aminobenzoic acid as a definite physiological entity, brings to light as concretely as any example three fundamental conditions (69) of structural analogue inhibitions. These are as follows: first, the antagonism between the two substances is competitive; second, one of the competitive pair of compounds is a metabolically required compound, that is, a native normal constituent of a biological system; and third, a molecular and structural similarity exists between the competing pair of compounds. Woods' work was followed almost immediately with the demonstration of nicotinic acid-nicotinic acid analogue competitions.

The studies of the structural antagonism of nicotinic acid activity have been carried out mainly with three analogues of nicotinic acid; pyridine-3-sulfonic acid, its analogous amide pyridine-3-sulfonamide and 3-acetyl pyridine. 3-Acetyl pyridine was prepared by Woolley, Strong, Elvehjem and Madden (70), in 1938, and tested for its vitamin activity in nicotinic acid deficient dogs where it acted as an inhibitor and actually increased the manifestations of that disease in the dog. This observation was made prior to the work of Woods (60) and the explanation was lacking at that time. Woolley (71) was able to bring about a deficiency disease in mice typical of nicotinic acid deficiences in other related animal species by feeding 3-acetyl pyridine to mice, but was not able to demonstrate that this analogue could inhibit the microbial species he tested. Neither could Auhagen (72) show real inhibitory powers for 3-acetyl pyridine in bacterial species.

McIlwain (73), who introduced pyridine-3-sulfonic acid and its amide as an inhibitor analogue in 1940, worked with Proteus vulgaris

and Staphylococcus aureus, both of which require external sources of nicotinic acid for growth, and showed that pyridine-3-sulfonic acid inhibited the growth of Proteus vulgaris when nicotinic acid was used as the growth factor, but that the analogue was not inhibitory when nicotinic acid amide was used as the growth source. With Staph. aureus the sulfonamide analogue inhibited the growth due to the nicotinic acid amide, but to a much lesser extent that due to nicotinic acid. Woolley and White (74) reported that the pyridine-3-sulfonic acid was ineffective as an inhibitor in mice. Euler (75) tested pyridine-3-sulfonic acid in an isolated enzyme system using lactic dehydrogenase, and showed that nicotinic acid itself was as inhibitory to the system as the pyridine-3-sulfonic acid.

Other analogues prepared by McIlwain (76) and Erlenmeyer and his collaborators (77, 78) have shown similar and apparently less inhibitory abilities.

Numerous analogues have been prepared of pantothenic acid and have been tested for their inhibitory properties. Roblin (79) has reviewed and integrated these extensive researches, and while the total number of analogues is large, the basic modifications have fallen into several definite structural patterns. Pantoyltaurine, first prepared and investigated by Snell (80) in 1941, is an analogue in which the carboxyl group of pantothenic acid has been replaced by the sulfonic acid group, and has been found to be a fairly active inhibitor of growth in many bacterial species. Several structural analogues of pantoyltaurine also show activity. The replacement of the carboxyl group with other groups than sulfonic acids has resulted in compounds with varying degrees of antagonistic activity. Woolley and Collyer (81) in substituting the phenyl ketone for the carboxyl produced a very potent antagonist to

pantothenic acid in all microbial species tested.

Modification of the basic structure of the pantothenic acid molecule such as effecting isomeric and homologous changes within the parent molecule, as typified by the work of Barnett and Robinson (82), has resulted in analogues of little or no antagonistic activity. However, simple isomeric modifications of the riboflavin molecule have resulted in riboflavin analogues possessing antagonistic activities toward riboflavin.

Kuhn, Weygand and Möller (83) prepared 6,7-dichloro-9-(d-1'-ribityl)-isoalloxazine, in which the methyl groups normally in the 6,7-positions were replaced by chlorine atoms in the analogue. With exception of yeast, this analogue was a potent antagonist of riboflavin in all bacterial species tested, and the antibacterial action was not dependent on whether or not the organism required riboflavin as a growth factor.

Emmerson's group (84, 85) have reported inhibition in rats with both 5,6-dimethyl-9-(d-1'-ribityl)-isoalloxazine and with 6,7-dimethyl-9-(d-2'-dulcityl)-isoalloxazine, the inhibitions being reversed with additional riboflavin in both cases. Foster (86) and Sarett (87) were unable to find any growth inhibition with Lactobacillus casei with the 5,6-analogue.

Woolley (88) prepared the 2,4-diamino-7,8-dimethyl-10-d-ribityl-5,10-dihydrophenazine which is also structurally related to riboflavin, and which can inhibit the growth of L. casei and also of rats, although sufficient amounts of riboflavin reversed this action.

Several biotin analogues of antagonistic activities have been described. For instance, Dittmer, Melville and du Vigneaud (89), and Lilly and Leonian (90) found that d-desthiobiotin, in which the $-\overset{|}{C}-S-\overset{|}{C}-$ linkage of biotin has been broken, and the sulfur replaced by two

hydrogen atoms, was able to reversibly antagonize biotin utilization by L. casei.

Dittmer and du Vigneaud (91) synthesized two analogues of desthiobiotin, the 4-(imidazolidine-2) caproic acid and 4-(imidazolidine-2) valeric acid. With yeast, which is apparently able to synthesize biotin from desthiobiotin (89) and is not inhibited by desthiobiotin, the caproic acid analogue was inhibitory, while in contrast, the valeric acid analogue was stimulating to yeast. Only the caproic acid analogue inhibited L. casei.

Rubin, Drekter and Meyer (92) observed that dl-desthiobiotin had but half the activity of d-desthiobiotin.

English and collaborators (93) prepared an analogue of biotin in which the -S- was replaced by $-CH_2 - CH_2-$. With few exceptions, this compound was an effective antagonist of biotin in both yeast and L. casei.

Apparent vitamin K antagonisms have been described and attributed to structural analogues of vitamin K. The hemorrhagic factor causing "sweet clover disease", 3,3'-methylenebis-(4-hydroxy coumarin) and some of its derivatives have elicited symptoms associated with vitamin K deficiency (94) in several animal species. Vitamin K will reverse these deficiency signs. It is interesting to note that in this case the parent inhibitor compound occurs in nature. Iodinin, an antibiotic pigment which resembles vitamin K in structure, has had its antibiotic properties reversed (95) by vitamin K in bacterial growth.

α-Tocopherol quinone, similar to vitamin K_1 in structure causes (96) some degree of deficiency in pregnant mice. In particular, uterine hemorrhages that might be associated with a vitamin K deficiency were observed. As further evidence of a direct relationship, these

conditions can be reversed by the administration of vitamin K_2.

The action of α-tocopherol quinone on mice really shows more of the characteristics of vitamin E deficiencies (96), including in addition to death and resorption of the embryos, excessive vaginal bleeding. Vitamin E, however, did not prevent these conditions while vitamin K_1 was able to overcome both the hemorrhage and resorption.

The antagonistic action of glucoascorbic acid, 2-3-enediol-d-gluco-heptono-1,4-lactone, to vitamin C in guinea pigs and the production of a scurvy-like condition in cotton rats and mice by this analogue of ascorbic acid have been reported by Woolley and Krampitz (97), and Woolley (98). Banerjee and Elvehjem (99) have not been able to confirm this work in rats, chicks or guinea pigs, but found that ascorbic acid itself produced similar though less severe symptoms in rats.

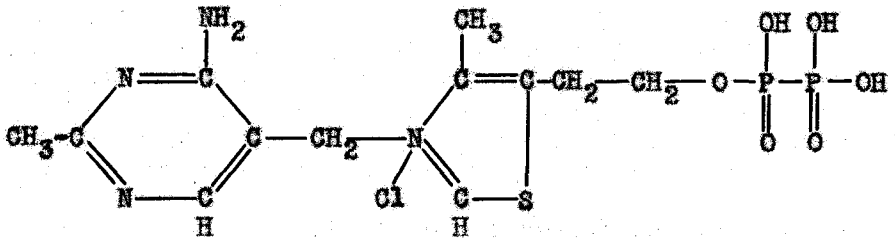
Sealock and Goodland (9) have used the glucoascorbic acid successfully as a specific competitive inhibitor in *in vitro* studies of an apparently ascorbic acid dependent enzyme system.

A derivative of folic acid, N-[4-{[(2-4-diamino-6-pteridyl)-methyl]-amino}-benzyl]-glutamic acid, has been prepared by Seeger, Smith and Hultquist (100) which in the case of *Streptococcus faecalis R.* has been shown to be a very potent inhibitor.

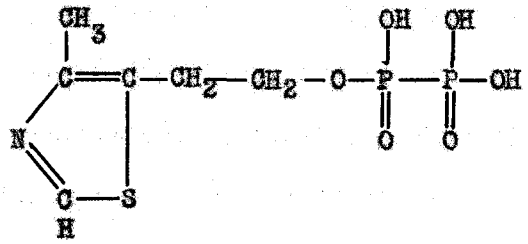
D. Inhibition of Thiamine Systems

The observation that the thiazole pyrophosphate portion of the cocarboxylase molecule inhibited the enzymatic decarboxylation of pyruvic acid by brewers' yeast was made by Buchman, Heegard, and Bonner (10) in 1940. In this inhibition, the importance of the pyrophosphate ester linkage was clearly demonstrated since the thiazole moiety alone,

its monophosphate ester, or sodium pyrophosphate produced no inhibition. The similarity of the "thiazole pyrophosphate" portion to the whole cocarboxylase molecule is evident in a comparison of the two structures.



Cocarboxylase



Thiazole Pyrophosphate

The inhibitions were studied by the Warburg manometric technique with the reconstituted yeast-carboxylase system, consisting of buffered alkaline washed brewers' yeast, manganese and magnesium ions, cocarboxylase, and excess sodium pyruvate as the substrate. With the amount of cocarboxylase added (4 μg.) as the limiting factor, the effect of the addition of the "thiazole pyrophosphate" to this system was studied. Significant inhibition as manifest by reduced CO_2 production was observed at "thiazole pyrophosphate" levels (Mn salt) of 4 and 8 μg., while at the 80 μ g. level CO_2 production was reduced to 10 per cent of the control level. The authors did not calculate mole ratios for their experiment but graphical calculation of their results places the mole ratio of thiazole pyrophosphate to cocarboxylase at which the CO_2 production is reduced to 50 per cent of the control level at approximately 12.5:1. Initial carbon dioxide production was reduced if the "thiazole

pyrophosphate" was added to the carboxylase system some time prior to the cocarboxylase. The "thiazole pyrophosphate" was used in the form of the magnanese salt, which was prepared from the silver salt of "thiazole pyrophosphate" described by Lohmann and Schuster (11). The method used for the preparation of the manganese salt was not disclosed.

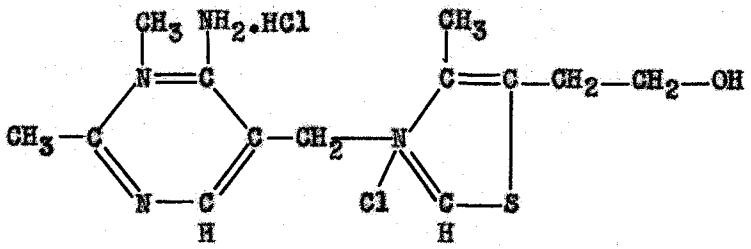
The authors suggested that this inhibition was due to a competition between cocarboxylase and the "thiazole pyrophosphate" for the specific carboxylase protein, and this view was substantiated by the observation that prior administration of the "thiazole pyrophosphate" reduced the initial CO_2 production. While the assumption was not made that the cocarboxylase linkage to the enzyme is exclusively through the pyrophosphate group, from the analogy that protein-inhibitor bond is similar to the cocarboxylase-protein complex, these experiments were interpreted as strong evidence that at least one of the cocarboxylase linkages to the carboxylase-protein was through the pyrophosphate group. This evidence is confirmatory to that proposed by Stern and Melnick (101) for the carboxylase linkage to the coenzyme through the pyrophosphate.

This work demonstrates clearly an enzymatic inhibition in which the competition is not between substrate and inhibitor for the enzyme, but between coenzyme and inhibitor for the specific enzyme protein.

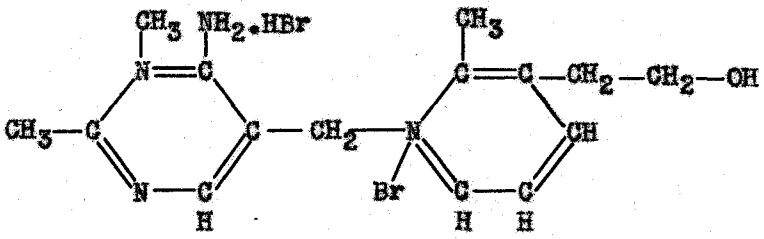
The restudy of the reported vitamin activity of heterovitamin B_1 led to the discovery that this compound actually had inhibitory powers rather than the vitamin activity previously ascribed to it. Since that time this analogue of thiamine, in which the thiazole nucleus has been replaced by the pyridine nucleus, has been extensively studied for its inhibitory powers.

Tracy and Elderfield (102) synthesized this pyridine analogue, the 2-methyl-4-amino-5-pyrimidylmethyl-(1)-2-methyl-(3)-β-hydroxyethylpyridinium

bromide hydrobromide, in 1940. The pyrimidine portion of the molecule is identical with that of the thiamine molecule while the pyridine portion differs from the thiazole of thiamine as the structural formulas indicate:



Thiamine



Pyridine analogue of Thiamine

In 1939, Schmelkes and Joiner (103), Schmelkes (104), and Baumgarten and Dornow (105) had synthesized a compound which they thought was the pyridine analogue of thiamine with the β-hydroxyethyl side chain, to which some vitamin activity had been ascribed by Schmelkes (104). Baumgarten and Dornow (106), in 1940, observed that in one of the reduction steps of their synthesis, a step common to both of the reported syntheses, the hydroxyl was formed on the alpha carbon of the ethyl side chain instead of the beta carbon, and therefore, the two compounds thought to be the β-hydroxyethyl analogues were in reality the α-hydroxyethyl analogues. Tracy and Elderfield (102) in a different synthesis actually obtained the β-hydroxy analogue.

Robbins (107), in 1941, set out to test the thiamine activity of

the β-hydroxyethyl analogue prepared by Tracy and Elderfield. He studied its action on three fungi, Phycomyces blakesleeanus, Pythiomorpha gonapodioides, and Phytophthora cinnamomi, which showed different and specific types of thiamine deficiences. These organisms were all able to utilize thiamine for growth, but their abilities to utilize or synthesize the pyrimidine and thiazole portions of the molecule varied.

The organisms were grown in liquid media, adequate for growth of the fungi if the required thiamine or intermediates were added to the media. The measure of growth after a designated period, was obtained by weighing the dried mycelium. The varying amounts of the pyridine analogue, thiamine or its component portions were added as desired.

In the interpretation of Robbins' results it must be kept in mind that he was actually testing for a growth function of the analogue, and therefore did not design experiments that would demonstrate to the best advantage the presence and extent of the inhibition of the fungi. Therefore, only a qualitative evaluation of these results is possible.

The effect of the pyridine analogue on the Phycomyces (able to combine pyrimidine and thiazole portions, but unable to synthesize either) revealed that slight inhibition resulted with low concentrations of the analogue with complete cessation of growth resulting at higher concentrations. Low concentrations of the analogue which produced little inhibition, were able in the presence of added thiazole to produce good growth. Added pyrimidine produced no effect since the fungi was not able to synthesize the thiazole. The Phytophthora, which required preformed thiamine was inhibited by the analogue much the same as was the Phycomyces. The addition of thiazole or pyrimidine portions of thiamine had no effect since the fungi could utilize neither, but added thiamine was able to reverse the inhibition. The Pythiomorpha, unable to synthesize the

thiazole and utilize it if pyrimidine is supplied, grew well in the presence of low concentrations of the pyridine analogue but was inhibited by higher concentrations. Both pyrimidine and thiamine were able to reverse the inhibition.

The results indicated that the pyridine analogue of thiamine was definitely inhibitory to thiamine in these fungi. Thiamine reversals, either from added thiamine or from the apparent splitting of the analogue of the pyrimidine and subsequent coupling to form thiamine, established the reversibility of the inhibitions. The Phycomyces and Pythiomorpha evidently were able to split the analogue and produce pyrimidine suitable for synthesis into thiamine for their own use.

Confirmatory evidence for the inhibitory powers of the pyridine analogue of thiamine in microorganisms was preceded a few months by the announcement by Woolley and White (108), in 1943, that a thiamine-like deficiency disease in mice could be produced by feeding the same pyridine analogue of thiamine that was used by Robbins (107) in his work with fungi. In this paper, Woolley and White coined the term "pyrithiamine" for the compound originally prepared by Tracy and Elderfield (102), and that term has been used synonymously with the chemical description since.

This thiamine-like deficiency in mice was unique, since in mice the typical manifestations of thiamine deficiency seen in other animal species do not appear when these animals are placed on a thiamine deficient diet. Normally the mouse will simply eat less and less, lose weight and die. The administration of pyrithiamine rather dramatically brought forth a disease in the mice which resembled the characteristic symptoms of thiamine deficiency seen in other species. Additional thiamine, and in some instances the removal of pyrithiamine, resulted in recovery from the disease, indicating reversal of the inhibition by

thiamine. Through experimentation with varying amounts of thiamine and pyrithiamine, it was shown that one mole of thiamine would counteract the effect of 40 molecules of pyrithiamine.

In their experimental tests, weanling mice of approximately 12 grams, were kept in individual cages with screen bottoms and were fed a ration shown by Woolley (109) previously to be adequate for mice. Since the average consumption of the ration was 2.0 grams per day for each mouse, it was calculated that each mouse received approximately 1.6 μ g. thiamine per day. In the later experiments, thiamine was eliminated from the basal diet and supplied orally. The pyrithiamine was administered orally in 0.02 ml. of a solution of the required concentration three times daily.

The disease elicited by pyrithiamine in mice was observed to be both striking and remarkably typical for each mouse in the group. In general, the following sequence of symptoms was observed to appear in each mouse in each group within 24 hours after the first was affected. The animals ceased to grow, lost weight, became inactive and assumed a hunched posture. Sometimes at this point, tremors and convulsions were noted. Spasticity of the legs was next observed followed by violent head jerking. The inability of the legs to support the body led to the last stage which was a characteristic spread-eagle position with the mouse lying on its belly. Death came within 24 hours after the onset of this last symptom. The general pattern of the disease in mice ran in direct proportion to the relative ratios of pyrithiamine and thiamine fed. In the first series of experiments where the thiamine intake was 1.6 micrograms per day, the administration of varying amounts of pyrithiamine ranging from 20 mg. to 0.1 mg. per day to groups of mice, characteristic deficiency symptoms were produced in all of the mice with

resulting death within three days of the onset of the symptoms. As the levels of pyrithiamine were reduced, the time for the onset of symptoms lengthened from 5 days in the case of the 20 mg. level to 12 days for the 0.1 mg. level.

The ability of thiamine to prevent and cure this disease was studied. In a series of experiments similar to those described above, the thiamine intake was increased to 60 μg. per day and in this case, the administration of 0.6 and 2.0 mg. of pyrithiamine evoked no deficiency symptoms and the growth and general appearance of these mice was as satisfactory as the controls. The ability of the thiamine to effect cures was investigated by observing the effect of the administration of relatively large doses of thiamine to mice rendered acutely ill by the daily administration of 2 mg. of pyrithiamine. Single doses of 1 mg. and 0.5 mg. of thiamine resulted in noticeable improvement in one hour, and with apparent complete recovery in 20 hours. However, continuation of the pyrithiamine at the same levels resulted in remissions that in turn resulted in death within 5 days. Under the same conditions, a single administration of 20 μg. of thiamine produced noticeable improvement for 24 hours, but death occurred shortly afterward.

One aspect of the effect of pyrithiamine on mice suggests possible toxic action by the compound. A definite delayed yet cumulative effect was encountered which made it possible to produce the disease, with its fatal conclusion, in the same time in two groups of mice; one which received 1.2 mg. of pyrithiamine for 3 days at which time administration was ceased, and one which received 1.2 mg. of pyrithiamine per day for the duration of the experiment. After 9 days, all of the animals in both groups had succumbed. Another group of 3 mice received 20 μg. of pyrithiamine and 2 μg. of thiamine for 14 days at which time the

pyrithiamine was eliminated. On the 19th day two mice died, but the third, though showing symptoms on the 19th day, did recover slowly and was alive when the experiment was terminated on the 28th day.

Detailed quantitative studies of the effect of pyrithiamine on microorganisms were simultaneously announced by Woolley and White (110) and Wyss (111) in 1943. Woolley and White (110) studied many species of bacteria, yeasts, and fungi for their ability to grow in the presence of pyrithiamine. The microorganisms were selected in accordance with their different specific thiamine requirements, and the media employed were those commonly used for each species in growth determinations. To the media of those microorganisms which required it, thiamine or its component parts was added, but none was added to the media of those species which did not require it in any form.

Antimicrobial potency of pyrithiamine was determined for each organism by the following procedure. Series of tubes (or flasks) of the appropriate basal medium were prepared with graded doses of pyrithiamine ranging from 5 mg. to 0.01 μg. per ml. Each tube was inoculated with a suspension of cells and incubated until good growth had occurred in the control tube. The extent of growth was determined quantitatively by titration of the acid produced, turbidity of the suspension, or the weight of dried mycelium, depending on the species tested. By a plot of the measure of growth against the pyrithiamine concentrations, a typical inhibition curve was obtained. By interpolation, the concentration of pyrithiamine required to cause a 50 per cent inhibition of growth was determined. From these data the "inhibition index" was calculated for the organisms tested. This inhibition index was defined as the mole ratio of pyrithiamine to thiamine that causes 50 per cent inhibition of growth. The various media used for the organisms

which required thiamine or its component parts for growth contained 0.01 μg. of thiamine per ml. while the media for the organisms that required no thiamine were assigned a thiamine value of 0.001 μg. per ml. for the purpose of calculation of the inhibition index. Microbiological assay revealed no thiamine in these media, but since the method itself was sensitive to only 0.001 μg., that figure was taken as the thiamine content of the media.

Table I

Inhibitory Power of Pyrithiamine for Various Microbial Species (110)

Organism	Inhibition Index*	Thiamine Requirement
Ceratostomella fimbriata	7	**Intact thiamine**
Phytophthora cinnamomi	12	" "
Endomyces vernalis	130	**Pyrimidine**
Mucor ramannianus	800	Thiazole
Saccharomyces cerevisiae	800	**Pyrimidine and thiazole**
Staphylococcus aureus	2,000	" " "
Escherichia coli	Greater than 2,000,000	**None**
Lactobacillus arabinosus	" 40,000	"
Lactobacillus casei	" 5,000,000	"

*Inhibition index = $\frac{\text{Pyrithiamine}}{\text{Thiamine}}$ to produce 50 per cent inhibition of growth.

Representative data from the authors' tabulation has been extracted and appears in Table I. Microorganisms from each class of thiamine dependency have been included. These data indicate that the susceptibility of the microorganism to pyrithiamine is closely correlated to its dependency on thiamine. For the two organisms requiring intact thiamine, the inhibition index is low. Endomyces vernalis, requiring pyrimidine, is quite sensitive to pyrithiamine, while those organisms requiring thiazole and pyrimidine and thiazole are much less sensitive.

The organisms requiring no outside source of thiamine or its component parts were not prevented from growing with large amounts of pyrithiamine. For those which were inhibited by pyrithiamine, the ability of thiamine to reverse the inhibition was demonstrated in each case. The complete failure of pyrithiamine to inhibit certain of these microorganisms in concentrations 500,000 times greater than that required to completely inhibit others was investigated. The possibility existed that these organisms synthesized enough thiamine during the period of growth to counteract the pyrithiamine. These organisms were grown in large quantities under similar conditions to those used in the inhibition studies and their thiamine content was determined, but the amount of thiamine synthesized was not sufficient to account for the failure of the pyrithiamine to prevent growth. Escherichia coli was grown in the presence of large amounts of pyrithiamine to see if pyrithiamine stimulated the production of thiamine, but the amount of thiamine synthesized was no greater than was normally formed in the absence of the analogue.

Contrasted to the inability of pyrithiamine to inhibit some species was its ability to stimulate others at subinhibitory levels. These stimulations were not large but definite, and were not related to any particular requirement for thiamine in the species stimulated.

Wyss (111) studied the competition between pyrithiamine and thiamine on Staph. aureus under the same general conditions as reported by Woolley and White (110). For the same inhibition index at half maximal growth a value of 700:1 was reported. With his strain of E. coli, the inhibition ratio was about 20,000:1. The reversibility of these inhibitions by added thiamine could be shown. The injection of noninjurious amounts of pyrithiamine into mice was shown to produce no antibacterial

concentrations in their blood.

Since the papers by Woolley and White (110) and Wyss (111) were submitted on the same date, the marked difference in the effect of pyrithiamine inhibition of E. coli was not discussed by the respective authors. It may be postulated that the difference in strains of the E. coli might account for the difference.

Continuing his work with pyrithiamine, Woolley (112) reported in 1944 that he had succeeded in developing a strain of Endomyces vernalis that would grow well in high concentrations of pyrithiamine. Through twelve successive transfers in increasing concentrations of pyrithiamine up to 20 μg. per ml. of media, plus thirty transfers of young cells at a pyrithiamine level of 25 μg. per ml. of media, Woolley obtained a strain of Endomyces vernalis which he called pyrithiamine-fast that would grow as well in a medium containing 25 μg. of pyrithiamine per ml. of media as it would in the absence of the compound. This amount of pyrithiamine was 25 times that amount that would inhibit the parent strain half-maximally.

The pyrithiamine-fast strain has the same thiamine requirements as the parent strain, that is, it grew very poorly in the absence of thiamine, but was stimulated by either the vitamin or its pyrimidine portion alone. In the absence of the vitamin, pyrithiamine acts as a growth factor for the fast strain, the pyrithiamine being 50 per cent as active as thiamine on the molecular basis.

The ability of this strain to grow well in high pyrithiamine concentrations as well as its ability to use pyrithiamine as a growth factor was explained in part by the demonstration that this organism could split the pyrithiamine molecule, thus reducing the pyrithiamine concentration and also making a supply of pyrimidine available as a growth factor.

The increased inhibitory powers of pyrithiamine on the diphosphothiamine stimulated growth over that stimulated with thiamine was observed by Sarett and Cheldelin (113) for Lactobacillus fermentum and Penicillium digitatum. For both organisms diphosphothiamine promoted faster growth than the thiamine, but in both cases pyrithiamine added in concentrations too low to inhibit growth stimulated by thiamine was very effective in the inhibition of the diphosphothiamine stimulated growth. For L. fermentum the inhibition index is about 50 when the organism is grown with thiamine and only 10 with diphosphothiamine, while with Penicillium digitatum, approximate indices are about 150 with thiamine and 50 with diphosphothiamine.

In attempting to arrive at the reason for the difference in inhibitory powers, the inhibition of equimolar mixtures of thiamine and diphosphothiamine was studied to establish the fact that a possible lack of free thiamine in the media due to the suppression of hydrolysis of the diphosphothiamine by the 6-aminopyrimidine portion of the inhibitor, might account for the difference in the observed inhibitions. They postulated that if the lack of free thiamine in the media was the cause of the increased sensitivity of the organism to pyrithiamine, the addition of some thiamine should then reduce the sensitivity of the system back to that observed for thiamine alone. The resulting indices were somewhat greater, that is, more pyrithiamine was required to obtain 50 per cent inhibition of the mixture, but not enough more to attach any significance to lack of free thiamine in the system.

Inhibitor studies were carried out using pyrithiamine, iodoacetate, fluoride, malonate, cyanide, and dinitrophenol to attempt to find if alternative metabolic pathways existed for the L. fermentum to use diphosphothiamine for growth or acid production. These studies indicated

that while different metabolic pathways exist, the effects of the inhibitors were similar with thiamine, monophosphothiamine, which was included as a possible intermediate, and the diphosphothiamine.

The effect of the 6-aminopyrimidines on the growth stimulations by thiamine and diphosphothiamine for L. fermentum were studied to see if the mechanisms for conversion of added thiamine or diphosphothiamine to the carboxylase enzyme differed. The 6-aminopyrimidines, 2-methyl-6-aminopyrimidine, 2-methyl-5-ethoxymethyl-6-aminopyrimidine and 2,6-dioxypyrimidine, were used to prevent the hydrolysis of diphosphothiamine to thiamine. Westenbrink and his group (114) had calculated that 150 to 200 μg. of the aminopyrimidine compounds prevented completely the splitting of 1 μg. of diphosphothiamine to thiamine. Sarett and Cheldelin (115) had shown that under the conditions of this test with L. fermentum (18 hours) none of the pyrimidines could be used for growth. The 2,6-dioxypyrimidine had no effect even at 200 μg. per tube, while the two aminopyrimidines at 100 μg. per tube inhibited growth with the diphosphothiamine but not with thiamine. In concentrations of 200 μg. per tube thiamine stimulated growth is inhibited slightly while the growth with diphosphothiamine is completely repressed. This effect of the 6-aminopyrimidines on the utilization of diphosphothiamine by L. fermentum is opposite to the stimulation of the yeast carboxylase by even smaller amounts of these substances (116, 117). The inhibitions of the added diphosphothiamine by the 6-aminopyrimidines was observed to be competitive, and that of thiamine at much higher concentrations was also competitive.

In evaluating their results Sarett and Cheldelin (113) summarized these points:

The fact that pyrithiamine inhibition of diphosphothiamine stimulated

growth is much greater than the inhibition of thiamine stimulated growth is contrary to expectation since the thiamine must be phosphorylated prior to taking part in its enzymatic role. The observed opposite effect required explanation.

The more rapid growth of the L. fermentum with diphosphothiamine did not indicate that cell permeability was different for the two forms of thiamine such as has been observed in brain tissue (13). The need for free thiamine by the bacteria was not shown to be important in this case, nor was the alternate metabolic pathway shown to be significant with respect to the two forms of thiamine. The competitive inhibition observed with the higher levels of 6-aminopyrimidines in which the diphosphothiamine stimulated growth was completely inhibited indicated that the diphosphothiamine was either prevented from reaching the carboxylase proteins or was displaced completely from these proteins by the 6-aminopyrimidines. The inhibition observed with the 6-aminopyrimidines on thiamine stimulated growth was slight indicating that the diphosphothiamine, which must have been produced from thiamine derived from the nutrient media, was more firmly bound. The competitive inhibitions observed with pyrithiamine for the thiamine and diphosphothiamine logically can be interpreted in the same manner.

The conclusions of Sarett and Cheldelin (113) drawn from these facts were that phosphorylation of the thiamine to thiamine pyrophosphate must occur after a firm linkage has been accomplished by the thiamine molecule on the carboxylase protein; in other words, the phosphorylation occurs on the carboxylase protein. If phosphorylation of the media thiamine occurred at a different site the thiamine pyrophosphate so formed could only associate with the carboxylase protein in the same manner as does the added preformed diphosphothiamine. This hypothesis

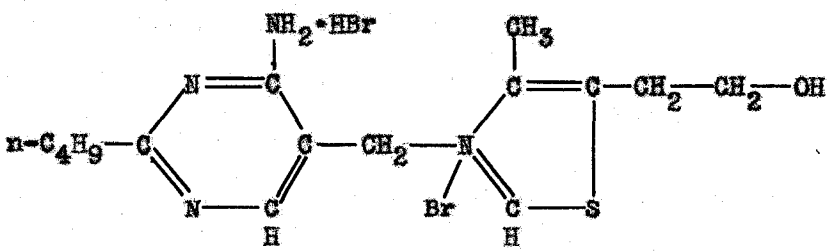
is supported by the work of Westenbrink, Willebrands and Kamminga (118) in their findings that in natural carboxylase the association is firm between the diphosphothiamine coenzyme and the protein while the complex formed by the addition of diphosphothiamine to the carboxylase protein results in a more highly dissociable linkage. These workers postulate that the cocarboxylase is attached to the protein at two points for the non-dissociated enzyme, one through the pyrimidine amino group to a protein carboxyl group (tightly bound) and the other by a salt-like linkage of the pyrophosphate group to an amino group (highly dissociated), and postulate the latter linkage for the case where preformed diphosphothiamine is added to the apoenzyme. The findings of Buchman, Heegard and Bonner (10), and Stern and Melnick (101) for their evidence of attachment through the pyrophosphate linkage mentioned earlier in this thesis are not in conflict with this hypothesis.

The hypothesis of Sarett and Cheldelin (113) concerning the demonstrated ability of the 6-aminopyrimidines to displace the easily dissociated preformed diphosphothiamine from the enzyme protein seems to assume and possibly require that the competition between these inhibitors and the diphosphothiamine centers about the "firmly associable bond" between the protein carboxyl group and the inhibitor or diphosphothiamine amino group. If this dissociable linkage were to occur elsewhere in the diphosphothiamine molecule the role of the 6-aminopyrimidines in a competitive inhibition would seem obscure, yet this same "firmly associable bond" between protein and amino groups is the one which they postulate holds the thiamine firmly, prior to and after phosphorylation. It would seem logical, therefore, that during phosphorylation of the thiamine attached to the carboxylase protein through this strong bond, there would be an instant, during which the newly

phosphorylated thiamine is leaving the phosphorylation center and becoming associated with the carboxylase protein, that it would be identical with the "loosely" bonded preformed diphosphothiamine supplied. The ability of this "loose" carboxylase to decarboxylate quite efficiently is assumed; therefore, the loosely bonded diphosphothiamine must be relatively well oriented in order to be able to function.

A good possibility does exist that the pyrophosphate is first attached to the carboxylase protein, then linked with the thiamine molecule, thereby forming a firmer link with the protein through the pyrophosphate than otherwise could occur.

The interesting observation by Emmerson and Southwick (119) that the 2-n-butylpyrimidine homologue of thiamine was able to produce characteristic deficiency symptoms in rats was made in 1945. This compound, the 2-n-butyl-5-(4-methyl-5-β-hydroxyethylthiazolium bromide)-methyl-6-aminopyrimidine hydrobromide, possesses the chemical integrity of both portions of the thiamine molecule, but is different from thiamine only in the length of the alkyl side chain on the 2-position of the pyrimidine portion of the molecule -- points which are well illustrated in the structural formula which appears below.



2-n-Butylpyrimidine Homologue of Thiamine

The experiment that was successful in bringing forth the antagonistic action of this 2-n-butyl homologue indicated both the antagonism of the

homologue at suboptimal levels of thiamine and the inability of the same concentrations to inhibit higher concentrations of thiamine, an indication of the reversibility of this inhibition.

The experimental animals, rats, were maintained on established thiamine deficient ration and were supplemented daily by stomach tube as follows: group 1, no supplement; group 2, 2.8 mg. of the inhibitor analogue; group 3, 5 μg. of thiamine; group 4, 5 μg. of thiamine and 2.8 mg. of the inhibitor; group 5, 50 μg. of thiamine; group 6, 50 μg. of thiamine and 2.8 mg. of the inhibitor. The 2.8 mg. of the n-butyl homologue is the molecular equivalent of 2.0 mg. of thiamine.

The summary of results obtained is shown in Table II extracted from the authors' paper.

Table II

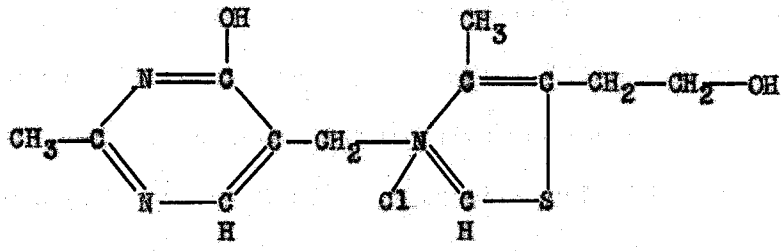
Response of Rats to Thiamine and 2-n-Butylpyrimidine Homologue of Thiamine (119)

Group No. (Eight rats each, males)		Average gain in weight 30 days	No. of survivors	Average gain in weight 50 days	No. of survivors	Average length of survival	Remarks
		gm.		gm.		days	
1	Control	21	8		0	38	
2	2.8 mg. homologue	23	3		0	29	
3	5 μg. thiamine	86	8	117	8		No polyneuritis
4	5 μg. thiamine 2.8 mg. homologue	51	8	37	3		All animals showed polyneuritis
5	50 μg. thiamine	130	8	193	8		
6	50 μg. thiamine 2.8 mg. homologue	114	8	166	8		

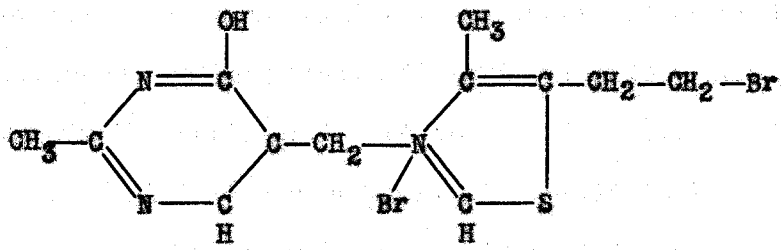
The comparison of groups 1 and 2 shows that the survival period is reduced in the thiamine deficient diet by the 2-n-butyl analogue. The

contrast between groups 3 and 4 maintained on the suboptimal levels of thiamine, clearly demonstrated the antagonistic powers of the analogue. The rats on 5 μg. per day of thiamine continued to grow and were free of symptoms of polyneuritis, while on the rats in group 4, the effect of the compound was manifest by the appearance of definite signs of polyneuritis in all animals and death of five of the eight in the group. The effect of the compound at the 50 μg. levels was manifest by a slightly reduced growth rate but the animals were apparently normal otherwise. On the basis of the above experiment, the authors calculated that one mole of thiamine counteracts the antithiamine effect of about 40 moles of the n-butyl homologue. This activity was approximately that reported for pyrithiamine in mice by Woolley and White (108).

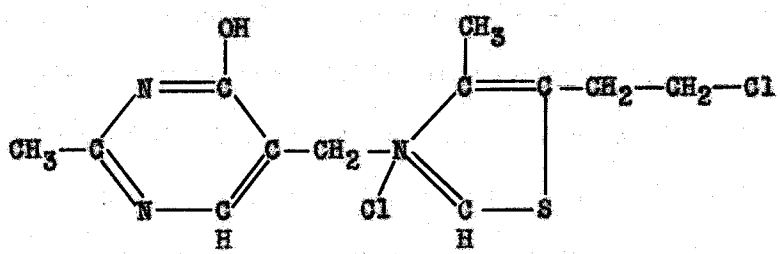
Oxythiamine hydrochloride, 2-methyl-4-hydroxy-5-pyrimidyl methyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chloride, had been reported as antagonistic to thiamine by Soodak and Cerecedo (120) in a note to the editor in 1944. The ability of this analogue to inhibit the Chastek-Paralysis Factor was also announced; however, experimental evidence for these two properties of oxythiamine hydrochloride was not forthcoming until 1947 when Soodak and Cerecedo (121) revealed the results of the thiamine inhibitory properties of this compound in mice. The authors also presented evidence indicating the necessity for the free hydroxyl group on the side chain in the thiazole portion of the oxythiamine for antagonistic activity, through the use of oxybromothiamine and oxychlorothiamine. These structures are compared below:



Oxythiamine



Oxybromothiamine



Oxychlorothiamine

The effective blocking of the hydroxyl group in the thiazole moiety is obvious.

Previously thiamine depleted groups of mice on a synthetic thiamine deficient diet received 1 μg. of thiamine per day plus daily supplements, through injection, of one of the following; 25, 50 or 90 μg. of oxythiamine, 100 μg. of oxychlorothiamine, or 139 μg. of oxybromothiamine, and controls were maintained on thiamine alone. Growth inhibition was noticed promptly in those mice receiving oxythiamine. A hunched back, severe loss of appetite and weight, and eventual death

resulted after varying lengths of time. The thiamine controls as well as the mice on thiamine plus oxychlorothiamine and thiamine plus oxybromothiamine continued to gain weight for the duration of the experiment. That the inhibitory ability of this analogue of thiamine seems to be quite dependent on the presence of the free hydroxyl group on the hydroxyethyl side chain is quite clearly demonstrated. The fact that the inhibitory powers of this analogue compare quite favorably in magnitude with those of pyrithiamine in mice is noteworthy since the amine group, postulated by Sarett and Cheldelin (113) as an important grouping in the inhibitory powers of pyrithiamine in L. fermentum is replaced by a hydroxyl group in oxythiamine. No mention was made in the latest paper of Goodak and Cereceda (121) about the effect of oxythiamine on the Chastek-Paralysis enzyme.

E. Inhibition of Chastek-Paralysis Enzyme

The enzymatic nature of the Chastek-Paralysis Factor has been established through the work of Krampitz and Woolley (122), and Sealock and his collaborators (123, 124, 125). Sealock and Goodland (124), employing inhibitors in their studies of the enzymatic nature of this factor, found that certain compounds, structural analogues of thiamine, were able specifically and competitively to inhibit the action of the enzyme.

The most active inhibitor observed was the benzyl analogue of thiamine, o-aminobenzyl-(3)-4-methylthiazolium chloride. Thiamine destruction was inhibited 100 per cent by the inhibitor at concentrations of inhibitor of 5.0×10^{-4} molar. The striking structural similarity of this analogue to thiamine was noted.

An analysis of this inhibition, its specificity and type, was made through the application of the experimental inhibition data to the Lineweaver-Burk modification of the Michaelis-Menten equation for enzyme substrate competitions. It was shown that this benzyl analogue specifically inhibited thiamine destruction by competing with thiamine for the enzyme.

The structural analogues utilized in this study were derivatives of 4-methyl thiazole and of 2-methyl-4-aminopyrimidine. Table III shows the structural relationships of these analogues and also the comparative results of the inhibition abilities of these compounds.

Table III

Inhibition of Thiamine Destruction (124)

No.	Compound	Concentration mols/liter $\times 10^4$	Inhibition in %
	4-Methyl-thiazolium Chloride Derivatives		
1	3-o-Aminobenzyl-	5.0	100.0
2	3-o-Nitrobenzyl-	5.0	0.0
3	3-β-Aminoethyl-	5.0	56.4
4	3-β-Phthalimidoethyl-	5.0	18.4
5	3-Ethyl-	10.0	9.3
6	3-Phenyl-	5.0	2.3
7	3-Ethyl-2-methyl-	5.0	0.0
8	3-Phenyl-2-methyl-	5.0	0.0
9	3-Methyl-5-β-hydroxyethyl-	5.0	0.0
	2-Methyl-4-aminopyrimidine Derivatives		
10	5-Bromomethyl-	5.0	34.8
11	5-Methylenesulfonicacid	10.0	19.7
12	5-Ethoxymethyl-	5.0	13.2

From these data it was noted that structure modification of the parent analogue produced definite differences in inhibitory ability. Blocking the $-NH_2$ group in compound 3 with the phthalimido group as typified by compound 4 produced a marked decrease in inhibition ability.

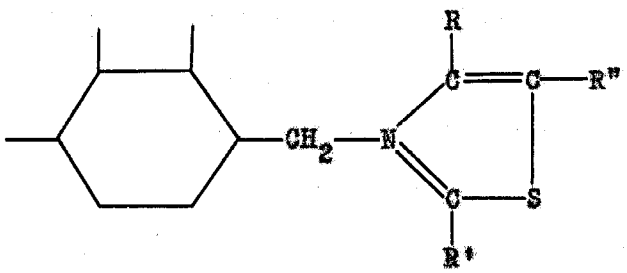
Substitution of the nitro group for the amino group in the parent inhibitor (compounds 1 and 2) resulted in complete negation of inhibitory powers. The importance of the free amino group for inhibition is indicated, though its position related to the thiazole ring does not seem to be so critical (compounds 1 and 3). The aminopyrimidines, all containing the essential pyrimidine moiety of thiamine, showed some inhibitory powers. In view of this, the authors postulated that the presence of the amino group in conjunction with the thiazolium portion of the molecule furnished the stronger inhibition. Their evidence indicated that the 5-β-hydroxyethyl side chain on the thiazole was not necessary for these inhibitions.

The demonstrated ability of the o-aminobenzyl-(3)-4-methyl thiazolium analogue of thiamine to compete with thiamine for the position on the enzyme is somewhat remarkable in one respect. Both the pyrimidine and thiazole rings have been markedly altered from those of the original thiamine molecule, yet the essential features for competition with thiamine for a position on the enzyme still remain. This type of analogue represents a simple combination of essential chemical and physical features with which to study enzyme mechanisms and action.

F. Benzyl-(3)-Thiazolium Compounds

The benzyl-(3)-thiazolium analogues of thiamine were first prepared in the laboratories of Clarke (126). Livermore and Sealock (127), recognizing the potentialities of these analogues as tools in the studies of enzymes and enzyme action undertook the systematic modification of the inhibitor molecule o-aminobenzyl-(3)-4-methylthiazolium chloride by the synthesis of its isomeric forms as well as many derivatives of the parent compound.

The benzyl-(3)-thiazolium nucleus as illustrated, shows that the



Benzyl-(3)-Thiazolium Nucleus

molecule lends itself to many substitutions. The amino group may be absent, or present in the o-, m- or p-positions of the benzyl nucleus. Other groups, such as nitro may be substituted for the amino in the same manner. Thiazolium substitutions can be made at R, R', or R". It is noted that the normal thiamine thiazole will evolve if R is substituted with a methyl group and R" consists of a β-hydroxyethyl group.

Livermore and Sealock (127) synthesized the following benzyl-(3)-thiazolium compounds. These included the benzyl, the o-, m-, or p-nitrobenzyl, and the o-, m-, or p-aminobenzyl quaternary salts of 2- or 4-methyl, 2-4-dimethyl, and the 4-methyl-5-β-hydroxyethylthiazoles. Crystallization was effected for all but two of these compounds; the o- and m-aminobenzyl-4-methyl-5-β-hydroxyethyl compounds could not be crystallized.

The possibility that benzyl-(3)-thiazolium analogues of thiamine could compete with thiamine itself in animal and microbial species presents interesting possibilities and potential analogies. Inhibitions observed in one system do not necessarily require that the same or similar analogues might inhibit in other situations. The brief remark by Soodak and Cereceda (120) mentioned previously, that their thiamine antagonist, effective in mice, was also effective in inhibiting the Chastek-Enzyme suggests that this possibility exists with the

benzyl-(3)-thiazolium analogues also.

The syntheses of benzyl-(3) analogues of thiamine with the 5-β-hydroxyethyl side chain on the thiazole ring result in analogues whose structural similarity to thiamine are even more pronounced, since the "thiamine thiazole" becomes common to both the benzyl analogues and thiamine.

III. EXPERIMENTAL

A. Compounds Tested

The investigation of the effects of the benzyl-(3)-thiazolium analogues of thiamine on several thiamine dependent systems required two major steps in the beginning. An adequate supply of structurally related analogues was assembled. These bore in addition to a common relationship of structure to thiamine, a structural relationship to each other. The reasonably available thiamine dependent test systems were studied and evaluated, and from them three were selected. Since the selection of the compounds for study was related to the test systems utilized, a brief discussion of the individual test systems is included with the discussion of the analogues.

Three thiamine dependent systems have been included. While essentially different in themselves, they have in common the fact that all are systems which lend themselves to thiamine assay. The rat, as an animal dependent on thiamine for life and growth, and Lactobacillus fermentum 36, a lactic acid bacterium whose growth under certain conditions becomes extremely sensitive to thiamine, were selected as means of evaluating the effect of these analogues on growth. Growth itself is the net result of adequate functioning of many or all of the enzyme systems in the animal or microorganism and can not be considered to be dependent upon thiamine alone. Thiamine, however, as a necessary member of several of these enzyme systems can be made the limiting factor to the growth functions of the rat and bacterium by controlling the amount made available. This fact has made possible the application of

the rat (128) and the L. fermentum (115) to assay procedures for thiamine, since under controlled conditions the amount of growth observed is dependent on the supply of external thiamine. The sensitivity of the growth response in these two assay methods to small increments of thiamine makes the L. fermentum and rat assay methods suitable thiamine dependent systems with which to evaluate the physiological effect of the thiamine analogues.

The third method employed depends upon the ability of added thiamine to stimulate the production of CO_2 by certain yeasts. Schultz, Atkin and Frey (129) have used this stimulating effect on bakers' yeast (Saccharomyces cerevisiae) to develop a yeast fermentation assay method for thiamine which has been widely used. Both the basal fermentation of the yeast, through its carboxylase enzymes and the effect of added thiamine on the stimulation of the basal fermentation are functions of the thiamine molecule which make this third method another thiamine dependent system that lends itself to analogue studies.

The rat assay method (128) and the L. fermentum assay method (115) have been used by other investigators (113, 119) in studying the effect of structural analogues of the vitamins on vitamin activity in these systems. The application of the method of Schultz, Atkin and Frey (129) to structural analogue studies has not been reported to the knowledge of the author. The usefulness of this method as a test system will be discussed in a later section of this thesis.

The L. fermentum and yeast fermentation tests, because of their relative rapidity and the fact that the actual amounts of analogues required were small, became ideal methods for the study and screening of a relatively large number of analogues for their physiological action; while in direct contrast, the rat assay method required three to four

weeks for an assay of a single analogue.

The benzyl-(3)-thiazolium analogues selected for investigation were of two general types as shown in Table IV. The first type retained the intact thiazole moiety of thiamine, the 4-methyl-5-β-hydroxyethylthiazole group, and varied the benzyl substituents on the (3)-position, the quaternary nitrogen of the thiazole ring. The second general type was characterized by the lack of the hydroxyethyl group in the 5-position of the thiazole ring in that the thiazole moiety of the analogue was the 4-methylthiazole, while benzyl moieties similar to those of the first group were attached through the quaternary nitrogen linkage to this thiazole.

These two types of analogues with various modifications of the benzyl moiety enabled, in addition to the simple test for activity, the study to be made of the effect of the presence, absence and position of the several groupings in the benzyl and thiazole portions of these analogues.

The studies on the physiological action of these analogues were supplemented in certain instances by parallel studies on the synthetic intermediates and derivatives of the analogues themselves, as an additional means of evaluating the effect of the analogue. While these "fragment analogues", as they might be considered, in themselves have little if any resemblance to the structure of the thiamine molecule, they are related indirectly through the analogue under study.

Several 6-aminopyrimidine compounds were included also since 6-aminopyrimidine compounds had been observed to exert an effect on the yeast fermentation method (130) and on the L. fermentum method (113) used in this investigation.

All of the compounds with which these studies were made are listed

in Table IV, which indicates in addition, the structural relationships of these analogues to each other. The source from which each compound was obtained is indicated by the footnotes of Table IV. The compounds were used in the forms as indicated with the exception of the silver salt of 4-methyl-5-β-hydroxyethylthiazole pyrophosphate. The preparation of the free "thiazole pyrophosphate" was accomplished in the following way.

The silver salt as obtained was contaminated with a small amount of the monophosphate ester of 4-methyl-5-β-hydroxyethylthiazole. In addition to removal of the silver ion it was also necessary to determine accurately the amount of the pyrophosphate ester present in the silver salt. For removal of the silver, 500 mg. of the salt was suspended in 10 ml. of H_2O in a 50 ml. centrifuge tube and titrated with 1.0 N HCl to remove the silver ion. When 2.38 ml. of 1.0 N HCl had been added, precipitation of silver was found to be complete. The precipitate was centrifuged and the supernatant liquid decanted through a small filter paper into a 50 ml. beaker. The precipitate was washed four times with 3.0 ml. of acidulated water (3 drops 1.0 N HCl + 9 ml. H_2O) followed by centrifugation and decantation of the supernatant washings into the filter paper. The filter paper was washed once with 3.0 ml. of the acidulated H_2O. This filtrate was adjusted to a pH of 7.0 with a Beckman pH Meter then transferred to a volumetric flask and diluted to 50 ml. This solution was kept under refrigeration and after determining the 4-methyl-5-β-hydroxyethylthiazole pyrophosphate concentration in this solution, it was used as the source of the "thiazole pyrophosphate" for this investigation.

The assay of the amount of "thiazole pyrophosphate" present was based on the following facts. The hydrolysis of the first phosphate group from the pyrophosphate ester of the 4-methyl-5-β-hydroxyethylthiazole

Table IV

Benzyl-(3)-thiazolium Analogues of Thiamine and Related Compounds Investigated for their Physiological Action in Thiamine Dependent Systems

Compound	Source*
(3)-4-Methyl-5-β-hydroxyethylthiazolium Derivatives	
Benzyl-chloride	(1)
o-Aminobenzyl-iodide hydroiodide	(2)
p-Aminobenzyl-chloride hydrochloride	(1)
o-Nitrobenzyl-chloride hydrochloride	(1)
m-Nitrobenzyl-chloride hydrochloride	(1)
p-Nitrobenzyl-chloride hydrochloride	(1)
γ-Phthalimidopropyl-bromide	(2)
Methyl-iodide	(4)
(3)-4-Methylthiazolium Derivatives	
Benzyl-chloride	(1)
o-Aminobenzyl-chloride	(1)
o-Nitrobenzyl-chloride	(1)
γ-Aminopropyl-bromide hydrobromide	(3)
γ-Phthalimidopropyl-bromide	(3)
Δ-Amino-n-butyl-bromide hydrobromide	(3)
2-Methyl-6-aminopyrimidine Derivatives	
5-Methylenesulfonic acid	(5)
5-Bromomethyl-dihydrobromide	(6)
5-Hydroxymethyl-	(5)
Other Derivatives	
4-Methylthiazole	(1)
2-Methylthiazole	(1)
2,4-Dimethylthiazole	(1)
4-Methyl-5-β-hydroxyethyl thiazole	(6)
4-Methyl-5-β-hydroxyethyl thiazole pyrophosphate (silver salt)	(5)
o-Aminobenzyl alcohol	(1)

*(1) These compounds were kindly furnished by Dr. R. R. Sealock from those prepared by Livermore and Sealock (127).
(2) These compounds were prepared and kindly furnished by Mr. R. L. Taylor.
(3) These compounds were prepared and kindly furnished by Miss H. K. Sarver.
(4) This compound was prepared and kindly furnished by Mr. N. C. Davis.
(5) These compounds were prepared and kindly furnished by Dr. R. R. Sealock.
(6) These compounds were commercial preparations (Merck & Co.).

to form the monophosphate ester "thiazole monophosphate" is rapid and complete in 30 minutes in the presence of 0.1 N HCl at a temperature of 100° C, while the second phosphate group is quite resistant to hydrolysis (131). This evidence indicated that an analytical method for the "thiazole pyrophosphate" could be based on the measurement of the difference in phosphate ion content of a solution of crude "thiazole pyrophosphate" before and after hydrolysis of the first phosphate radical from the "thiazole pyrophosphate". The fundamental assumption that must be made is that the phosphate ion so measured is the only phosphate split off and that its hydrolysis under the conditions employed is complete. Based on this assumption, the following scheme for analysis was devised. Three phosphorus analyses were made on both the silver salt and on an aliquot of the previously described "thiazole pyrophosphate" stock solution, one for free or ionic phosphate ion, one for total phosphorus and one for "acid hydrolyzable" plus ionic phosphorus. The difference between total phosphorus and ionic phosphorus gives the amount of combined phosphorus in the sample. The difference between the "acid hydrolyzable" plus ionic phosphorus and the ionic phosphorus gives the amount of "acid hydrolyzable" phosphorus present.

The analytical methods used were based in the main on the method of Fisk and SubbaRow as modified for the Klett-Summerson Colorimeter in the Klett Manual (132). The digestion step for the total phosphorus was based on the method for total acid soluble phosphorus as described in Hawk, Oser and Summerson (133). The hydrolysis of phosphate from the "thiazole pyrophosphate" was based on the method of Weijlard and Tauber (131). The stock reagents were prepared as described in the Klett Manual. The stock standard phosphate solution contained 1.3723 gm. of KH_2PO_4 per liter; the aminonaptholsulfonic acid, .5 gm.

1,2,4-aminonaptholsulfonic acid, 195 ml. of 15 per cent $NaHSO_3$, and 5.0 ml. of 20 per cent Na_2SO_3; the molybdate reagent contained 25 gm. of ammonium molybdate and 300 ml. of 10 N H_2SO_4 diluted to 1 liter; the 10 per cent trichloroacetic acid reagent contained 100 gm. of trichloroacetic acid per liter.

The methods used for the determination of total phosphorus, ionic phosphorus, and acid hydrolyzable plus ionic phosphorus in the thiazole pyrophosphate silver salt in thiazole pyrophosphate solutions were as follows:

Preparation of unknown — the amount of unknown estimated to contain approximately .05 mg. of P was added to a 25 ml. volumetric flask. Solution was affected by adding 22.5 ml. of 10 per cent trichloroacetic acid and the contents were diluted to the mark with distilled water.

Preparation of blank — 22.5 ml. of 10 per cent trichloroacetic acid was added to a 25 ml. volumetric flask and diluted to the mark with distilled water.

Preparation of standard phosphate — 2.0 ml. (0.6250 mg. P) of stock phosphate solution was added to a 25 ml. volumetric flask; 22.5 ml. of 10 per cent trichloroacetic acid was added and the contents of the flask were diluted to the mark with distilled water. This solution contains 0.05 mg. P per 2 ml.

Determination of total phosphorus — 2.0 ml. of each of the unknown, blank, and standard solutions was placed in micro-Kjeldahl flasks; 2.5 ml. of 5.0 N H_2SO_4 was added and the contents of the flasks were digested over a micro-burner until colorless. One drop of 30 per cent H_2O_2 was added to the cooled contents and the mixtures again digested. 5.0 ml. of water was added and the contents brought to boiling; on cooling, the contents of the flasks were transferred

quantitatively to a 25 ml. volumetric flask; 2.5 ml. of molybdate reagent was added and the solutions mixed. 1.0 ml. of the aminonaptholsulfonic acid was added, the solution diluted to volume, mixed and allowed to stand for 5 minutes; the colors produced were then read in the Klett-Summerson photoelectric colorimeter using the KS #66 filter.

Determination of ionic phosphorus -- 2.0 ml. each of the unknown, blank, and standard solutions was added to 25 ml. volumetric flasks. 10 ml. of 10 per cent trichloroacetic acid followed by 2.5 ml. of molybdate reagent and 1.0 ml. of aminonaptholsulfonic acid was added to each flask, the contents mixed and diluted to the mark. After 5 minutes standing, the solutions were read in the colorimeter as outlined above.

Determination of ionic plus acid hydrolyzable phosphorus -- 4.0 ml. of the unknown and blank solutions, and 2.0 ml. of the standard phosphate solutions were added to 25 ml. pyrex volumetric flasks. 2.0 ml. of trichloroacetic acid solution was added to the flasks containing the unknown and blank, and 4.0 ml. to the standard flask. Hydrolysis was effected in a boiling water bath for 30 minutes. The solutions were cooled and 5.5 ml. of 10 per cent trichloroacetic acid, 2.5 ml. of molybdate reagent, and 1.0 ml. aminonaptholsulfonic acid reagent were added to each flask, the contents mixed and diluted to volume. After 5 minutes of standing the solutions were read in the colorimeter.

The results based on the colorimeter readings were calculated as follows:

$$\text{Total P:} \quad \frac{(\text{reading unknown})}{(\text{reading standard})} (.05) \left(\frac{1}{2}\right) (25) = \text{mg. total P in original sample}$$

$$\text{Ionic P:} \quad \frac{(\text{reading unknown})}{(\text{reading standard})} (.05) \left(\frac{1}{2}\right) (25) = \text{mg. Ionic P in original sample}$$

Ionic P plus Acid Hyd. P: $\frac{\text{(reading unknown)}}{\text{(reading standard)}}$ (.05) $\frac{(1)}{(4)}$ (25) = mg. Ionic plus acid hyd. P in original sample

These calculations are derived to fit the specific conditions as outlined and read directly in terms of the amount of sample in the original preparation of the unknown for analysis. The methods themselves are general and may be used under different conditions and calculations made to fit the particular conditions used.

Two analyses, both in duplicate, were made to determine the amount of "thiazole pyrophosphate" present in the stock solution. The first was made on the silver salt of the "thiazole pyrophosphate" and the concentration of the stock solution calculated from these results, while the second was made on an aliquot of the stock solution itself.

The concentration of "thiazole pyrophosphate" in the stock solution was found to be: 2.3 mg. per ml. by the analysis of silver salt; and 2.4 mg. per ml. by the analysis of an aliquot of stock solution. The agreement between the two analyses is quite satisfactory and, though both results are calculated on the basis of the same assumption, the agreement does indicate that the assay is an adequate measure of the "thiazole pyrophosphate" present. For the purpose of the later studies this stock solution was assumed to have a concentration of 2.3 mg. of "thiazole pyrophosphate" per ml.

The analogues and derivatives of thiamine listed and discussed above were studied in one or more of the thiamine dependent systems investigated. The amounts available of some of the analogues were not large, and this dictated to a marked extent which analogues were used in the experiments requiring relatively large amounts of compound. Also several of the more effective analogues were prepared and made available

toward the conclusion of this investigation and were not available for study in all of the systems employed.

Chronologically the investigation of the physiological action of these analogues begins with the studies of their effect on the rat, and since the preliminary findings on the rat to a considerable extent influenced the trend of the investigation in the other thiamine systems, the action of certain of these analogues on the rat will be considered first.

B. Rat Assay Method

The physiological activity of three selected analogues was investigated in the animal species using the rat as the test animal. The function of thiamine in animal tissues as a component of several enzyme systems is well established, and the metabolic upset created by thiamine deprivation is acute. The rat therefore becomes, for the purpose of this study, a thiamine dependent system which can be manipulated within reason at the will of the investigator. With the rat as the test unit, the thiamine and analogue intake can be rigidly controlled and the responses of the animal can be measured and evaluated through well established criteria previously described for the response of the rat to varying levels of thiamine.

The test procedure for evaluation of the activity of the selected analogues was based on the rat assay method for thiamine determination (128). This procedure was selected since it included the desired features required for the tests and in addition presented a well established method for the assay.

Weanling rats of 26-28 days of age at weights of 50-60 grams were

maintained in individual cages on large mesh screen floors and received water and the following basal diet ad libitum: sucrose (commercial cane sugar) 69 gm.; vitamin-free casein (acid washed) 18 gm.; hydrogenated vegetable oil ("Crisco") 6 gm.; cod liver oil (Squibbs) 2.0 gm.; and a commercial salt mixture [G. B. I. Salt Mixture #2 U.S.P. XII (134) for experiments 1 through 6, and the Hawk-Oser salt mixture (135) for experiments 7 through 10], 5.0 gm.; riboflavin 0.5 mg.; nicotinic acid 10.0 mg.; pyridoxine-hydrochloride 0.2 mg.; d-calcium pantothenate 2.0 mg.; choline-chloride 10.0 mg.; and i-inositol 100.0 mg., per 100 gm. of basal diet.

Thiamine and analogue supplements were administered orally daily by means of a 1.0 ml. syringe fitted with an 18 gauge blunt needle. The supplements were made in the required concentrations in solutions containing 20 per cent alcohol and 0.1 per cent acetic acid. Fresh supplements were prepared each week and kept under refrigeration when not in use. Concentrations were adjusted so that the desired levels of thiamine and/or test compound would be contained in 0.1 ml. of supplement, which was the amount administered daily to each rat.

The rats, usually 3 to 4 to the group, were placed in four experimental test groups as follows: group 1, the thiamine-free controls received 0.1 ml. per day of the acetic acid-alcohol diluting solution; group 2, the thiamine controls received 0.1 ml. of supplement containing 5.0 μ g. of thiamine; group 3, the thiamine-compound test group, received in 0.1 ml. of supplement 5.0 μ g. of thiamine plus the amount of compound desired; group 4, the compound controls, received in 0.1 ml. the same amount of compound as was given to the animals in group 3.

The rats were weighed at the start of the experiment and on

alternate days until a definite deviation from the normal growth rate was noticed in any of the experimental groups; at this time daily weighings were begun.

The rats as needed were obtained from the two stock colonies maintained on the Iowa State College campus.[1] Adequate freedom of selection was allowed so that all rats unless otherwise noted conformed in age and weight to the figures outlined above.

The first compound tested was the benzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chloride analogue of thiamine, which was administered at a level of 50 μg. per day to a test group of twelve rats. This level of administration gave, in the case of the group 3 rats, an analogue/thiamine mole ratio of 12.5/1. The rats were grouped as previously outlined with those animals receiving the compound plus thiamine in group 3, and those on compound alone in group 4. The results of this experiment are given in Table V under experiment 1. The rats receiving no supplement showed insignificant gain in weight at the end of the experiment, though they actually gained weight for about 6 days and then gradually lost weight until the experiment was terminated. The rats in group 2, the suboptimal thiamine controls made a steady gain of weight characterized by the final average weight gain per day of + 1.51 grams. The animals receiving both thiamine and the test compound showed more erratic growth response though the final total response was positive in that all rats of the group demonstrated a definite weight gain, as shown by the group average of weight gain per day of +0.97 gm. The rats in

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[1] The author wishes to express his appreciation to Dr. B. H. Thomas, Director of the Animal Chemistry and Nutrition Subsection of the Iowa Agricultural Experiment Station, and to Miss Helen Clark of the Division of Home Economics for their generosity in making available from their colonies the rats used for these studies.

Table V

Response of Rats to Thiamine and Benzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium Chloride

Group Number	Rats in Group	Supplement Thiamine μg./day	Supplement Compound μg./day	Average Days on Expt.	Average Weight Gain gm./day	Average Deficiency Signs*
			Experiment 1			
1	3	0.0	0.0	21	+0.02	+++
2	3	5.0	0.0	21	+1.51	
3	3	5.0	50.0	21	+0.97	
4	3	0.0	50.0	12	-0.04	+++ P/3D[a]
			Experiment 1(a)			
1	3	0.0	0.0	28	-0.21	++
2	3	5.0	0.0	28	+2.08	
3	3	5.0	50.0	28	+1.88	
4	3	0.0	50.0	28	+0.01	+++
			Experiment 6			
1	3	0.0	0.0	18	+0.41	+++
2	3	5.0	0.0	18	+2.35	
3	3	5.0	50.0	18	+2.54	
4	3	0.0	50.0	18	+0.56	++

*Deficiency Signs
1st "+" indicates cessation of growth
2nd "+" indicates poor appearance, gradual weight loss
3rd "+" indicates rapid weight loss
"P" indicates symptoms characteristic of polyneuritis
"D" indicates death

[a]Fractional values indicate the number of rats in the group exhibiting the signs.

group 4, those receiving only the compound, survived for an average of 12 days with two rats succumbing on the 11th day and the third on the 14th day. The rat that survived until the 14th day showed some of the typical symptoms of thiamine deficiency -- body tremors and loss of equilibrium when picked up by the tail.

The interpretations based on this experiment have been made with some reservations since the administration of the daily supplements were not too satisfactory in the quantitative sense during the first week of the experiment. The presence of a definite physiological response was suggested, however, by the definite difference in growth response between groups 2 and 3, and between groups 1 and 4.

The reinvestigation of the effect of this compound and the added study of the effect of the o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethyl-thiazolium iodide hydroiodide were undertaken. The more detailed results of the experiment with the benzyl analogue are shown in Table VI. The difficulties in administration of the supplements encountered in the first experiment were overcome and in this experiment, as well as in the remainder of the experiments, the supplement administration was considered to be quantitative. The repeat study on the benzyl analogue, experiment 1(a), was made under the same conditions as the first experiment, with the thiamine and analogue levels being the same. Several differences in the experimental results were noted and can be seen by the comparison of the consolidated data for experiments 1 and 1(a) in Table V. The experimental period was twenty eight days and all rats survived the whole period. The effect of the benzyl analogue was not as marked as was observed before; the analogue effect on the group of rats receiving thiamine was only slightly inhibitory, while the effective shortening of the survival period of the rats on the

Table VI

Response of Rats to Thiamine and
Benzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium Chloride

Group Number	Rat Number	Supplement Thiamine μg./day	Supplement Compound μg./day	Days on Expt.	Weight Gain gm./day	Deficiency Signs*
1	13	0.0	0.0	28	-0.11	+++
1	14	0.0	0.0	28	-0.46	+++
1	15	0.0	0.0	28	-0.07	+++
1 (Ave.)		(0.0)	(0.0)	(28)	(-0.21)	(+++)
2	16	5.0	0.0	28	+2.14	
2	17	5.0	0.0	28	+1.89	
2	18	5.0	0.0	28	+2.21	
2 (Ave.)		(5.0)	(0.0)	(28)	(+2.08)	
3	19	5.0	50.0	28	+2.18	
3	20	5.0	50.0	28	+2.50	
3	21	5.0	50.0	28	+0.96	
3 (Ave.)		(5.0)	(50.0)	(28)	(+1.88)	
4	22	0.0	50.0	28	0.00	+++
4	23	0.0	50.0	28	+0.07	+++
4	24	0.0	50.0	28	-0.04	+++
4 (Ave.)		(0.0)	(50.0)	(28)	(+0.01)	(+++)

*Deficiency Signs
1st "+" indicates cessation of growth
2nd "+" indicates poor appearance, gradual weight loss
3rd "+" indicates rapid weight loss
"p" indicates symptoms characteristic of polyneuritis
"p" indicates death

thiamine deficient diet observed for the analogue in experiment 1 was completely absent. In fact, the rats receiving the analogue lost less weight and actually presented a better over-all appearance at the end of the experiment than did the thiamine free control rats. Since the supplementation technique was more accurate in this study, the apparent discrepancies of results obtained between groups 2 and 3 could be attractively explained on this basis; however, the rather marked differences between the effects of the analogue on the thiamine deficient rats cannot be totally reconciled to this explanation, in that, in the first experiment the period of supplement administration difficulties could only result in less, not more analogue being administered to the rats. This condition, at this point of the investigation, could hardly be expected to increase the physiological effect of the analogue on the rats as might be considered from the comparison of the results.

The physiological action of the o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium analogue of thiamine was studied and the results appear in Table VII. The o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium iodide hydroiodide was supplemented at a level of 93 μg. per day which is, in the case of the rats receiving both thiamine and analogue, an analogue to thiamine ratio of 12.5; the same mole level of analogue as administered with the benzyl analogue. The apparent effectiveness of this compound in inhibiting the effect of thiamine in the rat is quite striking in this experiment. All rats in group 3, receiving both thiamine and analogue, succumbed prior to the termination of the experiment -- the average survival period for this group being 21.7 days while the experiment was terminated on the 28th day. No signs of polyneuritis were observed in this group, possibly because of

Table VII

Response of Rats to Thiamine and
o-Aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium
Iodide Hydriodide

Group Number	Rat Number	Supplement Thiamine μg./day	Supplement Compound μg./day	Days on Expt.	Weight Gain gm./day	Deficiency Signs*
1	25	0.0	0.0	28	-0.11	+++
1	26	0.0	0.0	28	-0.46	+++
1	27	0.0	0.0	28	-0.07	+++
1	(Ave.)	(0.0)	(0.0)	(28)	(-0.21)	(+++)
2	28	5.0	0.0	28	+2.14	
2	29	5.0	0.0	28	+1.89	
2	30	5.0	0.0	28	+2.21	
2	(Ave.)	(5.0)	(0.0)	(28)	(+2.08)	
3	31	5.0	93.0	17	-0.24	+++ D
3	32	5.0	93.0	27	+0.07	+++ D
3	33	5.0	93.0	21	+0.67	+++ D
3	(Ave.)	(5.0)	(93.0)	(21.7)	(+0.17)	(+++ D)
4	34	0.0	93.0	28	+0.21	+++
4	35	0.0	93.0	28	0.00	+++
4	36	0.0	93.0	28	+0.11	+++
4	(Ave.)	(0.0)	(93.0)	(28)	(+0.11)	(+++)

*Deficiency Signs
1st "+" indicates cessation of growth
2nd "+" indicates poor appearance, gradual weight loss
3rd "+" indicates rapid weight loss
"P" indicates symptoms characteristic of polyneuritis
"D" indicates death

the manner in which death occurred. The rats in this group grew quite slowly for varying lengths of time and then suddenly began to grow very rapidly, gaining from two to four grams a day for several days; which action was followed by a precipitous loss in weight of 3 to 5 grams a day and terminated by death. These deaths were not predicted or expected from the observation of the general appearance of the rats. The general slow decline and deterioration of the rat on a thiamine deficient diet was not observed. Contrasted to the apparent effect of this analogue on rats receiving thiamine was the observed effect of the compound on the thiamine deficient rat. As was observed also in the second experiment with the benzyl analogue, the thiamine deficient rats apparently were stimulated slightly by the presence of the analogue since the decline in weight and the general over-all deterioration of the rats receiving the analogue alone was not as great as that in the case of the uncomplicated thiamine deficient rats. The observed effect resembles to some extent a "sparing-like action" of the compound on thiamine. The similarity of the observed deficiency signs of the rats in all groups except the positive thiamine control group (group 3) is apparent from the data presented in the column entitled "deficiency signs" of Table VII.

A preliminary interpretation of results from these first experiments indicated that the presence of the o-amino group in the benzyl-portion of the analogue was of considerable importance to the function of the o-aminobenzyl analogue as an inhibitor molecule. The importance of the presence of the free amino group in the ortho-position for the inhibitory powers of these benzyl-(3)-thiazolium analogues has been mentioned earlier in connection with the work of Sealock and Goodland (124). Their observation that the hydroxyethyl side chain in the 5-position of the

thiazole nucleus was not necessary for the inhibitory ability of the molecule, suggested that this group might not be necessary to the physiological action observed for the analogues that were being studied in the rat.

The experiment to test this hypothesis was initiated. The o-aminobenzyl-(3)-4-methylthiazolium chloride analogue was administered to rats in the same pattern as previously described at a level of 51 μ g. per day. This amount of analogue gave a mole ratio of analogue/thiamine of 12.5/1. Table VIII summarizes the results of this experiment. The weight gain of the positive thiamine controls, group 2, is greater than that of the group of rats receiving the test analogue, group 3, but the magnitude of the difference is hardly significant. The physical appearance of the rats in group 2 and 3 was much the same.

The effect of concentration on physiological activity was studied with the o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium iodide hydroiodide analogue. Administration of this compound at levels of 46.5 μg. per day was made in another experiment. The mole ratio in this case was 6.25/1 -- half that used in the previous experiments. The results of this test are summarized under experiment 5 in Table IX. The effect of the compound at this concentration is slight as shown by comparison of the weight gains of groups 2 and 3; certainly not of the magnitude of that observed earlier for this compound. A slight "sparing action" of the analogue was again noted as can be shown in the relative gains of groups 1 and 4.

The data on experiment 6 in Table V illustrate a check assay on the action of the benzyl analogue, which in this case showed a slight activating activity. The source of the analogue was identical with that previously utilized though the experimental period was shorter. The

Table VIII

Response of Rats to Thiamine and
o-Aminobenzyl-(3)-4-methylthiazolium Chloride

Group Number	Rat Number	Supplement Thiamine μg./day	Supplement Compound μg./day	Days on Expt.	Weight Gain gm./day	Deficiency Signs*
1	46	0.0	0.0	29	-0.41	+++
1	47	0.0	0.0	29	-0.38	+++
1	48	0.0	0.0	29	-0.07	+++
1	(Ave.)	(0.0)	(0.0)	(29)	(-0.29)	(+++)
2	49	5.0	0.0	29	+2.34	
2	50	5.0	0.0	29	+1.90	
2	51	5.0	0.0	29	+2.03	
2	(Ave.)	(5.0)	(0.0)	(29)	(+2.09)	
3	52	5.0	51.0	29	+1.69	
3	53	5.0	51.0	29	+1.48	
3	54	5.0	51.0	29	+2.03	
3	(Ave.)	(5.0)	(51.0)	(29)	(+1.73)	
4	55	0.0	51.0	29	-0.34	+++
4	56	0.0	51.0	29	+0.66	+++
4	57	0.0	51.0	29	-0.31	+++
4	(Ave.)	(0.0)	(51.0)	(29)	(0.00)	(+++)

*Deficiency Signs
1st "+" indicates cessation of growth
2nd "+" indicates poor appearance, gradual weight loss
3rd "+" indicates rapid weight loss
"P" indicates symptoms characteristic of polyneuritis
"D" indicates death

Table IX

Response of Rats to Thiamine and
o-Aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium
Iodide Hydroiodide

Group Number	Rats in Group	Supplement Thiamine µg./day	Supplement Compound µg./day	Average Days on Expt.	Average Weight Gain gm./day	Average Deficiency Signs*
			Experiment 2			
1	3	0.0	0.0	28	-0.21	+++
2	3	5.0	0.0	28	+2.08	
3	3	5.0	93.0	21.7	+0.17	+++ D
4	3	0.0	93.0	28	+0.11	+++
			Experiment 5			
1	3	0.0	0.0	26	+0.08	+++
2	3	5.0	0.0	26	+2.86	
3	3	5.0	46.5	26	+2.51	
4	3	0.0	46.5	26	+0.18	+++
			Experiment 7			
1	3	0.0	0.0	23	-0.23	+++
2	3	5.0	0.0	23	+2.13	
3	3	5.0	93.0	23	+2.31	
4	3	0.0	93.0	23	+0.26	++/3 + [a]
			Experiment 8			
1	3	0.0	0.0	11.3	-0.01	+++ 2P/3D
2	3	5.0	0.0	16	+1.75	
3	3	5.0	93.0	13	+1.33	1/3(+++ D)
4	3	0.0	93.0	14.3	+0.47	+++P/3 D/3
			Experiment 9			
1	3	0.0	0.0	11.3	-0.01	+++2P/3D
2	3	5.0	0.0	16	+1.75	
3	3	5.0	380.0	13	+1.08	2/3(+++PD)
4	3	0.0	380.0	16	+0.69	2+/3+2+/3

*Deficiency Signs
1st "+" indicates cessation of growth
2nd "+" indicates poor appearance, gradual weight loss
3rd "+" indicates rapid weight loss
"P" indicates symptoms characteristic of polyneuritis
"D" indicates death

[a]Fractional values indicate the number of rats in the group exhibiting the signs

"sparing action" of the analogue is again noted in comparing groups 1 and 4.

The data under experiment 7, Table IX, represents a check assay that was made on the action of the o-aminobenzyl analogue which gave results completely reversing the evidence presented earlier for the action of this analogue in which definite inhibitory powers were noted. No real explanation can be offered though several facts were noted which might explain this discrepancy. The analogue compound used was taken from a newly synthesized preparation; not the preparation used for the previous experiments. Several properties of this preparation were revealed later that cast some doubt as to its purity or possibly its identity. An investigation of the analogue was immediately made with the original preparation in two groups of rats, the results appearing in Table IX under experiments 8 and 9. The original level of 12.5/1 was used in experiment 8 while a level of 50/1 was used in experiment 9. Comparison of groups 3 and 4 of each experiment reveal some inhibitory activity. The rats available at the time this experiment was run were smaller than usual and apparently were quite susceptible to the change in diet and the deprivation of thiamine. Definite indications of thiamine deficiency appeared in groups 1 and 4, experiment 8 of Table IX, in about 10 days. The "sparing action" of the analogue is again apparent since the rats in group 4 were in much better condition than those of group 1. In experiment 9, Table IX, the "sparing action" of the analogue is evidenced at the higher analogue concentration while the inhibition effect is greater also.

The use of the o-aminobenzyl analogue at the higher concentrations introduces the factor of iodine intake of the rat far above the trace amount levels that iodine normally occupies in nutrition. The molecular

weight of the o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium iodide hydroiodide is 504.19, of which iodine itself, two atoms per molecule, is 253.84 units of the total molecular weight.

The administration of this analogue in levels comparable to those used by Woolley (108) and Emmerson and Southwick (119), in the one to two milligram range, would have resulted in an iodine intake up to 1 mg. per day. Repeated attempts to crystallize the o-amino analogue as the bromide hydrobromide or the chloride hydrochloride were made but without success. Similar unsuccessful attempts to crystallize this compound as the chloride have been made by Livermore and Sealock (127).

In order to make the o-amino analogue available in a concentrated form, the following procedure for removal of iodide was devised and used: 0.514 gm. of the iodide-hydroiodide analogue was dissolved in 3.0 ml. of 0.5 per cent HNO_3 in a 15 ml. pyrex centrifuge tube; 2.0 ml. of $AgNO_3$ solution (0.222 gm. $AgNO_3$) was added slowly with stirring to precipitate the iodide ion; prior to the addition of the last 2 drops, the precipitate was allowed to settle and the test was made for complete precipitation. In every instance precipitation was complete prior to the addition of the last drop. 1.0 ml. of HCl solution (0.00245 gm. HCl) was added slowly with stirring, and the last two drops reserved for testing for completeness of precipitation. Again in each instance precipitation was complete. The silver chloride and silver iodide precipitates were centrifuged down and the supernatant liquid containing the analogue taken off. The precipitate was washed three times with 0.5 ml. portions of the 0.5 per cent HNO_3. The combined washings and supernatant liquid were adjusted to a pH of 4.0, transferred quantitatively to a 10 ml. volumetric flask and diluted to the mark with distilled water. This solution contained 2.0 mg. of the o-aminobenzyl analogue, calculated as the chloride

hydrochloride per 0.1 ml.

With this concentrated preparation of the o-amino analogue the assay experiment was set up in the usual manner with the compound level at 1.0 mg. per day per rat. The results of this experiment are shown in Table X. The additional group 5 was added in this experiment with the rats receiving 50 μ g. of thiamine per day. The effect of the analogue was observed to be inhibitory as can be seen from the comparison of the average gains in group 2 and 3. The action of the analogue on the rats in group 4 was observed to exhibit the "sparing action" mentioned earlier. The general condition of the rats in group 4 was much better than that of the rats in group 1, two of which exhibited definite signs of polyneuritis.

The growth data for this experiment is presented in graphical form in Figure 1 as the general example of the type of growth response obtained in these experiments where inhibition was observed. The typical break in the growth curve of group 3 is clearly indicated on the graph.

In the case of this high level of compound administration a very characteristic and strong "thiazole-like" odor was noticed in and about the cages containing the rats receiving the high levels of the analogue. The relatively high content of thiazole or thiazole residues in the urine indicated that these rats were not retaining a considerable portion of the analogue administered, thus in effect reducing its practical concentration well below that administered. The comparable physiological effects both at the lower and higher concentrations indicate that after a certain practical point, the administration of more of this analogue compound will have little effect.

Table X

Response of Rats to Thiamine and o-Aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium Chloride Hydrochloride

Group Number	Rat Number	Supplement Thiamine μg./day	Supplement Compound μg./day	Days on Expt.	Weight Gain gm./day	Deficiency Signs*
1	108	0.0	0.0	35	-0.43	+++ P
1	109	0.0	0.0	35	+0.03	+++
1	110	0.0	0.0	35	-0.09	+++
1	111	0.0	0.0	35	-0.34	+++ P
1 (Ave.)		(0.0)	(0.0)	(35)	(-0.21)	(+++ P/2)[a]
2	112	5.0	0.0	35	+1.00	
2	113	5.0	0.0	35	+2.09	
2	114	5.0	0.0	35	+1.63	
2	115	5.0	0.0	35	+1.77	
2 (Ave.)		(5.0)	(0.0)	(35)	(+1.62)	
3	116	5.0	1000.0	35	+1.14	
3	117	5.0	1000.0	35	+1.17	
3	118	5.0	1000.0	35	+0.09	+++
3	119	5.0	1000.0	35	+1.23	
3 (Ave.)		(5.0)	(1000.0)	(35)	(+0.91)	(1/4(+++))
4	120	0.0	1000.0	35	+0.11	+++
4	121	0.0	1000.0	35	-0.06	+++
4	122	0.0	1000.0	35	-0.20	+++
4	123	0.0	1000.0	35	0.00	+++
4 (Ave.)		(0.0)	(1000.0)	(35)	(-0.03)	(+++)
5	124	50.0	0.0	35	+2.31	
5	125	50.0	0.0	35	+1.26	
5	126	50.0	0.0	35	+2.03	
5 (Ave.)		(50.0)	(0.0)	(35)	(+1.86)	

*Deficiency Signs
1st "+" indicates cessation of growth
2nd "+" indicates poor appearance, gradual weight loss
3rd "+" indicates rapid weight loss
"P" indicates symptoms characteristic of polyneuritis
"D" indicates death

[a]Fractional values indicate the number of rats in the group exhibiting the signs

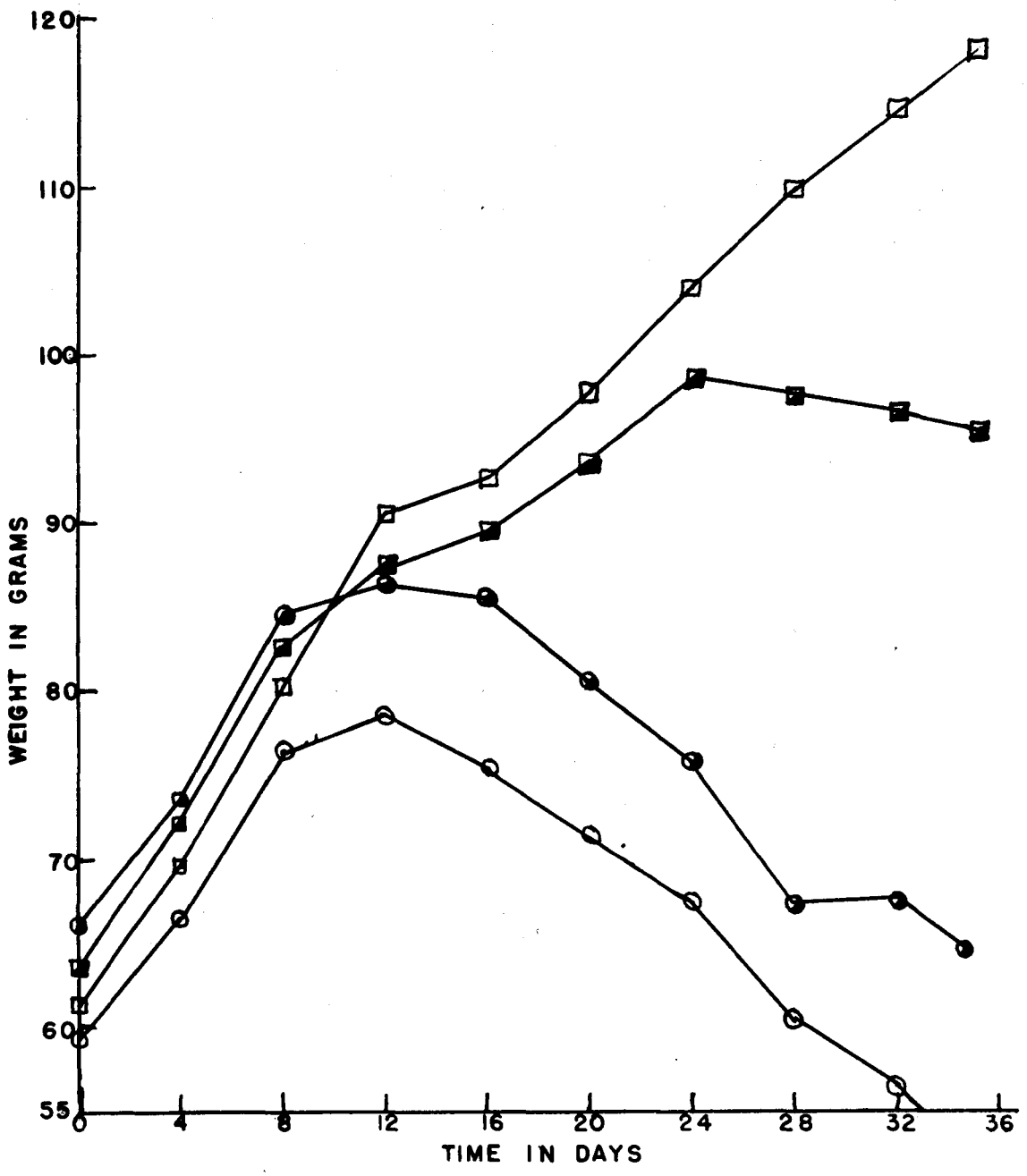

FIGURE I. GROWTH RESPONSE OF RATS TO THIAMINE AND o-AMINOBENZYL-(3)-4-METHYL-5-β-HYDROXYETHYLTHIAZOLIUM CHLORIDE HYDROCHLORIDE

○, Group I, No Supplement; □, Group 2, 5 μg. Thiamine per day; ◪, Group 3, 5 μg. Thiamine and 1mg. Compound per day; ◔, Group 4, 1mg. Compound per day.

From a survey of the data presented, it can be concluded that though some definite physiological response in the form of inhibition of thiamine activity has been observed for the o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium analogue at both low and high levels of administration it is apparently not a molecule of high inhibitory properties. The benzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chloride and the o-aminobenzyl-(3)-4-methylthiazolium chloride exhibit little if any inhibitory effect. These data can only suggest that the free amino group on the benzyl residue and the 5-β-hydroxyethyl group on the thiazole are necessary for inhibitions observed. The consistant observation in all but the first preliminary experiment of the small but definite "sparing action" of these analogues on the rats maintained on the thiamine deficient diet is significant and indicates that the analogues are exerting an effect in some manner toward thiamine in these animals. In this case with the analogues tested, structural specificity does not seem to be important. The qualitative observation that at high levels of administration of the o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium analogue much of the compound is promptly eliminated through the excretory system of the rat, either in its intact or modified form, is of importance since it suggests that there is a practical upper limit of administration of this analogue as far as the rat is concerned.

C. Yeast Fermentation Method

The application of the yeast fermentation assay method to the investigation of the benzyl-(3)-thiazolium analogues was undertaken to determine if this method could be used successfully to study the effects of the thiamine analogues. The method itself offered many desirable features for this type of study; it was sensitive to small amounts of thiamine, the amount of analogue required was small, and the experimental period was of short duration.

The thiamine dependency of the system was apparent since in the yeast system the carbon dioxide production was a function of the yeast carboxylase system, decarboxylating pyruvic acid to acetaldehyde and CO_2. The stimulation of the CO_2 producing ability of this system by the added thiamine, even though the exact mechanism of this stimulation has not been ascertained, was for the purpose of the proposed studies a function of the thiamine molecule. If a thiamine analogue was found that competed with thiamine in this system, it could be expected to exert an influence on the basal CO_2 production through the carboxylase system as well as on the stimulated CO_2 production through the stimulation process involving the added thiamine. The proposed method offered a sufficient number of desirable features to warrant this further investigation.

The fermentation method of Schultz, Atkin and Frey (129) and the apparatus required has been adequately described. Only the minor modifications indicated hereinafter were introduced. The smaller volume of the glassware used necessitated using only half as much yeast as recommended in the original method; that is, 0.25 gm. instead of 0.50 gm. It was also shown for the instrument used in these studies that a

shaking speed of at least 120 per minute was required to give acceptable precision. The precision of readings of gas volume could be made within 2.0 ml. difference between individual flasks, and in the majority of cases, it was possible to achieve differences of less than 1.0 ml. Considering the 2.0 ml. error at the basal fermentation level of about 35 to 40 ml. of CO_2, the maximum error that must be considered with this instrument was 5 per cent. Differences in CO_2 production resulting from the addition of analogues to the basal fermentation of less than 1.0 ml. were not considered as evidence of any action of the analogue. Increases or decreases in CO_2 production over 1.0 ml. in volume were considered as indicating activation or inhibition. However, unless the analogue gave a decided change in CO_2 production, it was not considered to be exerting a significant effect.

The effects of the analogues were studied on both the basal fermentation levels and the fermentation levels stimulated by 1.0 μg. of thiamine per flask. The various analogues were added at the desired concentrations to the reaction flasks containing the yeast and yeast plus 1 μg. of thiamine. The fermentation was started and at the end of the three hour period, the volumes of CO_2 produced in the flasks were read. The results for the analogue activities are given in activation per cent or inhibition per cent calculated by the formulas given below:

$$\text{Activation, per cent} = \left[\left(\frac{\text{ml. } /CO_2 \text{ unknown}}{\text{ml./}CO_2 \text{ control}} \right) \times 100 - 100 \right]$$

$$\text{Inhibition, per cent} = \left[100 - \left(\frac{\text{ml./}CO_2 \text{ unknown}}{\text{ml./}CO_2 \text{ control}} \right) \times 100 \right]$$

The results given in the tables are for individual flasks in almost all instances.

The summary of the results obtained with the benzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium analogues of thiamine is presented in Table XI. It is apparent from the data that none of these analogues at reasonably low concentrations exerts much effect. The o-aminobenzyl analogue was studied at several concentrations ranging from 9.4 μg. to 3.0 mg. of analogue per 50 ml. of solution. Mole ratios in the cases of the fermentations stimulated by 1 μg. of thiamine ran from 6.25/1 to 1500/1. Irrespective of the concentrations of this analogue employed, significant physiological effects were not noted. This analogue might have been expected to exert some effect since its effect on the rat growth had already been observed. In general the results show no real effect for any of this group of analogues at reasonable concentrations. Two analogues do show some interesting action. The benzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chloride was studied at several high concentrations. Its effect at the rather absurd mole ratio of 100,000/1 contrasts sharply with the effect at the lower concentrations tried. The p-nitrobenzyl analogue did show a rather remarkable stimulation at a mole ratio level of 1000/1 for both the basal and stimulated fermentations. The comparable stimulations of the 4-methyl-5-β-hydroxyethylthiazole included in Table XI for the sake of comparison are quite appreciable at the 1000/1 level.

In the case of these benzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium analogues that do show activation, the possibility exists that the 4-methyl-5-β-hydroxyethylthiazole becomes separated from the benzyl moiety under the conditions of the test and the resultant stimulation observed is because of liberated thiazole. Contrasted to this hypothesis is the fact that the p-aminobenzyl analogue especially, and the majority of other

Table XI

The Effect of (3)-4-Methyl-5-β-hydroxyethylthiazolium Analogues of Thiamine on the Basal and Thiamine Stimulated Fermentation of Bakers' Yeast

Compound	Conc. moles/liter x 10^{10}	Effect on Fermentation: Basal Per cent[a]	Thiamine Per cent	Stimulated Mole Ratio[b]
Benzyl-chloride	5.92	2.3 (+)		
	59.20	1.5 (-)	1.1 (+)	1000/1
	592.00	2.3 (+)	2.6 (+)	10000/1
	5920.00	34.3 (+)	17.1 (+)	100000/1
o-Aminobenzyl-iodide hydroiodide	0.37	1.3 (-)	3.7 (+)	6.25/1
	0.74	1.3 (-)	0.0	12.5/1
	1.48	0.0	0.9 (+)	25/1
	2.98	2.8 (+)	1.0 (+)	50/1
	5.95	2.3 (+)		
	15.10	0.6 (-)	2.3 (+)	250/1
	30.20	2.4 (-)	0.9 (+)	500/1
	59.50	0.0	0.6 (+)	1000/1
	89.20	1.4 (+)	0.0	1500/1
	119.00	2.2 (-)		
p-Aminobenzyl-chloride hydrochloride	6.24	1.6 (+)		
	59.20	0.6 (+)	1.5 (+)	1000/1
	592.00	0.0	0.4 (-)	10000/1
o-Nitrobenzyl-chloride	5.71	0.0		
m-Nitrobenzyl-chloride	5.71	0.0		
	58.40	2.2 (+)	3.0 (+)	1000/1
p-Nitrobenzyl-chloride	5.71	4.3 (+)		
	58.40	22.2 (+)	16.3 (+)	1000/1
γ-Phthalimidopropyl-bromide	59.60	8.7 (+)	2.4 (+)	1000/1
Methyl-iodide	59.40	3.6 (+)	2.6 (+)	1000/1
	594.00		5.4 (+)	10000/1
(4-Methyl-5-β-hydroxyethyl-thiazole)	3.00	4.0 (+)	5.6 (+)	50/1
	63.00	59.1 (+)	22.9 (+)	1000/1

[a](+) indicates activation in per cent; (-) indicates inhibition in per cent.

[b]Thiamine concentration for stimulation 0.06 x 10^{-10} moles per liter.

analogues do not show evidence of marked stimulation, indicating that the bond between the benzyl group and the thiazole tends to remain intact.

The studies of the analogues having the 4-methylthiazolium group common to all are presented in Table XII. Of these analogues the only

Table XII

The Effect of (3)-4-Methylthiazolium Analogues of Thiamine on the Basal and Thiamine Stimulated Fermentation of Bakers' Yeast

Compound	Conc. moles/liter x 10^{10}	Effect on Fermentation: Basal Per cent[a]	Thiamine Per cent	Stimulated Mole Ratio[b]
Benzyl-chloride	6.2	3.3 (+)		
	63.8	0.8 (-)	2.4 (+)	1000/1
o-Aminobenzyl-chloride	10.1	3.8 (+)		
o-Nitrobenzyl-chloride	59.0	2.5 (-)	0.8 (+)	1000/1
γ-Aminopropyl-bromide hydrobromide	24.0	4.0 (+)		
	60.4	7.5 (+)	5.7 (+)	1000/1
(4-Methylthiazole)	60.0	1.1 (+)	0.4 (-)	1000/1

[a] (+) Indicates activation in per cent; (-) indicates inhibition in per cent.

[b] Mole ratio of compound to thiamine; thiamine concentration 0.06 x 10^{-10} moles per liter.

significant effects are noted with the γ-aminopropyl compound. In this case the results show a definite stimulation that can not be attributed to the 4-methylthiazole moiety as the table shows. It is also noted in the one test that was made with the o-aminobenzyl-(3)-4-methylthiazole, that its ability to inhibit the Chastek-Paralysis enzyme is not duplicated in an ability to inhibit the physiological action of thiamine in yeast fermentation.

Three 2-methyl-6-aminopyrimidine moieties of thiamine were included in the fermentation studies. As is shown in Table XIII, these three

Table XIII

The Effect of 2-Methyl-6-aminopyrimidine Moieties of Thiamine on the Basal and Thiamine Stimulated Fermentation of Bakers' Yeast

Compound	Conc. moles/liter x 10^{10}	Effect on Fermentation Basal Per cent[a]	Thiamine Stimulated Per cent	Mole Ratio[b]
5-Methylenesulfonic acid	6.38	4.9(+)		
	60.20	12.9(+)	4.0(+)	1000/1
5-Bromomethyl-di-hydrobromide	59.40	35.2(+)	17.5(+)	1000/1
5-Hydroxymethyl-	3.0	23.6(+)	11.0(+)	50/1
	3.0	24.8(+)	13.0(+)	50/1
	12.0	19.3(+)	9.0(+)	200/1
	12.0	22.0(+)	9.7(+)	200/1
	60.0	58.1(+)	19.5(+)	1000/1

[a] (+) Indicates activation in per cent; (-) indicates inhibition in per cent.

[b] Mole ratio of compound to thiamine; thiamine concentration 0.06 x 10^{-10} moles per liter.

compounds differ only in the substituent substituted in the 5-methylene position; a bromine, a sulfonic acid and a hydroxyl group, resulting in the pyrimidine sulfonic acid, pyrimidine bromide and pyrimidine alcohol moieties of thiamine. Table XIII summarizes the results obtained with these compounds. On the comparable levels of assay, at mole ratios of 1000/1 the stimulating effect on the CO_2 production is evident for all of the compounds. The pyrimidine bromide and alcohol exhibit the greater stimulations. It is interesting to note that in the case of the pyrimidine alcohol moiety, the stimulation at a mole ratio of 200/1 is not as great as the stimulation at a mole ratio of 50/1. However, at the mole ratio of 1000/1 this compound shows increased stimulation.

The Schultz, Atkin and Frey method of thiamine assay is known (130) to be sensitive to 6-aminopyrimidine moieties of thiamine. While under the ordinary assay conditions for which the method was designed, the 6-aminopyrimidine concentrations in test solutions would never begin to approach those levels used, the fact that even the pyrimidine sulfonic acid shows an effect merits careful investigation if this method is to be applied to specific problems where an appreciable amount of 6-aminopyrimidine compounds might be present. The pyrimidine alcohol moiety has been shown by Krampitz and Woolley (122) to be one of the reaction products of the enzymatic splitting of thiamine by the Chastek-Paralysis enzyme. The ability of the 6-aminopyrimidines to stimulate the yeast carboxylase system has been adequately described (116, 117) in the literature also.

"Thiazole pyrophosphate" was found to be a definite stimulator of carboxylase function in this system as is evident from examination of the data in Table XIV. This stimulation was found to be in direct contrast to the observations of Bonner, Heegard and Buchman (10) in their study of the effect of "thiazole pyrophosphate" on the alkaline-washed reconstituted yeast carboxylase system. Their observation that the 4-methyl-5-β-hydroxyethylthiazole had no effect on their system, was also not consistent with the findings for the 4-methyl-5-β-hydroxyethylthiazole in this study. The data for this thiazole is shown in Table XIV with definite stimulations being observed at all concentrations tested. It was also observed that "thiazole pyrophosphate" was the more active stimulant of the two at the levels where the results are comparable.

The experimental conditions employed by Bonner, Heegard and Buchman (10) were materially different than those employed in this study which may account for the differences in the observed results.

Table XIV

The Effect of Thiazole Derivatives on the Basal and Thiamine Stimulated Fermentation of Bakers' Yeast

Compound	Conc. moles/liter x10^{10}	Effect on Fermentation Basal Per cent[a]	Thiamine Per cent	Stimulated Mole Ratio[b]
4-Methylthiazole	60.0	1.1(+)	0.4(-)	1000/1
	600.0	1.4(+)	2.5(-)	10000/1
2-Methylthiazole	59.4	1.7(-)	2.2(+)	1000/1
2,4-Dimethylthiazole	68.0	0.8(-)	2.0(+)	1100/1
4-Methyl-5-β-hydroxyethylthiazole	3.0	4.0(+)	5.6(+)	50/1
	12.0	23.2(+)	11.0(+)	200/1
	63.0	59.1(+)	22.9(+)	1000/1
4-Methyl-5-β-hydroxyethylthiazole pyrophosphate	3.0	13.2(+)	6.5(+)	50/1
	3.0	11.8(+)	6.2(+)	50/1
	6.1	18.8(+)	8.3(+)	100/1
	6.1	19.4(+)	6.0(+)	100/1
	12.1	35.3(+)	16.3(+)	200/1
	12.1	32.4(+)	12.4(+)	200/1

[a] (+) Indicates activation in per cent; (-) indicates inhibition in per cent.

[b] Mole ratio of compound to thiamine; thiamine concentration 0.06 x 10^{-10} moles per liter.

In an attempt to obtain additional information on this stimulation, a series of fermentation runs was made with equimolar concentrations of the 2-methyl-6-amino-5-hydroxymethyl-pyrimidine ("pyrimidine alcohol") and the "thiazole pyrophosphate" and with the 4-methyl-5-β-hydroxyethyl-thiazole. The results of these studies are presented in Table XV along with the results obtained for the pyrimidine and thiazoles alone. It is shown from the data in this table that the effects of the pyrimidine alcohol thiazole pyrophosphate and the pyrimidine alcohol plus the thiazole combinations are much greater than those with either the

Table XV

The Effect of Pyrimidine Alcohol plus Thiazole and Pyrimidine Alcohol plus Thiazole Pyrophosphate on the Basal and Thiamine Stimulated Fermentation of Bakers' Yeast[a]

Compound	Conc. moles/liter x 10^{10}	Effect on Fermentation Basal Per cent[b]	Thiamine Stimulated Per cent	Mole Ratio[c]
Pyrimidine Alcohol	3.0	23.6(+)	11.0(+)	50/1
Thiazole	3.0	4.0(+)	5.6(+)	50/1
Pyrimidine Alcohol	3.0	92.9(+)	36.0(+)	50/1
Thiazole	3.0			
Pyrimidine Alcohol	12.0	19.3(+)	9.0(+)	200/1
Thiazole	12.0	23.2(+)	11.0(+)	200/1
Pyrimidine Alcohol	12.0	84.6(+)	37.2(+)	200/1
Thiazole	12.0			
Pyrimidine Alcohol	3.0	24.8(+)	13.0(+)	50/1
Thiazole Pyrophosphate	3.0	11.3(+)	8.0(+)	50/1
Pyrimidine Alcohol	3.0	53.0(+)	25.7(+)	50/1
Thiazole Pyrophosphate	3.0			
Pyrimidine Alcohol	12.0	22.0(+)	9.7(+)	200/1
Thiazole Pyrophosphate	12.1	33.3(+)	14.0(+)	200/1
Pyrimidine Alcohol	12.0	75.4(+)	29.6(+)	200/1
Thiazole Pyrophosphate	12.1			

[a]Pyrimidine alcohol is 2-methyl-6-amino-5-hydroxymethylpyrimidine; thiazole is 4-methyl-5-β-hydroxyethylthiazole; and thiazole pyrophosphate is the pyrophosphate ester of 4-methyl-5-β-hydroxyethylthiazole.

[b](+) Indicates activation in per cent; (-) indicates inhibition in per cent.

[c]Mole ratio of compound to thiamine; thiamine concentration 0.06 x 10^{-10} moles per liter.

thiazoles or pyrimidine compounds alone. The pyrimidine-thiazole combination stimulates the basal fermentation more at a mole ratio of 50/1 than it does at a ratio of 200/1; however, this situation is definitely reversed in the case of the pyrimidine-thiazole pyrophosphate fermentations where the higher levels give the greater activation. The previously noted lesser stimulating effect of the pyrimidine alcohol at the 200/1 level might explain this effect with the pyrimidine alcohol-thiazole combination. Such an explanation is not supported in the case of the observed effect with the pyrimidine alcohol-thiazole pyrophosphate combination.

The increase in CO_2 production caused by the pyrimidine and the thiazoles separately do not account for the increase in CO_2 production when these two compounds are added to the yeast together. It is evident that these two halves of the thiamine molecule exert an additional effect because of their presence together. Since the ability of yeast to synthesize thiamine is well known (136) the possibility that the added effect may be caused by an actual thiamine synthesis certainly exists. The lesser ability of the thiazole pyrophosphate in the presence of the pyrimidine to effect the increase of CO_2 production suggests that it is not as available to enter into the stimulating process as the free thiazole portion of thiamine.

It became evident as these studies progressed that this method of assay was not lending itself to the tests of analogue activity as had been expected. One contributing factor, without doubt, was the modification of apparatus and methods required by the small sized gasometers with the resulting loss of accuracy of the experimental procedure. Effects of the analogue of small magnitude which might have been used as guides in designing experiments that would result in the demonstration

of measurable effects could not be observed. The definite activations that were observed with some of the analogues indicated that the yeast cells were permeable to the general type of analogue used in these tests.

Two general points of view may be taken in considering the effectiveness of this method for the assay of the action of thiamine analogues on the thiamine dependent portions of the yeast fermentation. The strong possibility exists that of the analogues tested there were none that showed real action with respect to activation or inhibition of the thiamine function in yeast, in which case the method gave a true evaluation of the properties of the analogues. The other point of view may be taken, that the yeast system as studied was not suitable for the testing of analogue-thiamine antagonisms. The three-hour test period really measures only the fermenting ability or capacity of the enzyme systems already present in the yeast cell at the time it is incorporated into the test. Under the experimental conditions little if any real growth activity is carried on in the yeast cell. The ability of any one analogue to effect the growth function over a reasonable period of time may differ markedly from its ability to influence an intact enzyme system already synthesized by the yeast and able to carry out its function if the proper substrate is made available to it. In the growing cells the new enzyme systems must be synthesized, or at least be assembled in part by the cells from component parts supplied to them. Therefore, it is entirely possible that the analogue might compete with the thiamine dependency for growth and enzyme synthesis where it will show no effect on the thiamine enzyme function after the enzyme has been assembled. The rather inconclusive results encountered with this method

were contrasted with the rather striking results obtained with the microbiological assay technique which will now be described.

D. Lactobacillus fermentum 36 Method

The successful use of the Lactobacillus fermentum 36 assay method for thiamine (115) in studying the analogue-thiamine relationships of pyrithiamine to thiamine was reported by Sarett and Cheldelin (113) in 1944. Their detailed investigation and the results and discussion of the relationship of pyrithiamine to the thiamine dependent system indicated that it might be equally sensitive to the proper benzyl-(3)-analogues of thiamine.

The L. fermentum method for thiamine assay has been thoroughly discussed in two papers by Sarett and Cheldelin (115, 137). A culture of L. fermentum 36 was obtained from the American Type Culture Collection, located in Washington, D. C. Upon receipt of the organism it was transferred to the glucose agar stabs as described in the assay method. Adequate precautions were taken to maintain a pure stock culture of this organism so that it would be available as needed. The L. fermentum assay method used in these studies was the exact method described by Sarett and Cheldelin (115, 137).

Preliminary assays were made to enable a check of the response of the organism to varying levels of thiamine and to establish a thiamine-growth relationship for the organism. In all the assays made with the analogues, representative levels of thiamine were included to insure that the particular assay was functioning properly with respect to thiamine in so far as the organisms, inoculum and the incubation were concerned.

The assay for the effect of the analogue was carried out in the following pattern: two levels of thiamine (0.04 μg. and 0.02 μg. per tube) and two levels of cocarboxylase (0.0568 μg. and 0.0284 μg. per tube) were run for five different levels of analogue in each determination; the thiamine and cocarboxylase levels were equimolar at both the high and low levels. The analogue concentrations were selected so that the following mole ratios of analogue to thiamine would be established in a series of tubes; 0/1, 1/1, 10/1, 100/1, 1000/1 and 10000/1, at the 0.04 μg. level of thiamine. The mole ratios of analogue to thiamine in the tubes containing 0.02 μg. per tube were thus twice those at the higher thiamine level. The same mole ratio relationship was set up for cocarboxylase in the other two series of tubes. All solutions of analogues as well as the other reagents were adjusted to a pH of 6.5 prior to addition to the test tubes.

The incubation period used was 16$\frac{1}{2}$ to 17 hours after which the organisms were removed from the incubator and placed in the refrigerator to stop growth. Turbidities were read in the Klett-Summerson photoelectric colorimeter using a KS #42 filter. Results were calculated in the following manner: the effect of the analogue was determined by comparing the turbidity due to growth of the thiamine control tube with the tube containing the analogue plus thiamine; basal turbidities (blank growth) were subtracted from all readings prior to calculation; all results were calculated by the basic inhibition or activation formulas illustrated in the fermentation section of this thesis. The next step involved a graphical calculation in which the activation or inhibitions in per cent were plotted against the logarithm of the mole ratio of analogue/thiamine for the concentrations described. The points on the graph were connected by the best curved line that could be drawn through

all reasonable points. From these data, by interpolation, the mole ratio of analogue/thiamine was determined at the level of analogue required to effect either a stimulation or inhibition of 50 per cent of the basal value. These data are shown in Tables XVI through XIX for the various general groups of analogues.

As the tables show, not all analogues stimulated or inhibited growth by an amount of 50 per cent of the basal values. The analogues in most cases exerted either a marked stimulation or inhibition of growth above the 50 per cent level or a very slight effect. The trend or direction of these sub-50 per cent activations or inhibitions are indicated in the tables but the actual values are not.

The analogues used in these tests were selected along the same general patterns as those used with the fermentation method. Several analogues not previously available were incorporated into the group used in this test.

The effect of the group of analogues characterized by the 4-methyl-5-β-hydroxyethylthiazole portion of thiamine on the growth of the L. fermentum at varying levels of thiamine and cocarboxylase is shown in Table XVI. Stimulation of growth of L. fermentum is characteristic of all analogues of this group. The inclusion of the thiazole moiety of thiamine, which is common to all of these analogues, in this table was for the sake of comparison of the effect of the thiazole portion of these analogues on the stimulation. Since the thiazole itself stimulates growth to a marked degree, it might be suggested that this growth stimulation by the other analogues of the group is due to breaking down of the analogue into the benzyl moiety and the thiazole with the thiazole moiety contributing the stimulating effect.

Table XVI

The Effect of (3)-4-methyl-5-β-hydroxyethylthiazolium Analogues of Thiamine on the Growth of Lactobacillus fermentum 36 in the Presence of Thiamine or Cocarboxylase

Compound	Effect on Growth Mole Ratio of Compound/Thiamine or Cocarboxylase required to Effect a 50 per cent Stimulation or Inhibition of Basal Growth Stimulated by:[a]			
	Thiamine per Tube moles x 10^{10}		Cocarboxylase per Tube moles x 10^{10}	
	0.595	1.19	0.595	1.19
Benzyl-chloride	5,900(+)	> 10,000(+)[b]	12,000(+)	4,000(+)
o-Aminobenzyl-iodide hydroiodide	71(+)	126(+)	135 (+)	79(+)
p-Aminobenzyl-chloride hydrochloride	100(+)	80(+)	100 (+)	34(+)
o-Nitrobenzyl-chloride	2,950(+)	4,370(+)	2,820 (+)	3,300(+)
m-Nitrobenzyl-chloride	(+)[c]	(+)	(+)	(+)
p-Nitrobenzyl-chloride	5,760(+)	7,950(+)	7,100 (+)	>10,000(+)
Methyl-iodide	4,080(+)	8,320(+)	4,170 (+)	>10,000(+)
(4-Methyl-5-β-hydroxyethylthiazole)	230(+)	145(+)	436 (+)	1,260(+)

[a] (+) Indicates stimulation of growth; (-) indicates inhibition of growth.

[b] (>) Indicates that 50 per cent activation or inhibition effect was almost achieved by the highest level of compound tested.

[c] Blank indicates 50 per cent activation or inhibition effect was not approached by highest level of compound tested; (+) or (-) indicates the direction of the effect.

This suggestion would appear as a distinct possibility if it were not for the fact that the two aminobenzyl analogues, the o-aminobenzyl and the p-aminobenzyl, exert even more pronounced stimulating abilities. Since these analogues were present in equimolar concentrations with each other and with the thiazole, it becomes apparent that other factors are involved in this stimulatory process. The importance of the benzylamino group whether it be in the ortho- or meta-position is also quite evident when the stimulating ability of the benzyl analogue is compared with the stimulatory abilities of the two amino analogues. In a comparison with the stimulatory abilities of the o- and p-nitrobenzyl analogues, the importance of the amino group to this effect becomes even more evident.

The effects observed with the nitrobenzyl analogues in relation to the position of the nitro group is rather startling since the m-nitrobenzyl analogue exerted almost no effect at all on the growth of the microorganisms while the other nitrobenzyl analogues did exert a marked stimulatory effect.

The group of analogues characterized by the 4-methylthiazole nucleus exert an even more interesting effect on the growth of the L. fermentum. Table XVII presents the data on these analogues. It becomes apparent in a glance that two of these analogues are definite inhibitors. Considering the benzyl group first, the benzyl and o-nitrobenzyl are both definitely stimulating in their action in the L. fermentum system. However, by the single structure modification of converting the o-nitro group to an o-amino group these stimulatory properties were completely reversed and the analogue became definitely inhibitory. The inhibition is definite and of reasonable magnitude. Referring to Table XVI and to the o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethyl analogue an opposite

Table XVII

The Effect of (3)-4-Methylthiazolium Analogues of Thiamine on the Growth of *Lactobacillus fermentum* 36 in the Presence of Thiamine or Cocarboxylase

Compound	Effect on Growth Mole Ratio of Compound/Thiamine or Cocarboxylase required to Effect a 50 per cent Stimulation or Inhibition of Basal Growth Stimulated by:[a]			
	Thiamine per tube moles x 10^{10}		Cocarboxylase per tube moles x 10^{10}	
	0.595	1.19	0.595	1.19
Benzyl-chloride	1,660(+)	3,160(+)	1,000(+)	6,300(+)
o-Nitrobenzyl-chloride	890(+)	10,000(+)	3,160(+)	9,550(+)
o-Aminobenzyl-chloride hydrochloride	5,012(-)	3,162(-)	7,245(-)	2,631(-)
γ-Amino propyl-bromide hydrobromide	2,000(-)	1,000(-)	6,310(-)	500(-)
γ-Amino propyl-bromide hydrobromide		2,571(-)	5,012(-)	1,820(-)
γ-Phthalimido propyl-bromide	2,820(+)	1,269(+)	(+)[b]	(+)
Δ-Amino-n-butyl-bromide hydrobromide	(+)	(+)	(+)	(+)

[a] (+) Indicates stimulation of growth; (-) indicates inhibition of growth.

[b] Blank indicates that a 50 per cent stimulation or inhibition effect was not approached by the highest level of compound tested.

effect is very evident. The only difference in the structures is the lack of the 5-β-hydroxyethyl group in the inhibitor analogue. With the group present the molecule has a stimulatory role while if the group is absent an inhibitory role is evident. In this case the only variable in the system is the presence or absence of the hydroxyethyl side chain in the thiazole group, in one instance the analogue appears to facilitate the action of thiamine while in the other it interferes with the growth

function. The effect of these two analogues with cocarboxylase as the growth stimulant is essentially the same; the analogue with the hydroxyethyl group stimulates growth while the analogue lacking this group is an inhibitor.

More information of interest is obtained when the γ-aminopropyl inhibition is observed, Table XVII. This compound has the amino group three carbons removed from the quaternary nitrogen of the thiazole nucleus as does thiamine and those benzyl analogues with the amino group in the ortho-position. This analogue as an inhibitor stresses again the importance of this amino group to the inhibiting action. Blocking the amino group with a phthalimido group reverses this tendency completely and the phthalimido analogue has growth stimulating properties. The δ-aminobutyl is actually a homologue of the γ-aminopropyl compound. In this case the free amino group is four carbons removed from the quaternary nitrogen. The data shows that this compound exerts no inhibitory activity. This evidence indicates clearly that the position of this amino group is important to the inhibitory powers of this type of analogue. The graphical presentation, Figure 2, of the differences in the action of these three alkyl analogues of thiamine illustrates even more clearly the importance of the free amino group and its separation distance from the thiazole ring.

Through the studies with these two groups of benzyl-(3)-thiazolium analogues several points have become clear. The free amino group three carbons removed from the thiazole nucleus is important to the inhibitory ability of these analogues. The 5-β-hydroxyethyl side chain is a contributing factor in determining whether the analogue acts as an activator or an inhibitor, its absence apparently being required for inhibitory powers in the analogues tested.

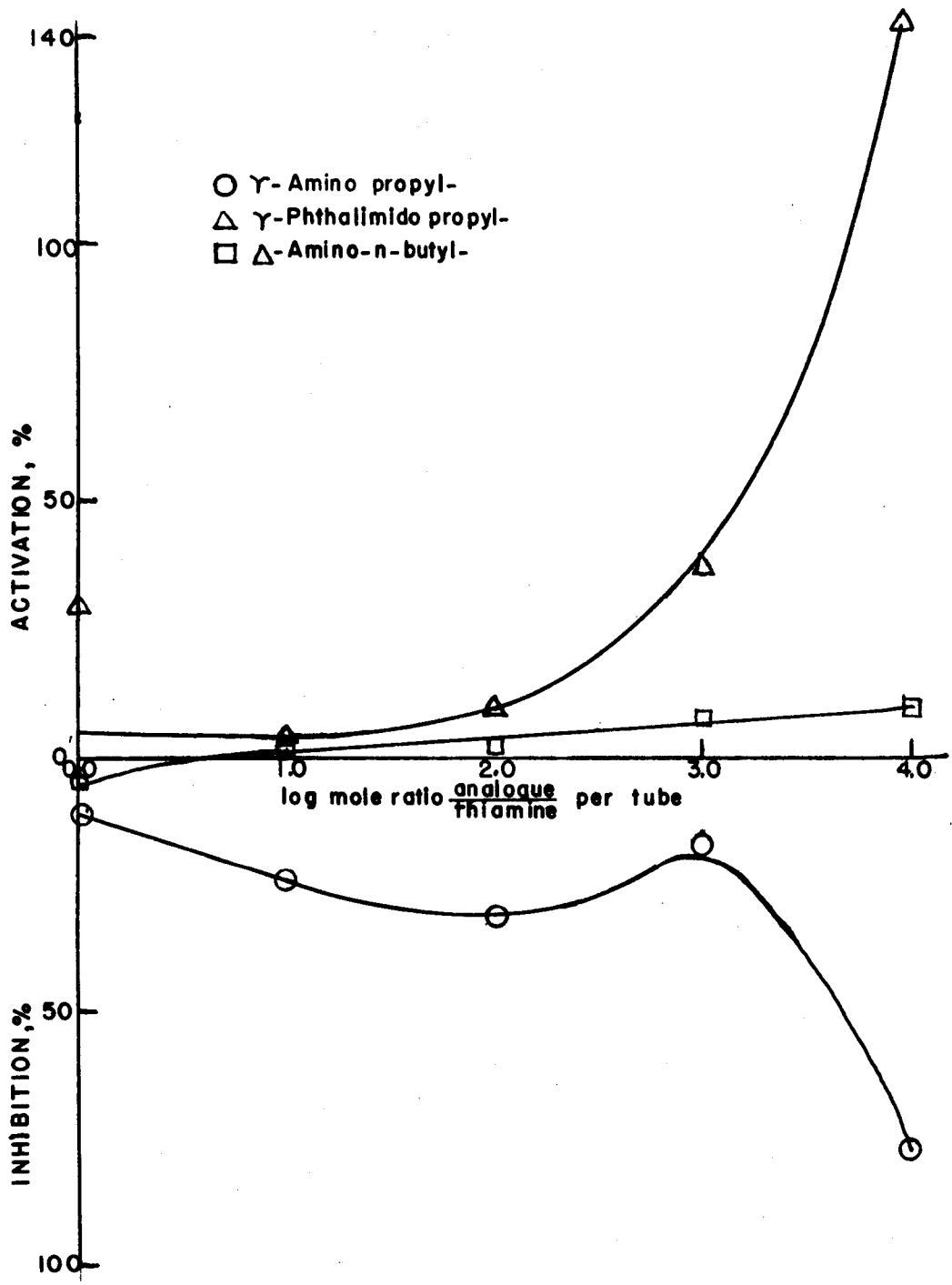

FIGURE 2. THE EFFECT OF AMINOALKYL AND SUBSTITUTED AMINOALKYL (3)-4-METHYLTHIAZOLIUM ANALOGUES OF THIAMINE ON THE GROWTH OF L. FERMENTUM 36.

The effects caused by the 6-aminopyrimidine analogues are given in Table XVIII. The effects were generally inhibitory though in several instances in the high concentrations of compound the growth was actually

Table XVIII

The Effect of 2-Methyl-6-Aminopyrimidine Analogues of Thiamine on the Growth of Lactobacillus fermentum 36 In the Presence of Thiamine or Cocarboxylase

Compound	Effect on Growth Mole Ratio of Compound/Thiamine or Cocarboxylase Required to Effect a 50 per cent stimulation or Inhibition of Basal Growth Stimulated by:[a]			
	Thiamine per tube moles x 10^{10}		Cocarboxylase per tube moles x 10^{10}	
	0.595	1.19	0.595	1.19
5-Hydroxymethyl-	14,130 (-)[b] (-) (+)	(-) (-) (+)	2,880 (-) (-) (+)	400 (-) (-) (+)
5-Bromomethyl-di-hydrobromide	3,020 (+) (-) (+)	2,630 (+) (-) (-)	440 (-) (-) 10,000 (+)	630 (-) (-) (-)
5-Methylenesulfonic acid	(+)	(+)	(+)	(+)

[a] (+) Indicates stimulation of growth; (-) indicates inhibition of growth.

[b] Blank indicates that a 50 per cent stimulation or inhibition effect was not approached by the highest level of compound tested.

stimulated. Sarett and Cheldelin (113) had reported that for the L. fermentum the 6-amino-pyrimidines were inhibitory in their mode of action.

Since these assays were being run at relatively high concentrations of analogues, the halogen ions (Cl^-, Br^- and I^-) contributed by the addition of the analogue to the nutrient media were quite large in relation to the amounts normally present. A series of assays were run to determine the effect of these halogen ions on the L. fermentum assay

under conditions and concentrations comparable to those that were present during the actual studies. The results of these experiments are given in Table XIX. All halogens stimulated the growth of the L. fermentum

Table XIX

The Effect of Some Analogue Components of Thiamine and Inorganic Salts on the Growth of Lactobacillus fermentum 36 in the Presence of Thiamine or Cocarboxylase

Compound	Effect on Growth Mole Ratio of Compound/Thiamine or Cocarboxylase Required to Effect a 50 per cent Stimulation or Inhibition of Basal Growth Stimulated by:[a]			
	Thiamine per tube moles x 10^{10}		Cocarboxylase per tube moles x 10^{10}	
	0.595	1.19	0.595	1.19
Sodium Chloride	(+)[b]	(+)	39,800(+)	(+)
Sodium Bromide	400(+)	2,500(+)	39,800(+)	(+)
Sodium Iodide	(+)	(+)	(+)	(+)
o-Aminobenzyl Alcohol	6,300(+)	(+)	12,600(+)	10,000(+)
4-Methyl-5-β-hydroxyethylthiazole	230(+)	145(+)	440(+)	1,260(+)

[a] (+) Indicates stimulation of growth; (-) indicates inhibition of growth.

[b] Blank indicates that a 50 per cent stimulation or inhibition effect was not approached by the highest level of compound tested.

with the effect of the bromide ion being much greater than that of the chlorine or iodine. Even though the bromide ion's effect is large, the γ-aminopropyl analogue, a bromide hydrobromide salt showed definite growth inhibiting activity.

The o-aminobenzyl alcohol moiety of the o-aminobenzyl analogues is in itself a stimulating molecule as shown in Table XIX. The results obtained with these studies show that the L. fermentum assay method does

respond to the benzyl-(3)-thiazolium and related analogues of thiamine with sufficient sensitivity to enable the study of the effect of the various groups and structural modifications of the molecule.

The results obtained with these benzyl and alkyl-(3)-thiazolium analogues show quite clearly they can enter into two different phases of the metabolism of the L. fermentum. The decided stimulation of growth observed with the aminobenzyl analogues containing the 5-β-hydroxyethyl group in the thiazole ring indicates that this molecular pattern enters into a thiamine system quite specifically in a manner that actually stimulates the growth of this microorganism. The lesser stimulations with other analogues in this group serves to emphasize the importance of the free amino group in this mechanism. The contrasted inhibition activity of some of these analogues indicates that with certain structural modifications, noticeably the elimination of the hydroxyethyl group on the thiazole moiety of the analogue, a compound is evolved which enters into the same or a different thiamine system in such a manner that the growth of the L. fermentum is actually inhibited. The specific role the hydroxyethyl group plays in eliciting these opposite effects is obscure, but its presence or absence in the molecule certainly alters the observed effect of the analogue. The importance of the free amino group in appropriate spatial relationship to the thiazole nucleus is an additional indication of the structural specificity required for the inhibition. The activation, though dependent on the free amino group, apparently does not require such rigid structural specificity for this effect.

E. Interrelationships

The results obtained in this investigation of analogue action on thiamine systems reveal that even though the systems as a whole are common in their dependency on thiamine for their physiological functions of growth and fermentation, the individual thiamine functions in these systems differ markedly in their reactions to the presence of these analogues. Consequently these observations serve to substantiate, by a different method, the known facts that the thiamine function itself is complex and is manifest in a similar yet specifically different manner in different species.

Where it has been possible to affect similar analogue-thiamine relationships in the three systems investigated, some qualitative comparisons can be made. In the case of the o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium analogue of thiamine, its interference with the thiamine functions of the rat and of the L. fermentum was established, while in the case of CO_2 production by yeast this analogue apparently did not inhibit significantly, if at all. The importance of the free amino group in the benzyl or alkyl portion of the analogue is evidenced in all systems where a definite effect, either stimulatory or inhibitory is noticed. Likewise the importance of the β-hydroxy alkyl group in the thiazole moiety to the activity of the analogue is evident in the rat and assumes a unique and striking importance in the L. fermentum system since its presence or absence dictates not only the presence or absence of activity, but specifically the direction or type of activity. The apparent ability of many analogues to specifically "spare" or stimulate the activity of the thiamine function in all three

test systems seems to be more common to the basic benzyl-(3)-thiazolium configuration rather than to a specific functional group in the molecule.

IV. DISCUSSION

The investigation of the action of benzyl-(3)-thiazolium analogues in the three thiamine dependent systems has been undertaken in order to gain additional information on the effects of these analogues on the physiological action of thiamine. The studies with the rats, and the L. fermentum 36 show that the benzyl-(3)-thiazolium compounds, as analogues of thiamine, did influence the physiological action of thiamine in these systems. The evidence for the effect of the analogues in the fermentation of bakers' yeast was less conclusive, though in isolated instances definite activity was observed.

The ability of these compounds to influence the thiamine systems is linked with their structural relationship to thiamine. The importance of specific functional groups, their position, presence, or absence to the effectiveness of these analogues has been demonstrated for several groups of analogues. The quite different physiological actions of a particular analogue in different species illustrates and confirms once more that thiamine function and thiamine enzymes are quite complex and differ in various species.

The broader aspects of this type of study are found in both the theoretical and applied or practical field of biochemistry and medicine. Basic knowledge of many of the intricate mechanisms of metabolism has been gained through the study of enzymes and enzyme inhibited reactions. One of the newer tools in this field, the use of the structural analogue inhibitors of enzyme action, has become quite firmly established. Systematic structure modification of analogues of vitamins has been

shown to give new insight into the mechanism and function of the vitamin enzyme systems involved. The systematic structural modification of the analogues of thiamine that affect the function of thiamine enzymes has not been pursued with the vigor that has been applied to some analogues of other vitamins. The importance of this type of study in the accumulation of fundamental knowledge of inhibitor action has been shown in the case of the oxythiamine analogue (121) as well as in the studies reported herein. Definite structure modifications that can negate or alter the action of an inhibitor molecule are details of importance in the basic studies of enzyme action.

The further application of these fundamental studies with structural analogues leads to the field of medicine and to one of its major tools -- the chemotherapeutic agents. The fundamental species differences observed in the study of the effects of analogues on animals and bacteria makes the study and selection of analogues of high antimicrobial potency and of low or insignificant effect on animal species one of major importance. An analogue of little effect on the host (man or domestic animal) and of high antibacterial potency becomes an extremely important chemotherapeutic agent in both human and veterinary medicine. The sulfonamide drugs as analogues of p-aminobenzoic acid are the striking example of this fundamental concept. The application of this important chemotherapeutic concept to other vitamins whose structure and metabolic importance is known, through structural modification and adequate biological testing will undoubtedly develop chemotherapeutic agents of even wider acceptance and use.

The practical application of structural analogues in medicine need not be limited to the antibacterial action of these compounds. The concept of anti-hormones in the control of hormonal upsets is becoming more

of a reality each day. Basic researches along the line of those carried out in developing the antivitamin concept may also be accomplished with the probable addition of a new general class of chemotherapeutic agents.

V. SUMMARY

The physiological action of a number of benzyl-(3)-thiazolium analogues of thiamine has been studied in three fundamentally different thiamine dependent systems.

The ability of certain of these analogues to inhibit the thiamine stimulated growth of the rat and the bacterium L. fermentum 36 has been demonstrated. The o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium analogue inhibits the growth of rats but not that of the L. fermentum. The o-aminobenzyl and the γ-aminopropyl-(3)-4-methylthiazolium analogues are definite inhibitors of the thiamine stimulated growth of the L. fermentum.

The ability to stimulate further the growth promoting effect of thiamine for the L. fermentum by many of these analogues has been shown also. Two analogues, the o- and p-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chlorides, are particularly effective in this respect and to the extent that it must be assumed that the stimulating abilities of these analogues are specific functions of their structure.

The specific physiological responses of these systems in the presence of several of these analogues can be correlated with their specific structures as related to themselves as well as to thiamine. A free amino group three carbons removed from the quaternary nitrogen was present in all analogues showing marked inhibition properties. This group in the analogue molecules has approximately the same spatial relationship to the whole analogue molecule as does the free amino group of thiamine to the whole thiamine molecule. The importance of this

amino group to both the thiamine and analogue molecules for their activity adds indirect but substantial evidence for the importance of the free amino group for thiamine function.

A new and rather peculiar function has been shown for the 5-β-hydroxyethyl group in the thiazole moiety common to both the analogues studied and the thiamine, in that its presence or absence seems to dictate for the o-aminobenzyl-(3)-4-methylthiazolium analogue of thiamine whether its physiological effect on the thiamine system of L. fermentum is one of marked inhibition or activation.

In connection with these studies a different method of assay, the fermentating ability of bakers' yeast, for analogue-thiamine investigation was introduced, tested and evaluated. The preliminary investigation indicated that this assay method is not of general application to analogue-thiamine competition studies.

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