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The mechanism of the fish thiaminase

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NOTE TO USERS

THE MECHANISM OF THE FISH THIAMINASE

by

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I. INTRODUCTION

That thiamine is a necessary component for the maintenance of a balanced diet for all living things seems to be an established fact. It therefore appeared as somewhat of an anomaly to find present in certain fish a factor which actively destroyed thiamine *in vitro*. Immediately interest was aroused as to the nature of this factor and its mode of action. Subsequent studies in several laboratories revealed the factor possessed protein-like character and that the destruction of thiamine was enzymatic.

These results led investigators to inquire whether the enzyme(s) was concerned *in vivo* with the synthesis or the destruction of this vitamin. The answer to this question would seem to be best obtained by kinetic studies of the system and attempted reversal of the reaction *in vitro*. This problem was complicated, however, by the demonstrated presence of two enzymes in the complete system. A more convenient approach could be made by using the stable, saline extract of acetone desiccated fish tissue which was known to contain only the first of the two enzymes. Even with this simple system it was necessary to know the products of the destructive reaction in order to attempt its reversal.

To best study the reaction for identification of these intermediates, or products, a purified enzyme system would be highly desirable. Purification was attempted but met with little success as the activity of the enzyme was either partly or completely destroyed. It then became evident that there was a need for more knowledge of the chemical nature of the enzyme and also of the substrate in order to better formulate a reasonable mechanism for its destruction. In this way a more logical choice could be made as to the possible products of the reaction and their chemical characteristics.

Thus a survey was made of the known chemical reactions of thiamine and the fish factor, and studies were undertaken in this laboratory to correlate these facts for the better understanding of the enzyme and the manner of its destruction of thiamine.

II. HISTORICAL

A. The Organic Chemistry of Thiamine

Any historical survey of thiamine needs only to be a continuation of the excellent monograph, "Vitamin B_1 and Its Use In Medicine", by Williams and Spies (1). Their review, published in 1938, is a complete and critical treatment of the knowledge of the vitamin up to that time including its chemistry, physiological action, and methods of assay.

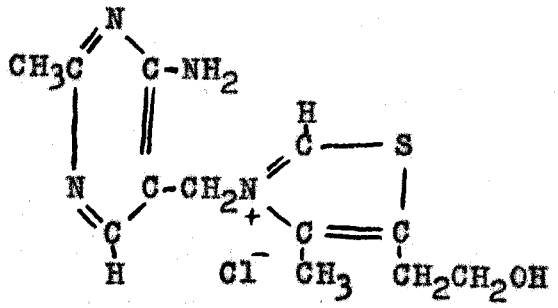
Two other excellent reviews on vitamins are also available, one by Rosenberg (2), and another by Eddy and Dalldorf (3).

The chemistry of thiamine, however, has not been investigated in a systematic manner except as it was required in its proof of structure and synthesis. Indeed, work done since 1938 has largely been concerned with its physiological action and assay. Most of its chemical reactions in these studies were more or less incidental and have been used for the most part in an empirical manner. Little or no knowledge is available as to their mechanisms, and in some cases even the products of the reactions are not known.

Since the reactions of thiamine lend themselves rather readily to a classification as to type, this approach will be

used in this paper rather than a strictly chronological one.

Thiamine has the following structure (4-12):



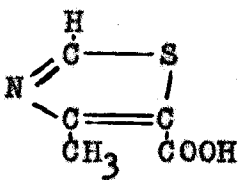
As can be seen, it is a complex, bicyclic molecule possessing several functional groups. Even in its physical properties this complexity is evident. It possesses three crystalline modifications, two of which have different melting points. The complete crystallographic data of all three modifications have been made available recently by the Armour laboratory in Chicago (13).

1. Cleavage reactions

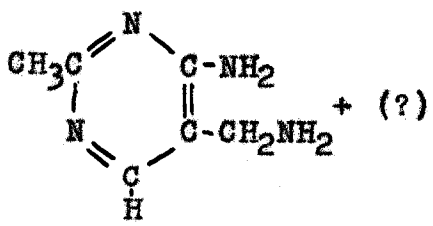
Early in the attempts at isolation of thiamine it was evident that the molecule was quite unstable and subject to inactivation by various chemical reagents and by heat. The inactivation by chemical means was found to be a result of a cleavage of the molecule and played an important part in the final elucidation of its structure.

a. Cleavage by chemical reagents. Windaus and co-workers (14) were the first to demonstrate the cleavage of

thiamine. From the action of nitric acid on the vitamin they were able to isolate two fragments. The first of these had the composition $C_7H_4N_3O_5$. They assumed it to be an esterified derivative of an alkylated dioxypyrimidine. The esterification, they thought, resulted from the use of ethyl alcohol as a solvent in the isolation procedure. While their surmise as to the nature of this fragment is probably correct, it has not been proven by synthesis up to the present time. The second cleavage product was isolated and shown to have the composition $C_5H_5NSO_2$. It has since been reproduced (15) and shown to be 4-methylthiazole-5-carboxylic acid.

(1) Thiamine + Nitric Acid $\longrightarrow$ Pyrimidine Derivative (?) + 

These workers also reported in a later paper the picrate of another base, unknown at that time, which had the following composition: $C_6H_{10}N_4$ (16). It was obtained from the vitamin by oxidation with barium permanganate. This was later synthesized by Grewe and identified as 2-methyl-5-aminomethyl-6-aminopyrimidine (17).

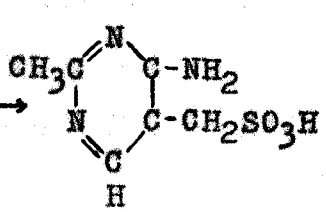
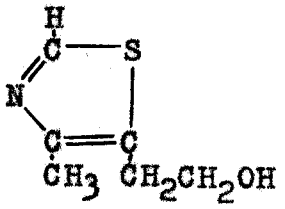
(2) Thiamine + Barium Permanganate $\longrightarrow$  + (?)

The second type of cleavage which was of even greater importance in proving the structure of thiamine is the quantitative splitting of the molecule by a neutral sulfite solution

at room temperature according to the equation (18, 19):

(3) $C_{12}H_{18}N_4SOCl_2 + Na_2SO_3 \longrightarrow \underset{\text{I}}{C_6H_9N_3SO_3} + \underset{\text{II}}{C_6H_9NSO} + 2\ NaCl$

Product I was later shown to be 2-methyl-6-aminopyrimidine-5-methylsulfonic acid (8). The other cleavage product, II, was also synthesized and identified as 4-methyl-5-(β-hydroxyethyl)-thiazole (20). The reaction is then

(3a) Thiamine + sodium sulfite $\longrightarrow$  + 

I II

This reaction was important because the point of attachment on the pyrimidine portion was marked by the position of the sulfonic group which was introduced during the sulfite cleavage. The point of attachment on the thiazole was not quite so obvious, and the reactions establishing this point will be considered later.

The cleavage by sulfite is affected by the pH of the solution. The reaction proceeds instantaneously at pH 6; at pH 5 it is complete overnight, and at pH 3 the vitamin may be kept in a solution of sulfite for several months without loss of activity (15).

Snyder and Speck (21, 22, 23) pointed out that the similarity of the benzene and pyrimidine rings made it seem

likely that this cleavage is another representative of the well-known lability of the carbon-nitrogen bond of benzyl amine derivatives. They employed such quaternary compounds as benzyl-phenyl-dimethylammonium chloride and found that its cleavage could be accomplished by various sulfur-containing salts. Among those used by these workers were sodium hydrosulfide, sodium sulfide, potassium thiocyanate, sodium hydrosulfite, and sodium dithionate. In all cases the benzyl group was split off just as the pyrimidyl group in the case of thiamine. The reactions and yield of products were as follows:

(4) $(CH_3)_2(C_6H_5CH_2)C_6H_5NCl + NaHS \rightarrow C_6H_5N(CH_3)_2$ (82%) + $(C_6H_5CH_2)_2S$ (54%) + $C_6H_5CH_2SH$ (18%).

(5) " " + $Na_2S \rightarrow C_6H_5N(CH_3)_2$ (28%) + $(C_6H_5CH_2)_2S$ (75%).

(6) " " + $KCNS \rightarrow C_6H_5N(CH_3)_2$ (68%) + $C_6H_5CH_2SCN$ (60%).

(7) " " + $NaHSO_3 \rightarrow C_6H_5N(CH_3)_2$ (40%) + $C_6H_5CH_2SO_3Na$ (63%).

(8) " " + $Na_2SO_3 \rightarrow C_6H_5N(CH_3)_2$ (63%) + $C_6H_5CH_2SO_3Na$ (82%).

(9) " " + $Na_2S_2O_3 \rightarrow C_6H_5N(CH_3)_2$ (99%) + Benzyl-dimethyl-phenyl-ammonium-S-benzylthiosulfate.

Their conclusions were: first, that the linkage between an alkyl group and the nitrogen atom which was attached to or

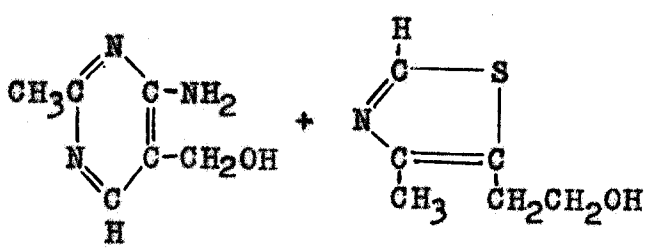
was part of an aromatic system was peculiarly labile to this type of cleavage and, second, of the reagents tested for action on these quaternary linkages sodium sulfide and sodium hydrosulfide were the most effective. The action of potassium thiocyanate on dimethyl-benzyl-phenylammonium chloride in the cold gave a precipitate which they identified as the thiocyanate salt. The salt rearranged on heating to give a 60% yield of benzylthiocyanate and a 68% yield of phenyl-dimethylamine. This, they felt, was a clue as to the manner in which the reaction proceeded although they did not elaborate on this statement.

b. Cleavage by heat. The second agent leading to cleavage of thiamine is heat. Its action is closely allied to the cleavage by chemical agents, but the products of its action are not known with the certainty of those of the latter reaction.

Many reports of the destruction of thiamine in foodstuffs by the action of heat at various hydrogen ion concentrations and temperatures are available (24-31). Their individual findings need not be detailed. It suffices to say that the common conclusion was that thiamine was relatively stable at pH 1 to 4 up to temperatures of 100° C. Increasing temperatures at this acidity caused a rapidly increasing destruction. Even more striking was the rapid increase in destruction of the molecule with increasing alkalinity even at lower temperatures.

The studies on solutions of the pure vitamin, while arriving at essentially the same conclusions, are of perhaps greater significance to this paper. According to Barger, et al (32), the amorphous free base undergoes decomposition when heated at 180° C. in a high vacuum. A crystalline solid, $C_9H_{10}N_3O_4$, was produced which exhibited an intense blue fluorescence in methyl alcohol. In acid media, pH 3.5-5.0, the resistance of the molecule to heat is considerable. This is demonstrated by the fact that 0.1% solutions will even withstand thirty minutes of heating in a sealed tube at 100° to 125° C. at pH 3 and pH 6. Autoclaving of the thiamine chloride crystals at fifteen pounds pressure for thirty minutes will, however, completely destroy its activity (1, p. 165).

Watanabe (33, 34) has reported that the heating of thiamine chloride at 120° C. in an aqueous solution leads to a hydrolytic cleavage and the production of the corresponding pyrimidine alcohol and the tertiary thiazole according to the following equation:

(10) Thiamine $\xrightarrow[120^\circ\ C.]{water}$ 

Farrer (35-37) has recently made a more detailed investigation of the effect of pH, buffer salts, and certain

metallic ions on the destruction of thiamine in aqueous solutions at 100° C. He found 100% destruction in fifteen minutes at pH 9 and in one hour at pH 8. At pH 7, 6, 5, 4, and 3 in one hour there was respectively 68, 53, 40, 20 and 16% destruction. In 1% hydrochloric acid there was no effect even after seven hours of heating at this temperature. This picture was modified though by the presence of buffer salts. In every case the velocity of destruction increased with increasing pH, but varied with different buffers. Farrer found the relation of reaction velocity to hydrogen ion concentration to be linear. The presence of copper ion in concentrations ranging from 2 ppm. to 20 ppm. accelerated the decomposition in direct proportion to the metal ion concentration in the presence of phosphate or phosphate-phthalate buffers. Yet, in the presence of tartrate, citrate, or glycine the destruction could be either decreased or increased. Iron, nickle, and zinc had no effect on the vitamin stability in the presence of phosphate buffer but did have an effect if citrate were present. Furthermore, the concentration of the buffer was also a determining factor. With buffer concentrations varying from 0.02 to 0.20 M., at pH 2.9-7.0, the reaction velocity was dependent on the phosphate concentration for all values below pH 6. Citrate suppressed the destruction while phthalate buffer magnified it. At a fixed hydrogen ion concentration, pH 5.9, the relation between velocity

coefficient and phosphate concentration varied continuously.

Beadle, Greenwood and Kraybill (38) reported the same situation. At pH 5.4, after heating for one hour at 100° C., there was 100% destruction in the presence of borate buffer, 57% with no buffer, 10% with acetate, and only 3.0% with phosphate buffer.

Booth (39), whose work was later corroborated by Farrer, had earlier stated that copper ion in concentrations of 2 ppm. catalyzed the destruction of thiamine, whereas iron, aluminum, zinc or tin ions had no effect. His work also included data for the destruction of cocarboxylase, the pyrophosphate ester of thiamine. Under similar conditions it was more rapidly destroyed than was the free vitamin. In neither case did the destruction appear to be oxidative.

McIntire and Frost (40, 41) studied the effect of the presence of amino acids and related compounds on the thermal destruction of thiamine. Their data seemed to support the conclusion that alpha- and beta-amino acids protected the molecule, whereas the delta-, epsilon-, and gamma-amino acids, and p-aminobenzoic acid enhanced its thermolability. Their investigation of these relations led them to conclude that the destruction of thiamine by heat was not a first order reaction but a complicated one subject to many influences other than pH and temperature, and that it could be inhibited or enhanced by certain compounds.

2. Ring opening

The rapidly increasing destruction of thiamine by heat with increasing alkalinity of its solution is an indication of another unusual reaction of this molecule. As pointed out above, the cleavage of the vitamin in more acidic solutions leads to derivatives of the respective pyrimidine and thiazole nuclei. In the alkaline range the destruction is more profound. It results in the evolution of hydrogen sulfide and other products of undetermined composition.

The peculiar stability of the sulfur atom in the basic cleavage product of thiamine with sulfite led H. T. Clark and Gurin to correctly postulate that it was a thiazole derivative (42). They added further evidence of this with a series of reactions involving thiamine itself, which likewise pointed to the presence of a thiazole derivative possessing a quaternary nitrogen atom. This, as mentioned before, was another important factor in proving the point of attachment of the pyrimidine and the thiazole nuclei. In collaboration with Williams' group they showed that in the thiamine molecule the sulfur atom was quite labile with respect to alkali. For example, with heat and strong alkali hydrogen sulfide was readily split out. With alkaline plumbite a precipitate of lead sulfide was formed, and the action of aqueous bromine was without effect. In the basic fragment from the sulfite

cleavage, the lability of the sulfur atom to these reagents was interchanged. The alkali and alkaline plumbite were without effect, whereas aqueous bromine gave sulfuric acid. Furthermore, if the methiodide of the basic fragment was used, the reactions were again the same as in the original molecule.

Meanwhile, Williams and Ruehle (43) had found that the electrometric titration of thiamine had certain unexpected peculiarities. The titration proceeded normally until it reached the point where 1 mole-equivalent of alkali had been added. From this point on to the end of the titration, which required 2 mole-equivalents more of alkali, the readings were difficult to obtain. With each addition of alkali there was a large initial increase in pH followed by a slow drifting to a lower value. Equilibrium, as indicated by a constant reading, was not attained for fifteen to twenty minutes after each addition. If the solution was immediately back-titrated, the titration curve was retraceable but with slightly lower values and losses up to 20% of the vitamin. If some time elapsed before back-titration, there were greater losses. Figure 1 illustrates the titration curves described above. Recalling the work of Mills, Clark and Aeschlimann (44) in the benzothiazole series and the presence of the quaternary nitrogen in the vitamin molecule, Williams (1, p. 164) postulated the following course of events to account for these properties:

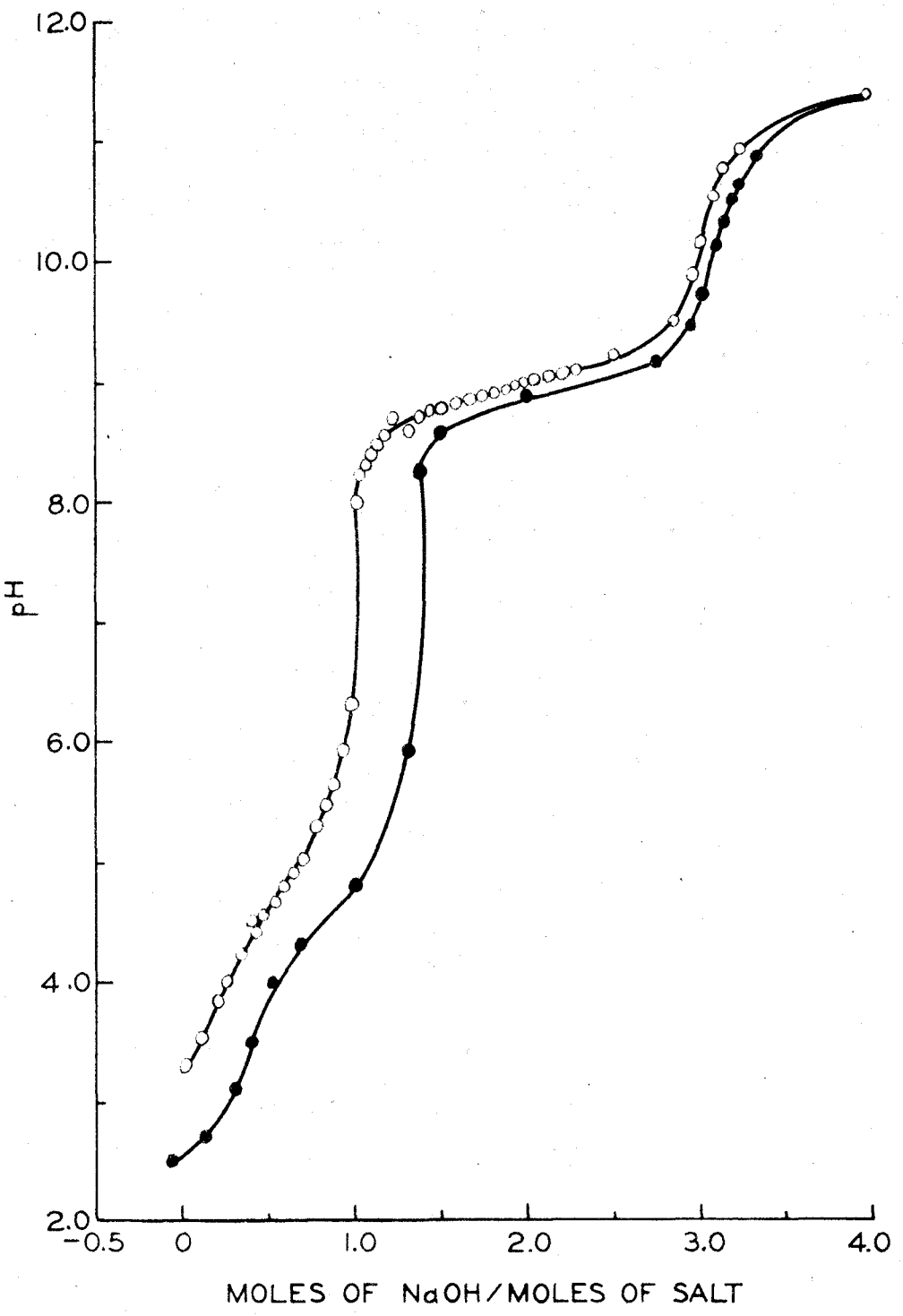
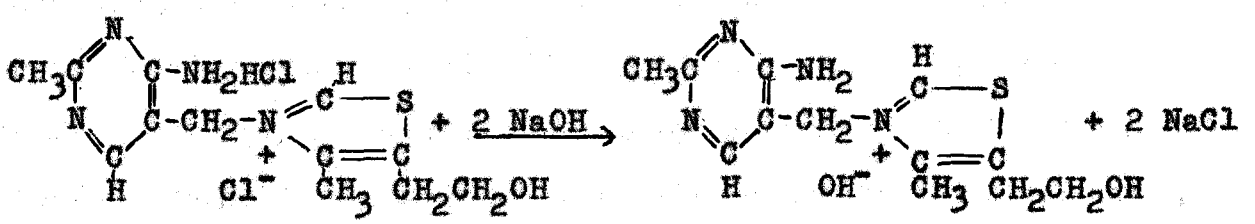
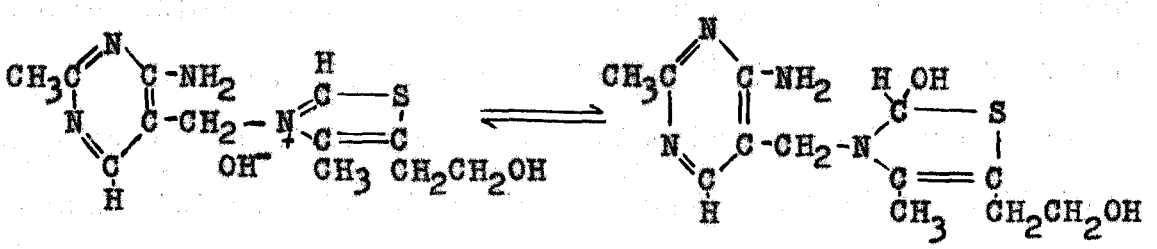
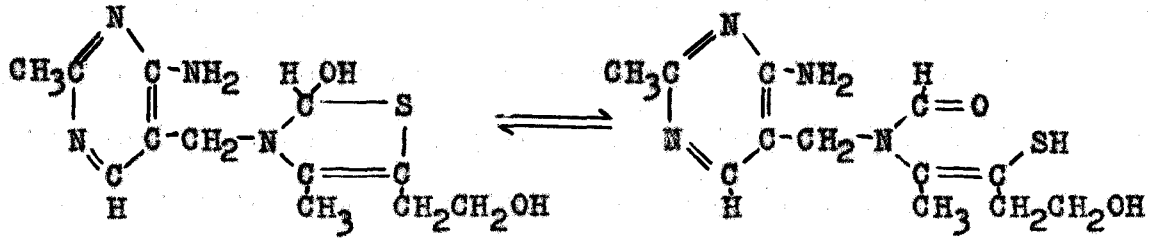
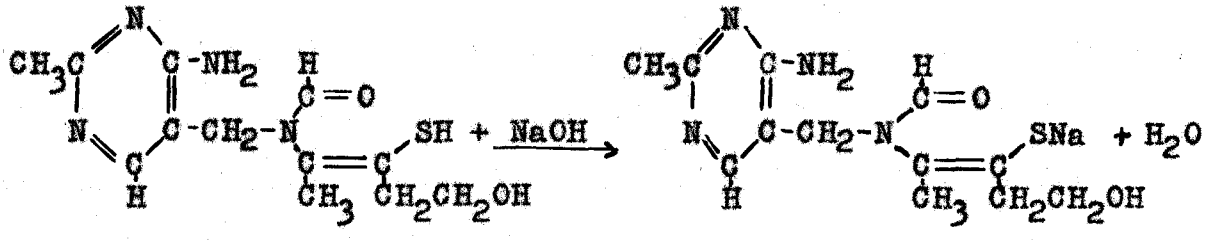


Fig. 1. Titration of Vitamin B_1 chloride hydrochloride; o-o titration with NaOH, o-o back titration with HCl. Williams, et al (1, p. 163).









They further showed that the methiodide of the thiazole moiety of thiamine behaved in exactly the same manner. The basic dissociation constants for thiamine and several thiazoles are listed in Table I.

Table I

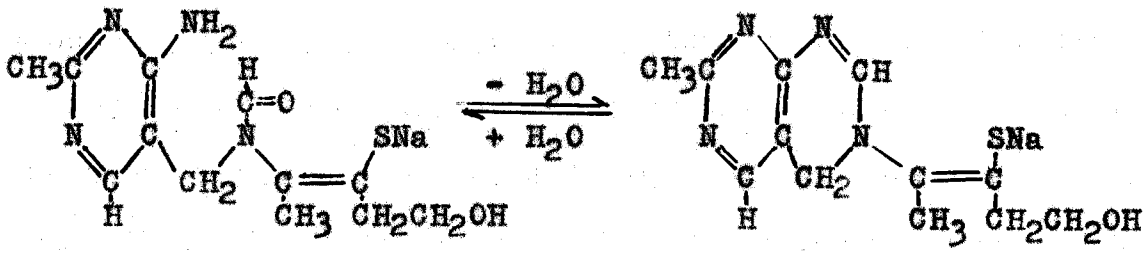
Basic Dissociation Constants of Some Thiazole Derivatives

Substance	pKb
Thiamine (stronger base)	5.0
(weaker base)	9.5
Basic cleavage product (quaternary)	4.1
(tertiary)	10.6
4-methyl thiazole (quaternary)	4.5
(tertiary)	10.4
2,4-dimethyl thiazole (quaternary)	2.8

Titration of the 2,4-dimethylthiazole with alkali results in the formation of a mauve color. This is due to the formation of a thiazole cyanine dye and prevents back-titration. The titration curve and the pKb for this compound indicate that its behavior is the same as that usually encountered with quaternary ammonium salts. He concluded that in this case the thiazole ring could not open as it did in the case of the other thiazole derivatives listed in Table I. Its inability to open is due to the methyl substitution in the 2 position.

In a later paper Zima and Williams (45) gave directions

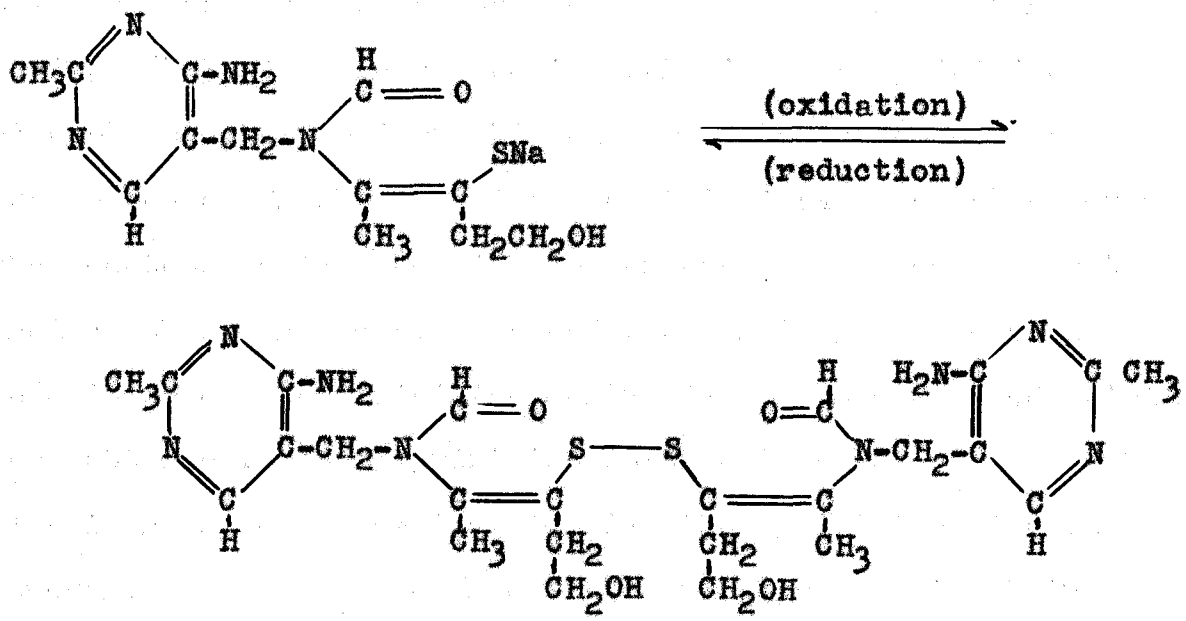
for the preparation of the open thiol form of thiamine pictured above. This product was obtained in good yields. They also reported that it could exist in two interchangeable forms as shown below.



The open thiol form did not give the sodium nitroprusside test as carried out by these workers, or as modified by Schöberl and Stock (46) in a further study of this type of reaction. It can be precipitated with salts of heavy metals such as mercury, silver and lead.

3. Oxidation-reduction reactions

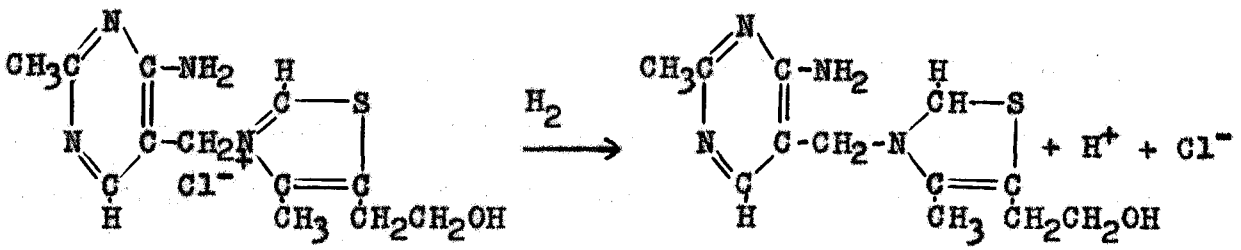
In the same paper (45) in which they reported the preparation of the open thiol form of thiamine, Zima and Williams also prepared and isolated the disulfide form of this compound by mild oxidation. The oxidation was produced by such reagents as iodine, hydrogen peroxide, and air on alkaline solutions of the vitamin. This reaction could be reversed and the disulfide reduced to the original thiol form by the action of hydrogen, tin and hydrochloric acid, and by addition of excess cysteine. This reversible oxidation-reduction reaction of thiamine furnished a basis for the attractive



hypothesis that the physiological functions of thiamine were intimately associated with its ability to undergo these transformations (47, 48). Thus it would act as a prosthetic group in enzyme systems whose purpose was the removal of hydrogen from some substrate molecule and the transference to some acceptor molecule.

The idea that thiamine might function in some such reversible oxidation-reduction system had been advanced previously by Lipmann along a different line (49). He pointed out the similarity of the quaternary nitrogen in thiamine to that in the pyridine dinucleotide coenzymes. In the case of these compounds the quaternary nitrogen atom had been shown (50, 51) to undergo a reversible oxidation-reduction in the performance of their physiological functions. In the case of thiamine, Lipmann reported that he had achieved its reduction by two

different methods. In the first, accomplished by hydrogen in the presence of platinum-black catalyst, the molecule took up the equivalent of 0.94 moles of hydrogen. In the second, and apparently different type of reduction, the molecule in the presence of sodium dithionate at pH 7.5 had released 2.76 moles of acid as determined manometrically. This corresponded to the uptake of one atom of hydrogen by thiamine and the production of one mole of acid. The reaction was pictured as (1, p. 162):



In the course of the reaction sodium hydrosulfite was released from the sodium dithionate, and it was necessary to show that no cleavage of thiamine resulted from its action. He stated that under the conditions of his experiment, pH 7.85, there was no evidence for a reaction between sodium hydrosulfite and thiamine such as Williams (15) had shown at pH 5.0. As further evidence he pointed out (52) the appearance of a transient green color in the course of the reduction, a phenomenon also observed when zinc and hydrochloric acid were used as the reducing agent. This he felt was the half-reduced (semiquinone) thiazole analogous to the semiquinones formed in

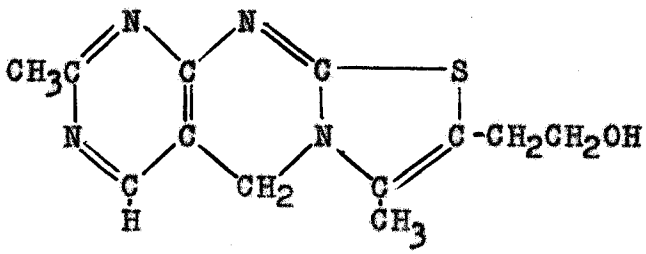
the 1 electron reduction theory of Michaelis (53). In a later paper Lipmann and Perlmann (54) studied the above two methods of reduction on the pyrimidine sulfonic acid (I), the thiazole portion of thiamine (II), thiamine (III), and the methiodide of II (IV). The catalytic reduction depended on time and pH and followed a different course than did the reduction with sodium dithionate in sodium bicarbonate buffer. In the latter case III and IV took up the equivalent of two atoms of hydrogen and released three moles of acid as reported before, while I and II were not reduced under these conditions. In none of the experiments were the products of the reduction isolated.

Despite the attractiveness of both of these hypotheses, the knowledge to date does not seem to support either of them. Barron and Lyman (55), in a study on the rates of reduction of both thiamine and diphosphothiamine, present evidence to refute the participation of either scheme in the actual function of thiamine in the body.

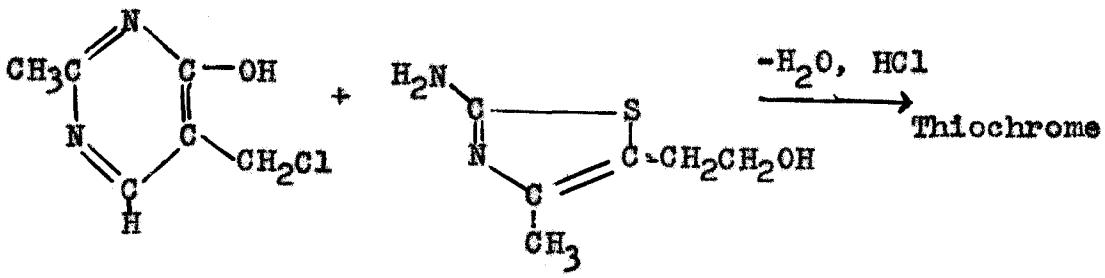
That thiamine is also capable of a type of irreversible oxidation of an entirely different sort is also known and has been abundantly verified. In fact, this reaction (thiochrome) is the basis for one of the most popular methods of assay for the vitamin.

Early investigators had noticed the appearance of a blue fluorescence in neutral or alkaline solutions of thiamine

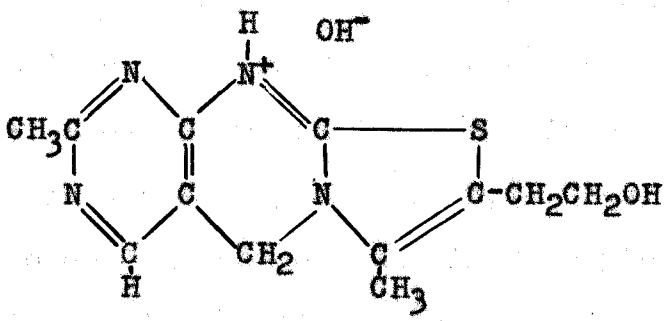
which had been exposed to oxidizing conditions. The compound responsible for this fluorescence was isolated from yeast by Kühn and co-workers in 1935 and given the name "thiochrome" (56). In the same year Barger, Bergel and Todd (57) were able to obtain the compound in fair yields by the oxidation of thiamine in alkaline solutions with potassium ferricyanide. From degradation studies and the similarity of composition to thiamine they correctly postulated its structure to be



Also in the same year these workers (58) were successful in synthesizing it according to the following equation:



In this paper and in another by Kinnersly and Peters (59) it was pointed out that the properties of thiochrome made it appear likely that in solution the molecule was hydrated to give a quarternary nitrogen, i.e.,



The literature on the development of this reaction as an assay method for thiamine is extensive, and, as it lends little knowledge as to the chemistry of the vitamin, it will not be covered here. It will suffice perhaps to mention a very recent and excellent review on this subject (60) and to point out the critical factors in the assay method as determined by Conner and Straub (61). These investigators found that the most important factor in the oxidation was the ratio of alkali to thiamine. The maximum oxidation was obtained if there were 0.01 moles of sodium hydroxide to 0.001 micromoles of thiamine regardless of the pH. The influence of the oxidizing agent was not of too great an importance if there was sufficient base, and 6 micromoles of the ferricyanide for the above ratio was optimum. If there was too much excess ferricyanide the thiochrome was destroyed. Under optimum conditions there was a conversion of some 67% of the thiamine to thiochrome.

4. Color reaction of thiamine

Along with the thiochrome test there is another reaction of thiamine which has found popularity as a chemical method of assay for this vitamin. This is a coupling reaction which the molecule undergoes with various diazotized aromatic amines to produce a characteristic dark red dye. The dye can then be extracted with organic solvents and compared colorimetrically with a standard.

In every case the coupling reaction occurs in an alkaline solution and, in view of the properties of thiamine in the presence of excess alkali, this should be kept in mind when considering the possible product of this type of reaction. Up to the present time the composition or structure of none of these colored compounds has been definitely proven.

Kinnersly and Peters (62) were the first to report such a reaction of thiamine. They used diazotized sulfanilic acid and added it to an acidic solution of the vitamin. Just prior to this addition, however, they added a small amount of formaldehyde (40%) to the acidic solution of the vitamin. This was done to prevent a fading of the dye encountered in earlier experiments. In order to effect the coupling, they also found that a pH of 8 or more was required. In a later paper (63) some improvements were made in this procedure. It is their opinion that the reaction of the formaldehyde is with the 6-amino group of the pyrimidine portion and that the

coupling with the diazonium salt occurs at this point.

In 1936 Prebluda and McCollum (64) suggested the use of diazotized p-aminoacetanilid or p-aminoacetophenone. Willstaedt in 1937 found that thiamine would also form a dye with a solution of diazotized 2,4-dichloro aniline and in the next year published further improvements on this reaction (65, 66).

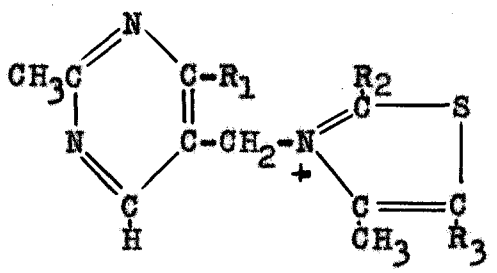
A later paper of Prebluda and McCollum (67) listed the properties of the dye formed from thiamine and diazotized p-aminoacetophenone and also indicated that the reaction will not proceed in an acid solution. The dye was found to be insoluble in water, concentrated alkali, dilute acids and petroleum ether. It was soluble in 95% ethanol, propyl, butyl and isobutyl alcohols, acetone, dioxane, glacial acetic acid, ether, carbon tetrachloride, chloroform, benzene, toluene and xylene. They also studied the action of the coupling reagent on the compounds listed. Their results are compared to those froma similar study by Bergel and Todd (68) for the Kinnersly-Peters Formaldehyde-Azo test.

Melnick and Field (69, 70) have subsequently modified the Prebluda test and made it more quantitative. If thiamine was deaminated, oxidized to thiochrome, or subjected to the action of sulfite or heat and alkali, no color was produced by this test.

More recently Marenzi and Vilallonga have added

Table II

Effect of Substituents on Colorimetric Tests and Thiochrome Test for Thiamine



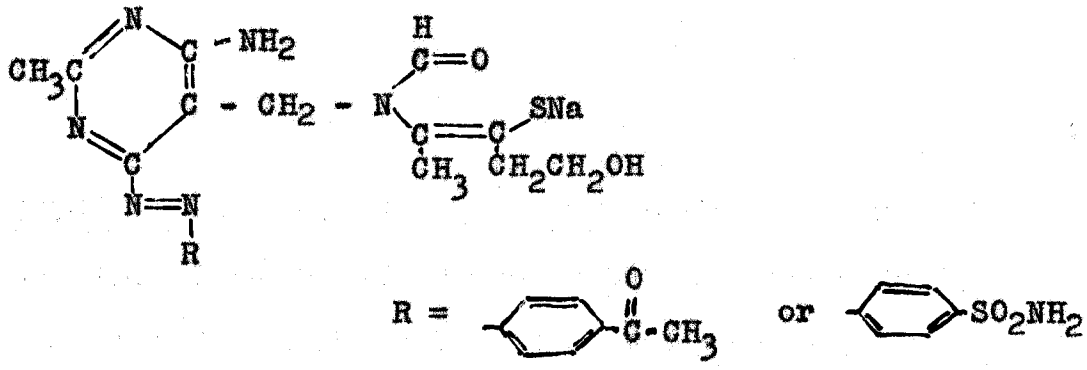
Substance	R_1	R_2	R_3	Azo Test	Prebluda-McCollum	Thiochrome
Thiamine	NH_2	H	C_2H_5OH	+	+	+
A	NH_2	H	H	-	-	+
B	OH	H	C_2H_5OH	-	-	-
C	OH	H	H	-	-	-
D	NH_2	CH_3	C_2H_5OH	-	(?)	-
2-methyl-6-amino-5-bromomethylpyrimidine					-	
4-methyl-5-β-hydroxyethyl-thiazole					-	
2-methyl-6-amino-5-ethoxymethylpyrimidine					-	
4-methyl-5-β-hydroxyethyl-thiazole methiodide					+	
4-methylthiazole					-	

diazotized p-nitroaniline to the list of reagents for determining thiamine (71).

Substances reported to interfere (63, 72, 73, 74, 75) with this coupling reaction are ascorbic acid, heavy metals, cysteine, uric acid, hydrogen sulfide, inositol, adrenaline, ferricyanide, and hydroxylamine.

Murata (76, 77) claims to have purified the dye formed from thiamine and the Prebluda reagent. The compound consisted of reddish-violet crystals which melted at 253-5.5° C. On reduction of the dye he was able to isolate p-amino-acetophenone. He also states, but without presenting specific evidence, that the same compound was obtained from the action of the Prebluda reagent on 4-methyl-5-(-hydroxyethyl)-thiazole methiodide as was obtained with thiamine. Murata concluded that the vitamin is split into its pyrimidine and thiazole components during the course of the reaction, and that it is the latter component which is responsible for the formation of the dye.

Runti in 1948 (78, 79) conducted a detailed investigation of the azo coupling reaction of thiamine and the nature of the product. He concludes that the problem is solved by his work, and that the coupling occurs at the 4-position of the pyrimidine. That is,



The open thiazole ring is indicated because of the strongly alkaline solution in which the reaction is carried out.

5. Miscellaneous reactions

In the initial investigations of the diazo coupling reaction, it was believed that the action of the nitrous acid was to diazotize the 6-amino group of the thiamine molecule. Indeed, this was reported as a test method for the presence of p-amino benzoic acid (80). The directions called for the diazotization of the thiamine solution, and this solution was then added to that suspected of containing the p-amino benzoic acid. Runti (78, 79) has proven that the actual course of events is the diazotization of the p-amino benzoic acid which then couples with thiamine exactly as in the Prebluda test.

Peters (81) had reported as far back as 1924 that nitrous acid under the ordinary low temperature conditions of diazotization had no effect on this vitamin. This observation has

been abundantly verified by other workers. More recently Soodak and Cerecedo (82, 83) claim to have isolated oxythiamine (analogue of thiamine in which the 6-amino group is replaced by a hydroxyl group) in 50-70% yields from the action of nitrous acid on aqueous solutions of the vitamin at a temperature of 40-50° C.

This is somewhat similar to the conditions that produce the chloroxythiamine (analogue of thiamine in which the 6-amino group is replaced by a hydroxyl group and the 5- - hydroxy group is replaced by chlorine). Buchman and Williams (20) obtained this compound when they subjected thiamine to the action of concentrated hydrochloric acid in a sealed tube at 150° C. for three hours.

Formaldehyde is said to inactivate the vitamin (84) as do ultraviolet light (85) and x-rays (86). The action of the ultraviolet light may be centered on the pyrimidine portion of the vitamin as Uber and Verbrugge (87) have shown that this agent completely destroys the absorption spectrum of 2-methyl-6-amino-5-ethoxymethylpyrimidine at a concentration of 6.65×10^{-5} M. in from ninety to one hundred and twenty minutes.

Tauber (88) has used p-dimethylaminobenzaldehyde and acetic acid as a color test for thiamine. His belief is that a Schiff's base is formed which gives the characteristic brick red color. This supposition seems to be well taken, as the same type of reaction occurs with this reagent and p-aminobenzoic

acid. Another such color test is that of Raybin (89) which employs 2,6-dibromoquinone-chlorimide. This reagent reacts with thiamine in aqueous borax buffer solution at pH 9.6 to give an orange color. He believes the thiazole portion to be responsible for the color.

Colored precipitates are given by the vitamin with picrolonic acid, gold or mercuric chloride, Mayer's reagent, and iodine (1). Silver, lead, tin, antimony, mercury or bismuth iodide in potassium iodide solution give crystalline products with thiamine (90, 91, 92). Crystalline products are also formed with potassium tetranitrodiaminocobaltate (rufianic acid) and with Reinecke's salt. Amorphous salts are precipitated by picric acid, alizarin sulfonic acid, β-anthraquinone sulfonic acid, trinitroresorcinol, potassium periodide, and perbromide (93). Feigl and Ribeiro (94) have used the catalytic action of the vitamin to release nitrogen from sodium azide as a micro-reaction for the detection of thiamine.

The adsorption of thiamine on activated charcoal (95) and synthetic ion-exchange resins (96) have also been studied.

B. The Chemistry of Thiaminase

To the chemistry of thiamine there has been recently added another reaction which may be intimately concerned with its physiological function in the body. This is the somewhat puzzling evidence for the existence of a factor, or factors, from several sources whose primary function appears to be the cleavage of the vitamin molecule in much the same manner as shown for the action of low valence sulfur salts and heat. As mentioned above, this is unexpected because of the demonstrated necessity of thiamine for the maintenance of the integrity of the body functions. If, as would seem more logical, the true function of this factor is the synthesis of the vitamin rather than its destruction, there is no evidence to date which supports this view.

Naturally enough, the discovery of this apparent anomaly excited general interest and extensive studies of the factor were initiated in several laboratories.

The purely historical aspect of the discovery has been completely reviewed elsewhere (97-98), and we will concern ourselves here only with the organic and physical chemical studies of the thiamine destroying factor (also called thiaminase, aneurinase, fish factor, Chastek-paralysis factor) found in carp.

With the demonstration by Sealock, Livermore, and Evans

(99) that the factor concerned an enzymatic system, the possibility of investigation of its nature was considerably advanced. The characteristic thermolability of its protein nature had previously been shown (100). Heating, or cooking, the factor-containing fish completely destroyed its action. Sealock, et al, established an in vitro method of assay for the Chastek-paralysis factor and outlined a method of preparation for a more stable acetone desiccated fish powder preparation. In this paper they also defined a unit of activity for the factor to permit better comparative measurements and made some studies of its properties. It was soluble in 10% sodium chloride and very little soluble in water or aqueous ethanol. The reaction velocity of thiamine destruction was affected by pH and temperature. The optimal conditions found were pH 9.1 and 37.5° C. The Q_{10} values for the factor were 2.3 and 2.1 for temperatures of 30°-40° C. and 40°-50° C., respectively. There was a marked decrease of activity at 60° C. or greater for the crude preparations, and complete inactivation of the factor resulted upon heating to 100° C. for twenty to thirty minutes. With more dilute, or purified preparations, complete inactivaction at this temperature was reached in five minutes. These reactions indicated the protein nature of the factor as did its behavior with protein precipitating agents, such as trichloroacetic acid, alcohol, acetone, ammonium sulfate, picric acid, and metaphosphoric acid, all of which acted to

precipitate it.

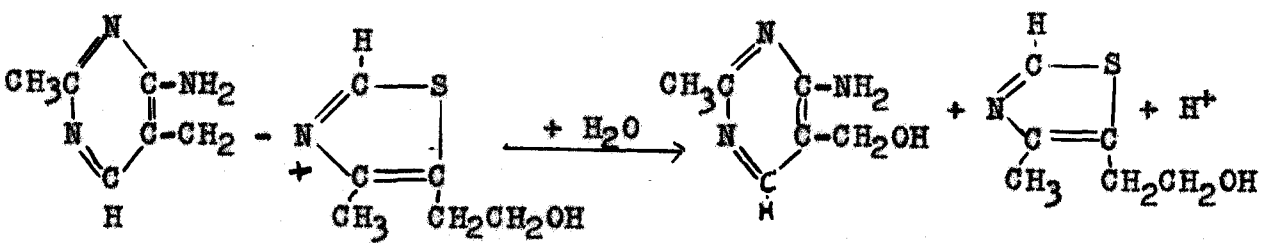
Time-course measurements were made and the destruction reaction was found to fit fairly well the graph of a first order reaction.

Bhagvat and Devi (101), in their preliminary studies, found that the factor was extractable with chloroform-water mixture. The factor was partially inactivated by dialysis and completely inactivated by boiling or incubation at pH 2.0 or 10.0. The activity of the dialyzed material could be partly restored by addition of the boiled dialysate to the dialyzed preparation. Its enzymatic action was not affected by potassium cyanide or sodium fluoride.

The inhibition of thiamine inactivation was investigated by Sealock and Goodland (102). It was strongly inhibited by copper, zinc, and ferric ions. Cyanide, fluoride, iodoacetate, sulfhydryl and sulfite ions were less effective. Of greater interest, perhaps, were their studies on competitive inhibition. Compounds resembling thiamine in one or more features were used. Among these, o-aminobenzylthiazolium chloride, and β-aminoethyl-4-methylthiazolium chloride were found to be strongly inhibitory. Alteration of the amino group of either compound almost completely destroyed their inhibitory action. The Michaelis constants were determined as 0.831×10^{-5} mole / liter for thiamine and 0.0197×10^{-5} for the o-aminobenzyl analogue. Up to this time no insight as to the manner of the

thiamine destruction had been obtained until Krampitz and Woolley (103, 104) succeeded in isolating the pyrimidine alcohol and the tertiary thiazole from the action of a suspension of fresh-fish tissue on thiamine. They also found that acetone desiccated fish powder used by Sealock did not produce these products. Instead the tertiary thiazole and some intermediate pyrimidine derivative of undetermined origin were obtained. This pyrimidine intermediate on boiling with alkali or on long standing did give the pyrimidine alcohol however. They concluded from this evidence that in their fresh-fish tissue suspension there were present two enzymes, but only the first of these was contained in Sealock's preparations.

Upon considering the overall reaction of the fresh-fish tissue suspension with thiamine, Sealock and Livermore (105) pointed out that it was an enzymatic hydrolysis with the release of a hydrogen ion, i.e.,



Accordingly, they reasoned that the course of the reaction could be followed manometrically in the Warburg instrument by the resultant release of carbon dioxide from the action of the hydrogen ion on a bicarbonate buffer. They successfully

accomplished this and measured 85.7, 56.5, and 61.3% of the theoretical amount of carbon dioxide as calculated from the loss of thiamine in the reaction.

In 1944-1945 four papers were published by Agren (106-109). He found the Chastek-paralysis factor present in 10 of 21 fresh-water fish tested. Nine of the 10 varieties containing the factor were of the carp family. Nine varieties of salt-water fish were tested and only one contained the factor. Agren reported that the factor could be inactivated by hydrogen peroxide in the cold and by dialysis. In either case glutathione would reactivate the inactive material. Twenty fold purification was achieved and the principle behaved as an albumen with an isoelectric point of pH 5. Later he reported a further 10 fold purification of the factor by means of electrophoretic separation and dialysis. Another electrophoretic study of the undialyzed material has been made by Beloff and Stern (110).

In 1946 Hennessy and Warner (111) reported in abstract the isolation of a pyrimidine derivative from the action of the thiamine inactivating factor in raw clams. It had a composition of $C_8H_{16}N_4O_3S.2HCl$, and the colorless needles obtained darkened at 235° C. and decomposed suddenly at 242° C. A crystalline picrate melted sharply at 175° C. The absorption maxima were characteristic of a 6-amino pyrimidine showing 2 maxima at 236mu and 279mu in dilute alkali, and

showing a single peak at 246mu in dilute acid. As yet there has been no elucidation of the structure or identity of this compound.

Murata (112) advanced a new idea on the inactivation of thiamine. He used a 0.1N disodium phosphate extract of a type of fresh-water mollusc, corbicula atrata. These extracts inactivated thiamine at pH 6.1 and 20-37° C. in one hour. Murati attributed the inactivation to a deamination of the pyrimidine portion of thiamine. Unfortunately only the abstract of his paper is available and details of his experiments cannot be checked.

Jacobsohn and Azevedo (113) found a thiamine destroying factor in several aquatic species, including shrimps and mussels.

The coenzyme of thiaminase was studied by Engelhardt and Tatarskaya (114). Their work supports that of Krampitz and Woolley (104) and demonstrates the loss of thiaminase activity by dialysis. This activity can be regained by addition of the dialysate. They concluded that the coenzyme was not an ion, and that it is very stable in the presence of strong acids and bases. It was insoluble in ether but soluble in butyl alcohol. In testing for its presence in other materials, they found the coenzyme widely distributed throughout the animal kingdom.

A publication of Reddy, Giri, and Das (115) was

particularly interesting in view of Krampitz and Woolley's work (104). They gave evidence that the thiamine destroying system in Lamellidens marginalis, a fresh-water mussel, is composed of two enzymes. They purified this system by absorption techniques using alumina C . One of the enzymes has an optimum activity at pH 3.5 and the other at pH 6.5. Fifty per cent of the activity of this second enzyme is lost on dialysis (time of dialysis not stated), and the activity of the other is not affected. The two enzymes also differed in stability at different hydrogen ion concentrations, each being most stable at its own optimum pH. Neither enzyme is affected by 0.01 M. sodium fluoride, 0.001 M. ferric sulfate, or 0.01 M. magnesium sulfate. Iodoacetic acid at 0.01 M. concentration inhibited the enzyme of lower optimum pH but not the other one. Copper sulfate at a 0.001 M. level inhibits both enzymes and 0.001 M. manganous sulfate activated both. Manganous sulfate also completely activated the dialyzed enzyme mentioned above.

Sealock and Livermore (116) continued their competitive inhibition studies employing aminobenzylthiazolium salts to analyze the structural features of the inhibiting molecule in relation to its effectiveness. The high inhibition attained with O-aminobenzyl-(3)-4-methylthiazolium chloride was mentioned earlier. If the phthalimido derivative of this compound or one in which the amino group was replaced by a

nitro group were used, the inhibition practically disappeared. The methyl group of the thiazole portion was shown to be of little importance to the inhibitory activity of the molecule. In studying the effect of changing the position of the amino group on the benzene ring, they were surprised to find that whereas the o-amino derivative was a good inhibitor of the thiamine destruction, the m-amino analogue was a very good activator of the system. The p-amino compound had little effect. The results of the action of these compounds are shown graphically in Figure 2. Furthermore, variation in the inhibiting power of the o-amino analogue with different fish-powder preparations led them to suspect the presence of some other influencing factor in the system. This evidence for such an influence was increased when they found that the addition of manganous ion increased the inhibition of a given concentration of the o-amino compound. With preparations of low activity, where larger amounts of the acetone-desiccated fish powder were needed to achieve comparative thiamine destruction, the effect of the manganous ion on the inhibition by the o-aminobenzyl-(3)-4-methylthiazolium chloride was small. On more active preparations where smaller amounts of the powder were used, the inhibition was materially increased. They found the effect of the manganous ion on the activation by the m-amino analogue was negligible.

The results are presented in Table III.

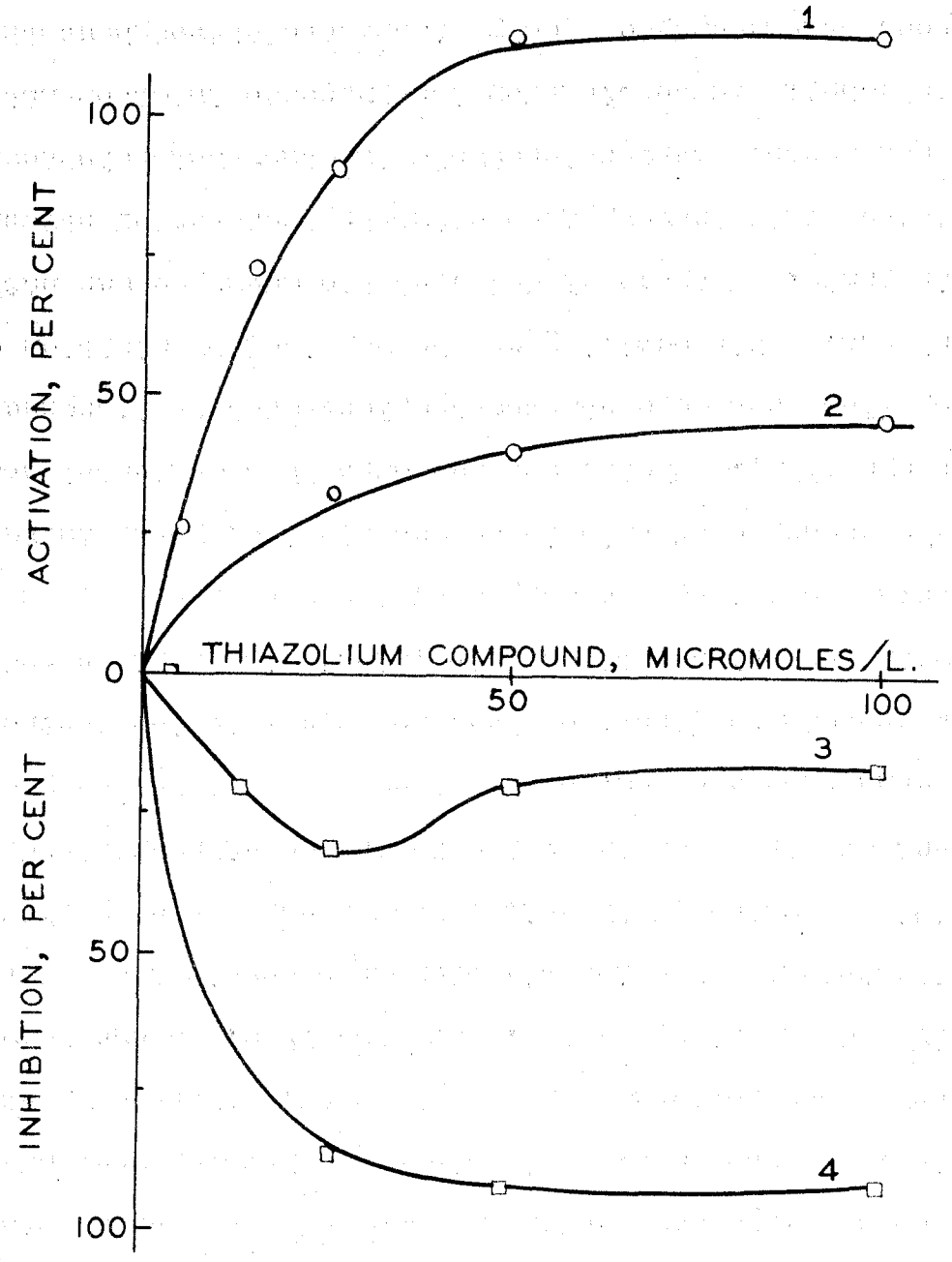


Fig. 2. Effect of benzylthiazolium chlorides on thiamine destruction. The m-aminobenzyl-2-methyl compound was used for Curves 1 and 2 and the o-aminobenzyl-2-methyl compound for Curves 3 and 4. Sealock and Livermore (116, p. 560).

Table III

Effect of Manganous Chloride on Inhibition

Experiment I. Enzyme equivalent to 25 mg. of Preparation 1-142-III-A. Experiment II. Enzyme equivalent to 37.5 mg. of Preparation 1-142-I-A.

	Additions	Thiamine Destroyed micromoles	Inhibition per cent
I.	None	1.83	
	1 ml. 25×10^{-4}M. inhibitor	0.43	77.5
	1 " 25×10^{-4}" " + 1 ml. 0.001M. $MnCl_2$	0.29	84.2
	1 ml. 0.001M. $MnCl_2$	1.76	3.7
II.	None	1.78	
	1 ml. 25×10^{-4}M. inhibitor	1.10	38.2
	1 " 25×10^{-4}" " + 1 ml. 0.001M. $MnCl_2$	0.82	53.9
	1 ml. 0.001 M. $MnCl_2$	1.84	-3.0

The effect of the manganous ion in increasing inhibition and displaying no action on the activation, they believed, pointed to two different types of action of these compounds.

In view of the Michaelis-Menten equation (117) or the Lineweaver-Burke modification (118), this increase in inhibiting power of the o-aminobenzylthiazolium salt is somewhat difficult to understand. Nevertheless, these results have been duplicated many times, and the effect is real.

The ultimate problem of establishing the physiological function of the various thiamine-destroying factors must await a better understanding of the properties and chemistry of the factor and the mechanism involved in thiamine destruction. Before this problem could be logically approached, there was a need for further information about the dialysis of the enzyme and the effect of activating factors, such as the manganous ion, compounds of the m-aminobenzylthiazolium chloride type and of their interrelation. It was also necessary to gain a better understanding of the nature of the destructive reaction, particularly in relation to the products from the initial destruction. It was the purpose of this investigation to establish the necessary information concerning these questions with the hope of bringing the final question nearer to solution.

III. EXPERIMENTAL

A. Methods

1. Preparation of acetone-desiccated fish powder

For convenience and reproducibility of results, acetone-desiccated powder was used throughout this work as a source of the thiamine destroying enzyme. These powders were prepared by the method of Sealock, et al, (99). The fresh frozen carp were opened and the viscera and gills separated from the swim bladder, fat and eggs. The clean viscera and gill mass was ground in a meat chopper, and the resultant viscous liquid treated with 6 volumes of ice-cold acetone by mixing in a Waring blender. The proportions used were 50 gms. of minced material to 300 ml of acetone. The combined suspensions were stirred frequently in the cold acetone over a period of thirty minutes. At the end of this time, the insoluble material was filtered off and crumbled into 4 volumes of cold acetone. The insoluble portion was again separated by filtering and broken up and spread in a thin layer to air dry. The last traces of acetone were removed from this powder by drying in a vacuum desiccator over phosphorous pentoxide. When thoroughly dry, the powder was transferred to a brown-glass bottle and stored in the ice

box. About 0.16 gms. of powder were obtained per gram of viscera. Prepared and stored in this manner, it will maintain its activity up to 18 months.

The activity of each powder was determined in respect to the unit defined by Sealock, et al, (99) as that amount which under standard assay conditions, described below, will cause the disappearance of one micromole of thiamine. This unit activity for a given preparation varies with concentration for a given powder.

2. Preparation of enzyme extracts and method of assay

Extracts of the acetone-desiccated powder were prepared according to the directions of Sealock, et al, (99). A weighed amount of powder was added to a 0.2 M. sodium and potassium phosphate- 10% sodium chloride buffer adjusted to pH 7.4. After standing for thirty minutes, with frequent stirring, the mixture was centrifuged and decanted through cotton into a graduated cylinder. The residue was further extracted with 3 more portions of buffer and the combined centrifugates diluted to the desired volume with buffer. The properly diluted enzyme solution was again adjusted to pH 7.4 before use.

In experiments involving the isolation of the intermediates of the enzymatic destruction of thiamine, the above procedure was inconvenient because of the high concentration

of the enzyme solutions. This difficulty was obviated by blending the powder and buffer in a Waring blendor, adjusting the resulting mixture to the proper volume and pH, and using it without attempting centrifugation and filtration. Extracts prepared in this way invariably had a higher activity than those prepared in the usual manner.

For assay, 2 ml. of the extract were mixed with 2 ml. of water and 1 ml. of a thiamine solution containing 2.5 micromoles of thiamine. This mixture was then incubated at 37° C. for 2 hours. At the end of this time the protein was precipitated by the addition of an equal volume, 5 ml., of 10% trichloroacetic acid; 2 N. hydrochloric acid or 95% ethanol may also be used. Controls were prepared in the same manner, except that the trichloroacetic acid was added immediately after the thiamine solution and there was no incubation period.

After standing for thirty minutes to assure complete flocculation of the protein, the mixture was filtered through Whatman #1 filter paper and the clear filtrate used for assay by the Melnick and Field method as described below.

In experiments involving the addition of other constituents the water was partly or totally eliminated. In every instance, however, the final incubation volume was 5 ml. and the mixture 0.08 M. in buffer.

3. Melnick and Field assay for thiamine

The colorimetric method of Melnick and Field (69) was used for the analyses of thiamine. A stock solution of p-aminoacetophenone was prepared by dissolving this compound in 1 N. hydrochloric acid to give a 0.05 M. solution. Five ml. of this reagent were cooled to 0-5° C. in an ice bath, and to it was added, with stirring, 5 ml. of a freshly prepared, cooled 4.5% solution of sodium nitrite. The mixture was stirred in an ice-bath for ten minutes and then 20 ml. more cold nitrite solution added and stirring continued for twenty minutes more. At the end of this time, 20 ml. of the cold diazo solution were withdrawn and added to 275 ml. of a cold solution of sodium hydroxide-sodium bicarbonate buffer (0.5 M. and 0.35 M. respectively), and the resulting solution was stirred for thirty minutes. The reagent was then ready for use. It was made fresh every day but can be safely used up to three or four hours after preparation if stored at 0-5° C.

For the analysis of thiamine, 2 ml. of the protein-free filtrate, described above, were transferred to a 40 ml. centrifuge tube and 10 ml. of an alcohol-phenol solution (2.5 gms. of phenol per liter of 50% ethanol) were added. Then 10 ml. of the diazotized p-aminoacetophenone solution were run in with gentle but thorough mixing. The mixture was allowed to stand at room temperature for at least thirty minutes to

insure complete development of color. After the allotted time the red-violet dye formed was extracted from the water by vigorous shaking with 15 ml. of xylene. The xylene-water mixture was then centrifuged and the xylene layer, containing the dye, was drawn off and read in the Klett-Summerson photo-electric colorimeter with filter #520. The instrument was of course first zeroed with solvent.

The following equation could then be used to calculate the amount of thiamine present:

$$\text{Thiamine, micromoles} = \frac{\text{Reading, Incubated}}{\text{Reading, Zero Time}} \times 0.5 \times \frac{\text{Total Volume}}{\text{Aliquot Volume}}$$

This was usually expressed as the per cent thiamine destroyed, i.e.,

$$\text{Thiamine destroyed, \%} = \frac{\text{Thiamine added} - \text{Thiamine present}}{\text{Thiamine added}} \times 100$$

4. Analytical method for determination of manganese

The method adopted here was a combination of that reported by Richards (119) and one used by Skinner and Peterson (120). This was necessary to eliminate the interference of the high concentration of chloride ion present in the saline extracts.

The powder, or saline extract evaporated to dryness, was ashed in a muffle at cherry red heat and the sodium chloride removed by two evaporations with 5 ml. of 33% sulfuric acid

which had previously been boiled for twenty minutes with potassium periodate. The resulting solid, semi-transparent mass was then treated with 5 ml. of 85% phosphoric acid and 40 ml. of water and placed on a steam bath to digest for thirty minutes to insure thorough extraction. Most of the mass dissolved. The mixture was cooled and filtered into a 50 ml. volumetric flask and diluted to volume. A test for chloride ion proved negative.

Aliquots of this solution were then taken and diluted further to known volumes for oxidation of the manganese to the permanganate ion. To each was added 15 ml. of a mixture of 5 ml. of 85% phosphoric acid and 45 ml. of water and then 0.3 gms. of potassium periodate. The resulting solution was heated to boiling until maximum pink color had developed (usually ten to fifteen minutes), diluted to volume, and heated five minutes more. These solutions were read in the Klett-Summerson photoelectric colorimeter (filter 520) which had previously been zeroed with the solvent treated in like manner. The readings were then converted into milligrams of manganese by comparison with standard solutions of known permanganate concentration which had been reduced with sulfite and reoxidized by the above method. The chart for the standard solutions, showing a straight line function of manganese concentration vs. color value, is shown in Figure 3.

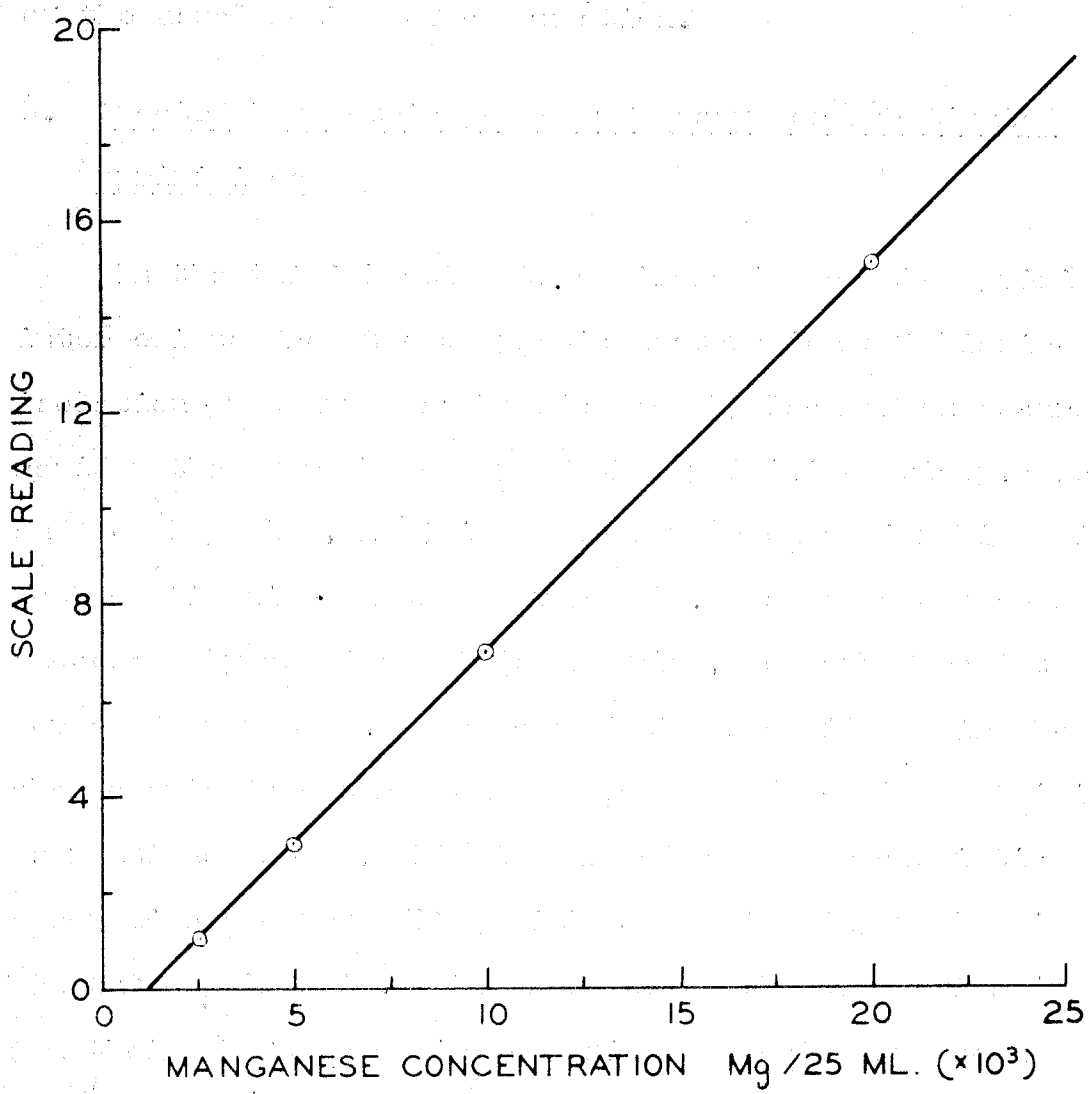


Fig. 3. Interpolation graph for determination of manganese concentration in thiaminase preparations. Standard solution of potassium permanganate reduced by sodium sulfite and oxidized to permanganate by method described in text and aliquots diluted to concentrations indicated. Read in Klett-Summerson Photoelectric colorimeter with filter 520.

5. The Bratton-Marshal test for aromatic amines

In the experiments involving activation of the enzyme by m-nitroaniline, it was desirable to know the loss of the aromatic amine during incubation, as well as the loss of thiamine in order to compare their disappearance. The loss of the aromatic amine was determined by the Bratton and Marshall test (121).

These reagents were required: 0.5 N. hydrochloric acid, 0.1% solution of sodium nitrite freshly prepared, 0.5% solution of ammonium sulfamate, and a 0.1% solution of N-(1-naphthyl)-ethylene diamine.

Two ml. of the protein-free trichloroacetic acid filtrate were diluted to 10 ml. with water and 2 ml. of the diluted solution, or an appropriate aliquot, were placed in a 8 in. test tube. To this was added 10 ml. of the hydrochloric acid solution and then 1 ml. of the nitrite solution. After thorough shaking the mixture was allowed to stand three minutes and then 1 ml. of the ammonium sulfamate solution was added to destroy the excess nitrous acid. Again the mixture was shaken and allowed to stand for two minutes when 1 ml. of the N-(1-naphthyl)-ethylenediamine solution was added. The color was allowed to develop for at least one hour, and then the color value was read in the Klett-Summerson photoelectric colorimeter which had previously been zeroed against a blank containing all of the above reagents except the trichloroacetic

acid filtrate. This was replaced by an equal volume of 5% trichloroacetic acid.

The amount of amine present could then be calculated from a comparison with a standard of known concentration by means of the usual method of calculation.

6. Spectrophotometric method of assay for substituted 6-amino-pyrimidines

In the initial attempts at isolation of the pyrimidine intermediate from the enzymatic destruction of thiamine the isolation procedure was handicapped by lack of an accurate and rapid method for the assay of the pyrimidine moiety. Krampitz and Woolley (103, 104) in their work used a microbiological method utilizing Endomyces vernalis. As we were unable to successfully cultivate this organism, we were forced to look for another method. Such a method was available in the characteristic absorption spectra of the 6-aminopyrimidines. As mentioned above, thiamine also has a characteristic absorption spectrum (122) with two maxima in an alkaline media and but one in acid. The presence of the two maxima in alkali and their shift to a single maximum in acid have been attributed to the presence in the molecule of the 6-aminopyrimidine nucleus (122, 123). This shift in absorption spectrum with change of pH is qualitatively true of all the 6-aminopyrimidines, and as this spectral shift is very characteristic for this

type of molecule (122-126), we felt that this could be used as an assay method instead of the absorption spectra as employed by Tinker and Brown (127) for the purines and pyrimidines.

One difficulty arising here is the close similarity of the spectra of variously substituted 6-aminopyrimidines. This, however, does not detract from the method as one for attempting to follow this type of molecule in an isolation. It merely denies the possibility of its use as an identification test for a particular one.

The method employed then was to dilute with buffer an aliquot of the solution to be tested to such a volume that it was approximately in the order of 2×10^{-5} M. with respect to the pyrimidine portion and to adjust the pH of this solution to 8.0. A complete ultra-violet spectrum reading (215-350 mu) of the solution was then made in the Beckman spectrophotometer. Because of the high background arising from the protein breakdown products in the incubated solutions, it was also necessary to make a similar reading of control solution containing only the incubated enzyme solution. These control values were then deducted from the above readings and the delta values were plotted and investigated for the characteristic 6-aminopyrimidine absorption spectrum. If this was evident, a further check could be made by adjusting the solutions to pH 5.0 and investigating the solution for the shift to a single maximum

characteristic for these compounds.

In some cases, a purification of the solution by absorption on acid-activated Folin Decalso and elution with 1 N. hydrochloric acid- 25% potassium chloride solution was accomplished by a procedure in use in the laboratory in other connections.[1]

B. Isolation and Identification of N-(2-methyl-6-aminopyrimidyl-5-methyl)-m-nitroaniline

1. Isolation from a m-nitroaniline activated reaction

Sealock and Livermore (116) have shown that certain compounds such as m-aminobenzyl-4-methylthiazolium chloride activate the enzymatic destruction of thiamine, and that these compounds, like thiamine, decreased in concentration during the course of the reaction. This fact, coupled with the evidence that another component than the enzyme protein and the manganous ion was necessary for the enzymatic system, led to the hypothesis that these m-amino analogues functioned in the system as "acceptors" of the pyrimidine moiety of thiamine as the latter was split by the enzyme. They thus increased the velocity of the decomposition of the enzyme-substrate complex, i.e.,

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[1] Sealock, R. R., and White, H. S., Unpublished data.

$$ES + H_2NR \rightleftharpoons E + \text{Pyrimidine-N(H)-R} + \text{Thiazole} + H^+.$$

If this was the case, then the similarity of structure of these compounds to thiamine might not be as important to their function as the presence of their activated amino group. To test this hypothesis, m-nitroaniline and m-aminobenzoic acid were chosen as logical activators of the system. The enzyme extract was prepared as described above and after adjustment to pH 7.4 and dilution to the desired volume, 2 ml. aliquots, representing or equivalent to the weight of powder indicated, were used. Water and the activator solutions were added to make a total volume of 4 ml.; 1 ml. of 2.5×10^{-3} M. thiamine (2.5 micromoles) solution was added and the solution incubated for two hours at 37.5° C. The reaction was stopped with the addition of 5 ml. of 10% trichloroacetic acid and the thiamine remaining determined by means of the Melnick-Field method and the aromatic amine by the Bratton-Marshall test -- comparison in each case being made with an identical unincubated solution. Incubation of the thiamine and the amine under identical conditions showed no measurable loss of either compound. The activation was calculated in the usual manner.

Activation of 78 and 114% were measured with 2.5 and 5.0 micromoles of m-aminobenzoic acid per assay tube and 91 and 107% activation for similar levels of m-nitroaniline when

there were 50 mg.-equivalents of fish powder present. If but 25 mg.-equivalents of the powder were used with the lower level of reactor, activations of 185 and 258% were found for m-aminobenzoic acid and m-nitroaniline respectively. These results are tabulated in Table IV. In Table V the results of

Table IV

Activating Effect of m-nitroaniline and m-aminobenzoic Acid on Undialyzed Enzyme Preparations

Compound Added	Concentration micromoles	Thiamine Destroyed micromoles	Amine lost micromoles	Activation per cent
None		1.12		
m-amino-benzoic acid	2.5	2.00	0.94	78.0
"	5.0	2.40	1.51	114.0
m-nitroaniline	2.5	2.15	1.11	91.0
"	5.0	2.32	1.53	107.0
None*		0.41		
m-aminobenzoic acid	2.5	1.18	0.63	185.0
m-nitroaniline*	2.5	1.48	0.90	258.0

*Indicates 25 mg.-equivalents of fish powder. Otherwise 50 mg.-equivalent.

activation with graded amounts of m-nitroaniline are shown. In this experiment the lower level of powder was used and the thiamine concentration was 2.5 micromoles per assay tube throughout while the concentration of the reactor was varied from 0.25 to 5.0 micromoles as indicated. The destruction increased regularly from 18% at the lowest level of m-nitroaniline up to 88% at the highest. The ratio of micromoles reactor lost to micromoles of thiamine destroyed also increased regularly from 21% up to 80%.

Table V

Fish Powder III-110 at 25 mg.-equivalent Level Per Assay Tube

m-nitroaniline Added micromoles	Thiamine Destroyed		m-Nitroaniline Lost		Ratio*
	per cent	micro-moles	per cent	micro-moles	
0	18.0	0.45	---	---	
0.25	32.5	0.81	67.3	0.17	0.21
0.50	41.7	1.04	66.3	0.33	0.32
1.00	55.0	1.37	66.3	0.66	0.48
2.00	73.2	1.83	58.0	1.16	0.64
2.50	77.7	1.94	53.7	1.34	0.69
5.00	88.6	2.22	35.4	1.78	0.80

*Ratio is for micromoles m-nitroaniline lost/micromole thiamine destroyed.

With the demonstrated activating effect of these two amines and their loss during the incubation, it became important to test their postulated mode of action by attempting to isolate the proposed intermediate from an activated incubation mixture. Examination of the results in the preceding experiments indicated that this "unnatural" reactor might be competing with some natural ones, as evidenced by its increased loss with increasing concentration. Logically then, the most promising conditions for isolation of the "unnatural pyrimidine intermediate" would be those which included a high concentration of the added reactor so that it could overwhelm the reactors already present and give a high yield of the desired intermediate. The m-nitroaniline reactor was chosen for the isolation experiment because of its yellow color which might facilitate the isolation procedure by affording a visual method of following the fractionation.

The enzyme preparation for this experiment was made by extracting 25 gms. of carp powder III-110 with 10x50 ml. portions of phosphate buffer. The combined, filtered extracts were then diluted to 1800 ml. with buffer and transferred to a 10 l. precipitation jar fixed in a constant temperature bath. To the extract was added slowly a solution of 6.9 gms. ($5x10^4$ micromoles) of m-nitroaniline in 2 l. of water containing sufficient hydrochloric acid to dissolve the compound. Additions of the amine solution were alternated

with additions of 2 N. sodium hydroxide (total of 70 ml. added), so that the mixture was maintained between pH 6.0 and 8.0 at all times. The final mixture, pH 7.6, was allowed to come to 37.5° C. and a solution of 1.685 gms. ($5x10^3$ micromoles) of thiamine in 1 l. of water was added to give a total volume of 4,870 ml. The solution was thoroughly mixed, and a 3 ml. aliquot was quickly removed for zero time assay. Then 130 ml. of buffer were added to bring the total volume to 4,997 ml. and the mixture was stirred and incubated at 37.5° C. for four hours. At the end of this time the mixture was removed from the bath and the reaction stopped by the addition of 2 l. of 1.5 N. hydrochloric acid. The precipitated protein was allowed to flocculate over a period of two hours and then filtered off by gravity filtration. A 10 ml. aliquot removed from the clear, dark-brown filtrate was assayed by the Melnick-Field method and indicated the destruction of 73.3% (3.67 millimoles) of thiamine.

The deproteinized filtrate was made alkaline (pH 8.5) by the addition, with stirring, of a saturated sodium hydroxide solution. The alkaline filtrate was then extracted with 2x1 l. and 14x500 ml. of n-butanol. The butanol extracts were centrifuged to remove emulsified material and the clear centrifugates combined and concentrated under vacuum at 60-65° C. to approximately 1.5 l. During the concentration acetic anhydride (3 ml. to every 500 ml. of butanol centrifugate) was

added inadvertently to the concentration flask. The concentration was continued with frequent addition of water (total of 5 l. added) to avoid bumping and to displace the butanol. The final water concentrate, containing a yellow precipitate, was again made alkaline (pH 8.0) by the addition of 6 N. sodium hydroxide and transferred to a separatory funnel. It was then exhaustively extracted with benzene until very little yellow color was evident in the benzene extracts (2x500 ml. and 8x100 ml. portions). The clear, yellow benzene extracts were combined and evaporated to dryness under an air stream and designated as fraction 1.

The benzene-extracted water layer was next extracted with chloroform (1x250 ml. and 5x100 ml. portions) and the chloroform extracts, along with the yellow solid material suspended in it, were combined and evaporated to dryness as fraction 2.

The doubly-extracted water layer, fraction 3, was discarded after it was tested and found to contain none of the desired material.

Fractions 1 and 2 were each treated with 5 ml. of 6 N. hydrochloric acid and the insoluble material filtered off and combined as were the filtrates containing the hydrochloric acid soluble materials. The hydrochloric acid insoluble residue was dissolved in the minimum amount of absolute ethanol, cooled and filtered. The crystalline material which

had formed on cooling (2.75 grams) melted at 154° C. and proved to be m-nitroacetanilid. A further yield (3.2 grams) was recovered by concentrating and cooling the filtrate from the first crop. The final filtrate when evaporated to dryness yielded 0.40 gms. of impure m-nitroaniline melting at 149-150° C.

The hydrochloric acid soluble material was recovered by concentrating the combined hydrochloric acid filtrates and cooling the concentrate in an ice bath. In this way 0.9 gms. (74.5% yield) of crystalline material was obtained which melted, with decomposition, at 211-212° C. (corrected), and which was later shown to be the dihydrochloride of N-(2-methyl-6-aminopyrimidyl-5-methyl)-m-nitroaniline. The free base, which crystallized on addition of alkali to the acid solution, was a yellow crystalline material and melted at 227.5-228.5° C. (corrected). These melting points are identical with those obtained with the above secondary amine synthesized as described below.

The proof that the compound was the dihydrochloride salt was obtained by electrometric titration of a solution of the material with alkali. The titration curve is shown in Figure 4. Thirty milligrams of the compound required 18.50 ml. of 0.01017 N. sodium hydroxide for neutralization. This corresponds to 22.08% HCl whereas the theoretical value for the above dihydrochloride is 21.97% on the basis of a

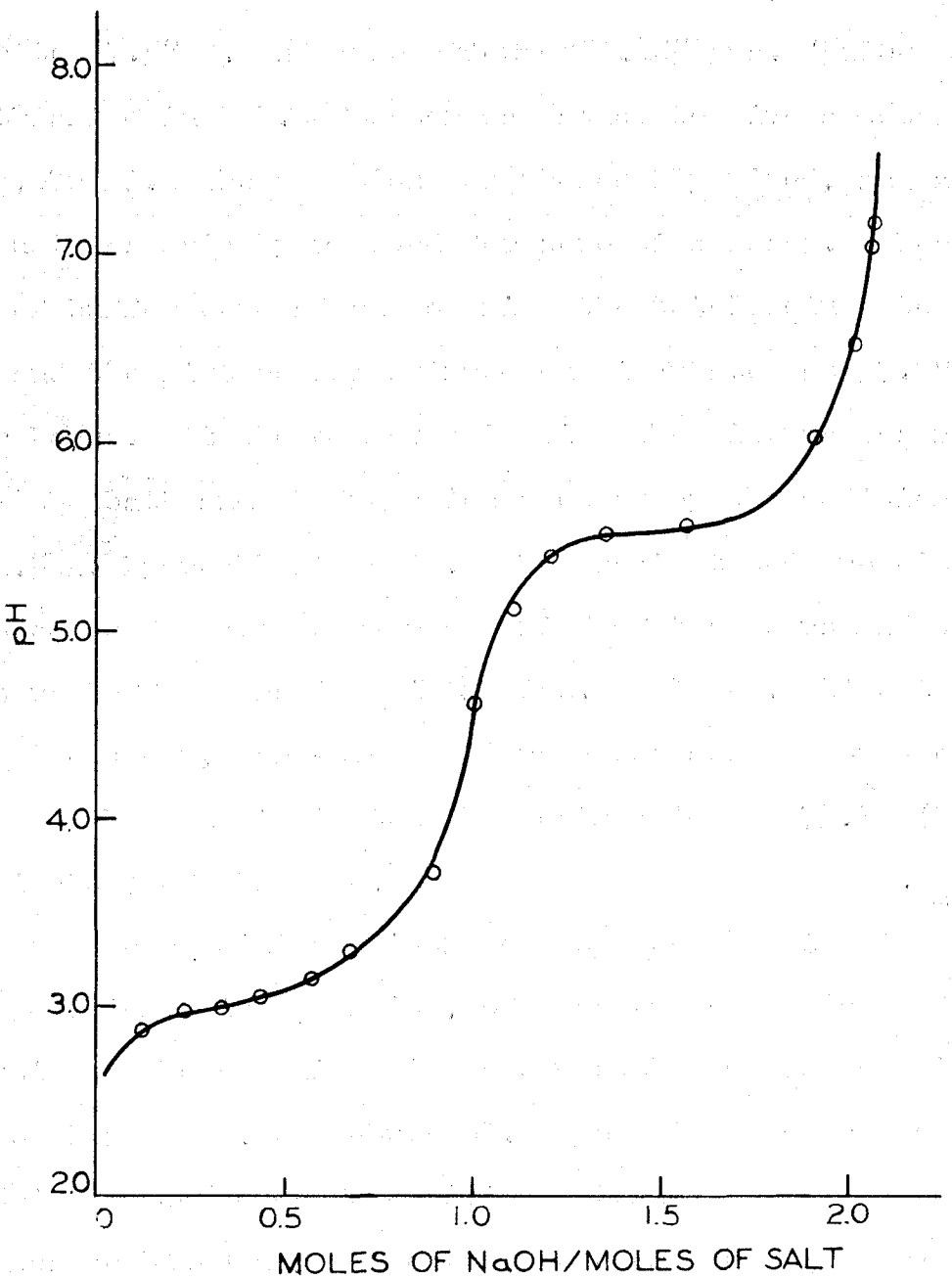
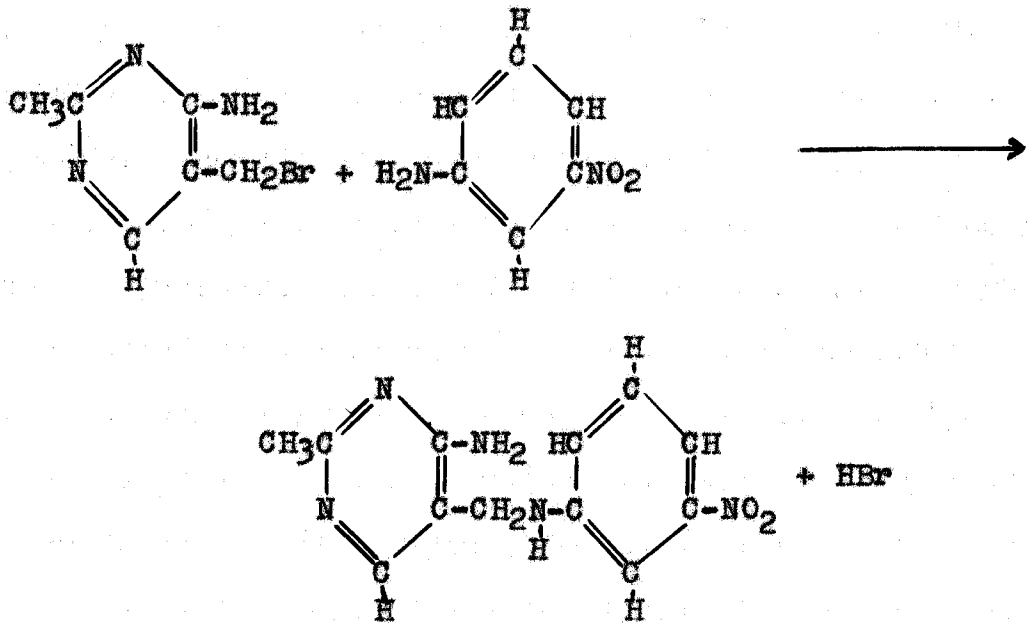


Fig. 4. Titration of N-(2-methyl-6-aminopyrimidyl-5-methyl)-m-nitroaniline dihydrochloride with NaOH.

molecular weight of 332.2 gms. The pKb values as taken from the titration curve are pK_1 = 8.45 and pK_2 = 11.0.

2. Synthesis of N-(2-methyl-6-aminopyrimidyl-5-methyl)-m-nitroaniline

To verify the identity of the material isolated from the enzymatic mixture, this compound, pictured below, was synthesized as shown in the accompanying scheme:



The 2-methyl-6-amino-5-bromomethylpyrimidine (3.63 gms., 0.01 moles) was added in portions, with shaking, to a solution of m-nitroaniline (5.52 gms., 0.04 moles) in 100 ml. of 95% ethanol. The mixture was acid to congo, so an excess of sodium bicarbonate (3.36 gms., 0.04 moles) was added to the warmed mixture until the solution was faintly alkaline to litmus. The mixture was then warmed for thirty minutes and filtered to remove precipitated sodium bromide and excess

sodium bicarbonate. The clear, orange-brown alcoholic filtrate was cooled in the ice-box for three days. The yellow-orange crystals which had formed were filtered off and air dried. The air dried product was washed with ether until no further yellow color showed in the ether washes and then dissolved in the minimum amount of 6 N. hydrochloric acid and the acid solution cooled in an ice bath. No precipitate formed, so concentrated hydrochloric acid was added dropwise until cloudiness developed and the solution again cooled in an ice bath for thirty minutes. A yellow-tan crystalline precipitate formed and was filtered off and air dried (1.16 gms., 35% yield). The melting point and mixed melting point were identical with the material obtained from the enzymatic mixture and their identity was further established by comparison of their ultraviolet absorption spectra in aqueous solution at pH 5.0 and 7.0 and in 0.1 N. sodium hydroxide (maximum at 2420-2430 A.). The absorption spectrum is reproduced in Figure 5.

C. Attempted Isolation of the "Natural" Intermediate

The isolation of the "unnatural" pyrimidine derivative from the m-nitroaniline activated enzyme mixture renewed our interest in isolating the "natural" derivative. This isolation had been previously attempted unsuccessfully in this laboratory. However, as mentioned before, subsequent work by

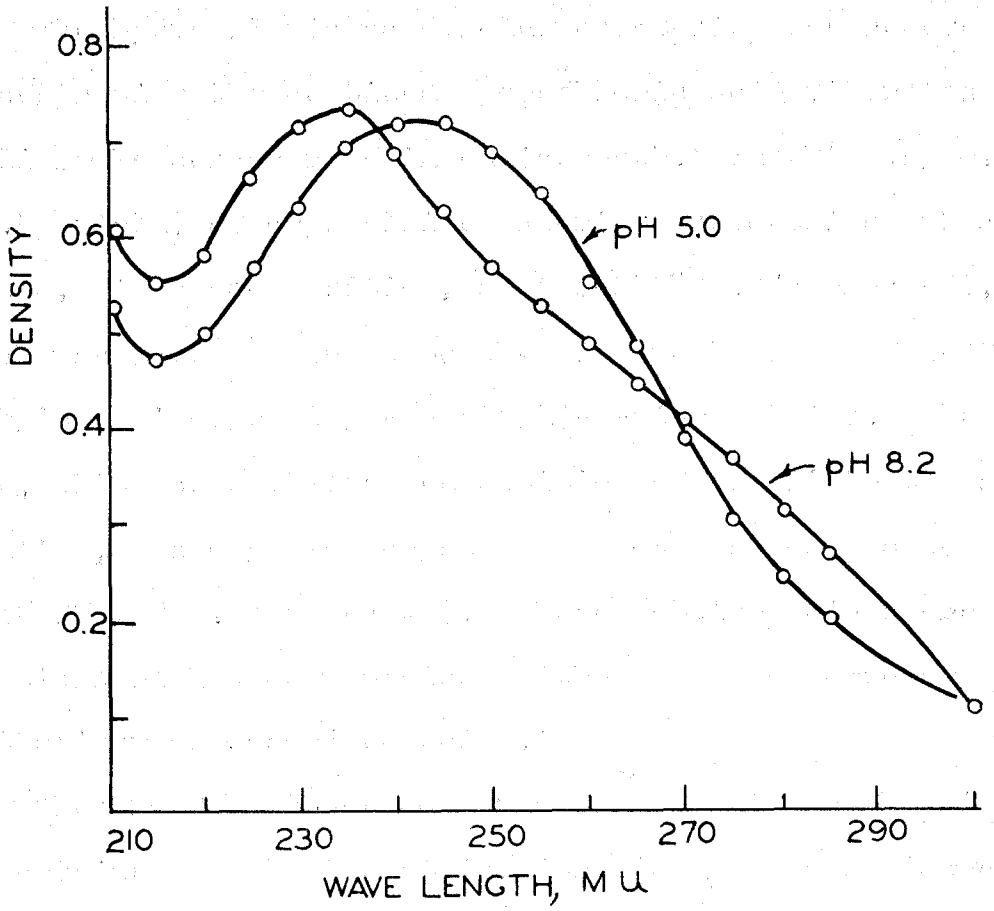


Fig. 5. Absorption spectrum of N-(2-methyl-6-aminopyrimidyl-5-methyl)-m-nitroaniline, 3×10^{-5} M. in 0.04 M. phosphate buffer.

Hennessy (111) and Tinker and Brown (127) suggested two useful methods for isolation of pyrimidine derivatives. These latter investigators applied the counter-current distribution method of Craig (128) to the difficult problem of separation of such pyrimidines as thymine, uracil, and cytosine. In this paper they stated (127, p. 585),

> Adequate criteria are not available for precise characterization of purines and pyrimidines. These compounds do not possess suitable melting points for precise characterization, do not readily form simple derivatives except salts, and nitrogen analysis are never adequate to detect the presence of small amounts of similar impurities. Even the characteristic ultra-violet absorption spectra are generally unsatisfactory owing to similarities of spectra and difficulties in detection of small contaminations of one by another.

Their work, however, demonstrated that the Craig counter-current distribution method was a convenient and precise one for the qualitative and quantitative characterization of these individual compounds and of simple mixtures. This paper presented a good assay method, if only qualitative, for following the 6-aminopyrimidine derivatives in any fractionation technique. That is, as pointed out previously, the characteristic two peaked absorption spectrum of this type of compound and its shift to one peak in acid media. It also offered a means of purification, or proof of purity, of any such compound isolated.

The first attempted isolation was a combination of Hennessy's silver salt precipitation method (111) and a

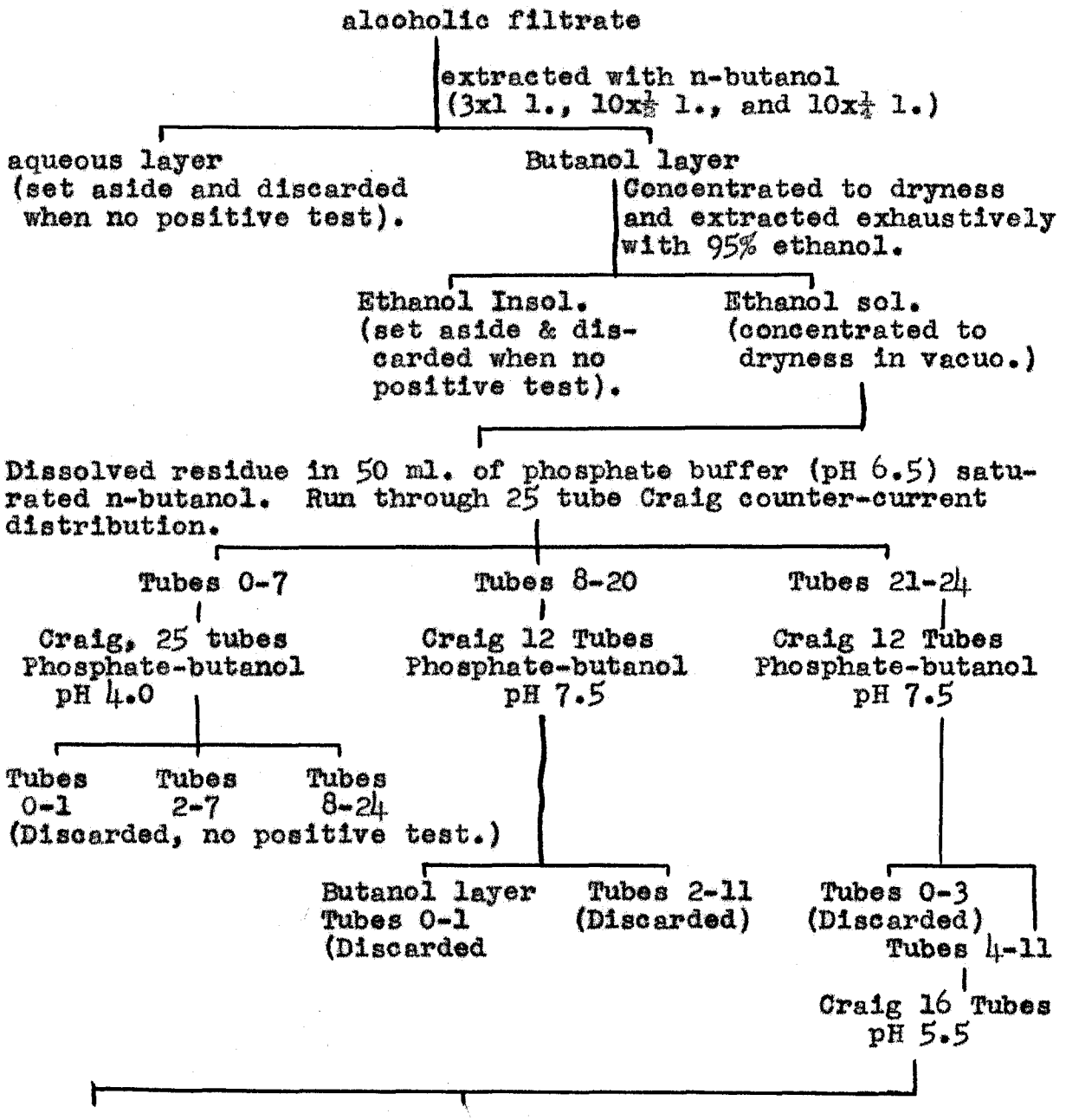
primary purification through extraction of the alkaline reaction mixture with n-butanol.

The butanol extracts were evaporated to dryness and the residue, which remained after concentration, was then treated with hot 95% ethanol. The alcohol soluble material, after evaporation of the solvent, was dissolved in water and subjected to the action of silver nitrate. Silver salt precipitates were removed at pH 5.0 and 8.0. These two fractions, after decomposing the silver salt with hydrochloric acid, along with the mother liquor from the silver salt precipitates were assayed for presence of 6-aminopyrimidine but in none of them was there evidence of the desired compound.

The second method for attempted isolation was repeated some six times with minor variations in the procedure. In all cases there was a complete lack of success. The details of this method will not be given, rather a general plan of procedure is shown below in Figure 6. The initial method of treatment is the same as in the first procedure but instead of the silver salt precipitation the counter-current distribution method of Craig (128), as used by Tinker and Brown (127), was employed. This method seemed logical since work carried out by Sealock and White[1] in this laboratory indicated the pyrimidine intermediate was extractable from an alkaline

[1] Sealock, R. R., and White, H. S., Unpublished data.

Extract of powder II-29-D, 77.5 gms. blended in 614 ml. of 5% sodium chloride-0.1 M. phosphate buffer. Adjusted pH 7.4 and brought to 37.5° C. in water bath, (20 ml. removed for control) Added 1 gm. of thiamine, (2.97×10^3 micromoles) in 891 ml. of water. Incubated 4 hours. Reaction stopped by addition of an equal volume, 1484 ml., of 95% ethanol. Cooled and filtered.



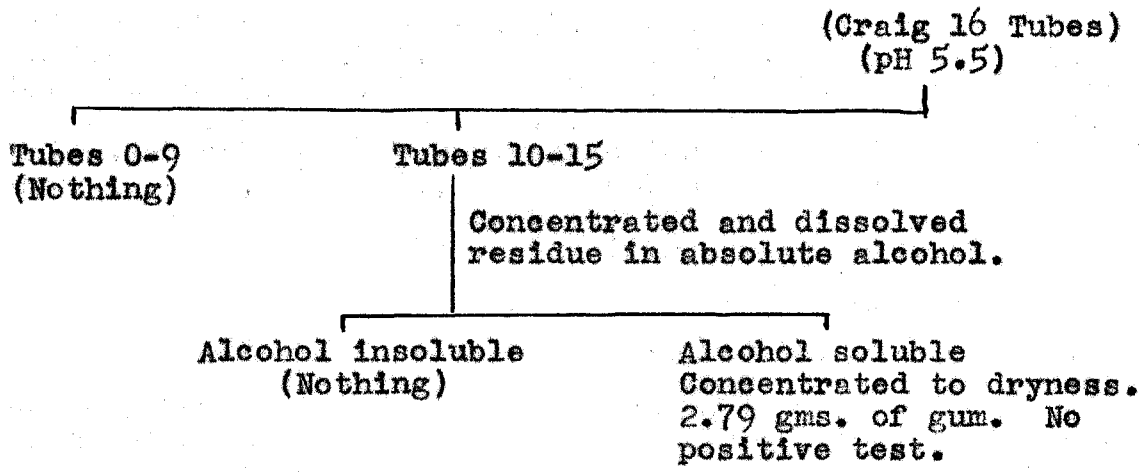


Figure 6. Scheme for isolation of "natural" pyrimidine intermediate.

aqueous solution by n-butanol. The counter-current distribution of this intermediate between n-butanol and phosphate buffer at various determined hydrogen ion concentrations was therefore expected to permit purification and isolation of this intermediate.

The main variation was a purification step in some of the experiments in which a previously mentioned procedure worked out by Sealock and Sarver was used. This purification was achieved by adsorption of the desired material on acid-activated Folin's Decalso and elution by means of a 1 N. hydrochloric acid-25% potassium chloride solution. This technique, employed on the aqueous solution of the butanol extracted material, showed great promise on small scale pilot-runs as indicated by the absorption spectrum assay. However, on a large scale, necessary for a sufficient amount of material to work with, difficulty was encountered in neutralization of the eluate for subsequent treatment. Upon treatment with alkali, a copious, gelatinous precipitate of alumina invariably formed, and the clear centrifugate or filtrate from this precipitate showed no evidence of 6-amino-pyrimidine in any of the experiments. In the later attempts this step was omitted as we felt it might be responsible for the loss of the desired material by adsorption on the alumina. Instead the Craig counter-current distribution techqniue, as used by Tinker and Brown, was employed directly on the butanol

soluble material. The immiscible pair used was 1 M. sodium phosphate-potassium phosphate buffer at pH 6.5, or as indicated, and n-butanol; the two being mutually saturated. Assay, as before, was made by means of the ultraviolet absorption spectrum at pH 8.0 and 5.0, using the Beckman Spectrophotometer. In only one instance was there evidence of the sought for 6-amino pyrimidine absorption spectrum, and in this instance the amount of material isolated was too small for further manipulation.

D. Dialysis and Activation of the Enzyme Preparations

Sealock and Livermore (99) pointed out in a previous paper that the enzyme system is dialyzable, and that two metallic ions, manganous and cobaltous, can partially reactivate the dialyzed system. Other ions, such as iron, tin, nickel, etc., were found to have no effect or to be inhibitory.

In the hope of characterizing the components of this enzyme system and thereby making possible its purification, and perhaps crystallization, this aspect of the problem was given considerable attention.

Even in the initial work in this respect some puzzling features arose. Whereas the above workers obtained almost complete loss of activity on dialysis of the enzyme extract for from four to eight hours, we found the loss of activity by dialysis to be quite variable for different powder-extracts,

and even for different extracts of the same powder. Seldom was there more than approximately 50% loss of activity even on prolonged dialysis. In addition to these peculiarities, the effect of added manganous ion showed a similarly variable action. In neither respect could there be found a correlation between the loss of activity on dialysis, or activation by manganese, and the activity of the extract. This led us to consider the importance of the conditions of dialysis. All dialysis experiments were carried out at low temperatures, 0-10° C., and since little difference could be found between static dialysis and one in which the dialyzing solution was constantly stirred, the majority of the cases used the more convenient static conditions.

The first variable taken into consideration was that of pH of the dialyzing solution. In Table VI the results from 2 such experiments are tabulated.

Obviously the optimum pH for dialysis is 6.5-7.5, since in this range there is a minimum loss of activity of the enzyme on standing, and the greatest loss of activity due to dialysis.

Table VI

Extract of III-A Dialyzed at 50 mg. Level for 7 Hours Against Distilled Water. Assayed at 100 mg. Level.

pH of treatment	Thiamine Destruction in Per Cent	
	Undialyzed	Dialyzed
5.5	50.0	28.6
6.5	79.0	42.0
7.4	79.0	44.0

(Activity of original extract - 86.5% destruction of thiamine.)

As above but dialyzed 2 hours against phosphate buffer at 15 mg. level and assayed at 30 mg. level.

4.0	15.3	11.5
5.0	16.5	14.6
6.0	26.5	13.3
7.0	34.5	20.0

(Activity of original extract - 34.5% destruction of thiamine.)

The second variable altered was the concentration of the enzyme extract to be dialyzed. From the above table and from Table VII below, it can be seen that dialysis of the more dilute preparation leads to greater loss of activity and greater activation by manganese.

The time variable was of little importance for periods past six to eight hours. At the end of eight hours there was the maximum loss of activity due to dialysis with the minimum of loss due to enzyme-protein denaturation. This point will

Table VII

Extract of III-A Dialyzed at: A. 100 mg., and B. 25 mg. Levels for Six Hours against Phosphate Buffer. Dialysate changed every half hour. Manganous ion concentration is $1x10^{-3}$ M in incubation tube.

Preparation		Thiamine Destroyed per cent	Thiamine Destroyed u moles	Activity per cent	Activation per cent
A.	U.D.	96.7	2.44	100.0	---
	D.	50.5	1.26	51.6	---
	D. + Mn	54.5	1.36	55.7	8.0
B.	U.D.	84.8	2.12	100.0	---
	D.	22.7	0.58	27.4	
	D. + Mn	55.0	1.38	65.1	138.0

U.D.: Undialyzed; D.: Dialyzed

be illustrated below in a time-activity dialysis experiment comparing the loss of activity to the loss of manganese. Accordingly, except as indicated for a few experiments, six or eight hours was chosen as the time of dialysis.

As mentioned before, Sealock and Livermore found 2 ions, the manganous and cobaltous ions, to be activators of the dialyzed enzyme preparations. In our work we found that concentrations of metallic ions higher than $1x10^{-3}$ M. in the incubation mixture led to interference and low values in the Melnick-Field assay method for thiamine. While in every case these readings were made against a control tube which had been treated in exactly the same manner as the incubated tubes, we still were somewhat hesitant about drawing any conclusions from such experimental values. Table VIII illustrates

this point.

Table VIII

Activation of Dialyzed and Undialyzed Extracts by Metallic Ions. Powder IIIA extracted in usual manner so that 1 ml. of extract is equivalent to 50 mg. of powder. Dialysis against distilled water for 8 hours at 5° C. and pH 7.4. Dialysate changed every half hour. All assays at 25 mg. level. Metallic ions added as chlorides at concentration indicated.

Preparation				Klett Summerson Reading Filter 520 Incubated/Zero time	Thiamine Destroyed per cent
Undialyzed				197/284	30.5
"	+ 2×10^{-3}	u moles	Mn^{++}	151/180	26.0
"	+ 2×10^{-2}	"	"	208/294	29.3
"	+ 2×10^{-1}	"	"	195/286	33.0
"	+ 2	"	"	196/292	34.0
"	+ 2x10	"	"	191/280	33.0
"	+ 2	"	Co^{++}	155/240	35.4
"	+ "	"	Ni^{++}	210/286	26.5
"	+ "	"	Fe^{+++}	226/284	20.5
"	+ "	"	Mg^{++}	196/286	21.4
"	+ "	"	Cr^{+++}	244/294	17.0
Stored				187/250	25.0
Dialyzed				230/284	19.0
"	+ 2×10^{-3}	u moles	Mn^{++}	193/222	13.0
"	+ 2×10^{-2}	"	"	210/256	18.0
"	+ 2×10^{-1}	"	"	230/274	16.0
"	+ 2	"	"	230/272	16.0
"	+ 2x10	"	"	238/272	13.0
"	+ 2	"	Co^{++}	158/180	12.0
"	+ "	"	Ni^{++}	224/250	11.0
"	+ "	"	Fe^{+++}	252/280	10.0
"	+ "	"	Mg^{++}	230/280	18.0
"	+ "	"	Cr^{+++}	270/288	6.0

In the experiments where the Melnick-Field values were low for the standards, there was always a precipitate formed on the addition of the manganous solution. As this limited the effective concentration range of added metallic ion, we investigated the possibility of another buffer system.

An acetic acid-sodium acetate buffer showed little interference with the assay method, even up to 1 M. concentration of the manganous ion, but the activity of the extract was considerably lower than with the phosphate or a veronal buffer, and therefore was not used again. The veronal buffer extract exhibited good activity but the interference with the color values was similar to that in the phosphate buffer, and since the latter was the more convenient, it was henceforth used. The results shown in Table IX indicate the activating effect of the manganous ion in these three buffers on the undialyzed extract.

Considering the possibility that phosphate concentration, by mass-action effect, might be responsible for the precipitation of the manganous phosphate and interference with the color values, the concentration of the phosphate buffer was varied in a series of experiments. The results shown in Table X for 0.067, 0.0016 and 0.0008 M. phosphate buffers for dialyzed preparations indicate that even at the lowest phosphate concentrations there was interference if the concentration of the manganese exceeds 1×10^{-3} M. in the incubation mixture.

Table IX

Manganous Ion Activation of Undialyzed Enzyme Preparations in (A) Sodium Chloride-Sodium Acetate buffer, and (B) Sodium Chloride-Veronal buffers.

Preparation	Klett-Summerson Readings	Thiamine micromoles	Destruction per cent	Activation per cent
A. Extract of powder IIIA by 0.1 N sodium acetate-10% sodium chloride solution so that 1 ml. of extract is equivalent to 25 mg. powder. 2.5 umoles thiamine per tube; Mn^{++} as indicated. Incubated 2 hrs., 37.5° C., at pH 7.4. Assayed at 25 mg. equivalent level.				
Original Extract	270/325	0.42	17.0	----
" $1x10^{-4}$M in Mn^{++}	270/325	0.42	17.0	00.0
" $1x10^{-3}$M in "	240/310	0.58	23.0	38.0
" $1x10^{-2}$M in "	220/300	0.68	27.0	62.0
B. Extract of II-196 in 0.02M Veronal-10% sodium chloride buffer. Otherwise, same as above.				
Original Extract	238/325	0.93	37.0	----
" $1x10^{-4}$M in Mn^{++}	238/330	0.95	38.0	1.0
" $1x10^{-3}$M in "	168/310	1.15	46.0	24.0
" $1x10^{-2}$M in "	52/166	1.73	69.0	86.0

Table X

Effect of Phosphate Concentration on the Activation of Dialyzed Enzyme Preparations by the Manganous Ion

Extract of powder IIIA by (A) 0.2M phosphate-10% sodium chloride buffer, (B) 0.067M phosphate-10% sodium chloride buffer, (C) 0.0016M phosphate-10% sodium chloride buffer and (D) 0.0008M phosphate-10% sodium chloride buffer. (A) and (B) dialyzed at 150 mg. level of powder, and (C) and (D) at 25 mg. level. Dialyzed 6 hours at 0-5° C., pH 7.4, static conditions changing dialysate every one-half hour. All assayed at 75 mg. level with addition of Mn^{++} as indicated.

Preparation	Addition molarity of Mn^{++}/ tube	Klett-Summerson Readings 2 hrs. inc./ zero time	Thiamine Destruction per cent	Activation per cent
A. Undialyzed	none	150/315	52.4	
Dialyzed	"	214/300	28.7	
"	$4x10^{-5}$	214/300	28.7	
"	$4x10^{-4}$	214/310	31.0	4.5
"	$4x10^{-3}$	138/148	16.8	-22.8
B. Undialyzed	none	146/305	52.0	---
Dialyzed	"	222/305	27.4	---
"	$2x10^{-4}$	226/300	25.0	-8.5
"	$4x10^{-4}$	214/305	30.0	8.5
"	$6x10^{-4}$	236/305	22.5	-18.0
"	$8x10^{-4}$	240/298	20.0	-27.0
"	$1x10^{-3}$	242/315	23.0	-16.0
"	$2x10^{-3}$	240/290	17.3	-37.0
"	$3x10^{-3}$	232/262	11.5	-58.0
C. Undialyzed	none	108/305	64.6	---
Dialyzed	"	268/310	13.5	---
"	$5x10^{-4}$	268/310	13.5	0.0
"	$1x10^{-3}$	254/305	16.8	25.0
"	$2x10^{-3}$	224/270	17.2	27.0
D. Undialyzed	none	138/300	54.0	---
Dialyzed	"	235/300	22.0	---
"	$5x10^{-4}$	234/305	23.2	5.0
"	$1x10^{-3}$	210/284	26.0	18.0
"	$1.5x10^{-3}$	142/248	42.8	94.0

In Figure 7 the results from several experiments are summarized to show the activating effect of the manganous ion on dialyzed enzyme preparations. Only those values in which the metal concentration is $1x10^{-3}$ M. or lower are given and in which there was no significant interference with the color development in the assay method. From these results, two facts are readily apparent; first, that the dialysis is more effective at the lower concentrations of enzyme, and second, though the activation by manganese is variable even in different preparations of the same powder, it is none the less real. Manganese is in some way associated with the mechanism of the enzymatic reaction.

These conclusions are also supported by results of experiments designed to study the interrelation of the manganous ion and m-nitroaniline as activators of dialyzed and undialyzed enzyme preparations. In the undialyzed preparations both activators exerted their greatest effect when initial activity was low. M-nitroaniline had by far the greater effect, and in every case except one the manganous ion increased this effect (Figure 8). In the dialyzed preparations the same situation was evident. In the one instance where complete inactivation by dialysis was achieved the manganous ion alone had no effect whereas m-nitroaniline exerted a very significant activation and this was materially increased by the addition of the metallic ion. These results

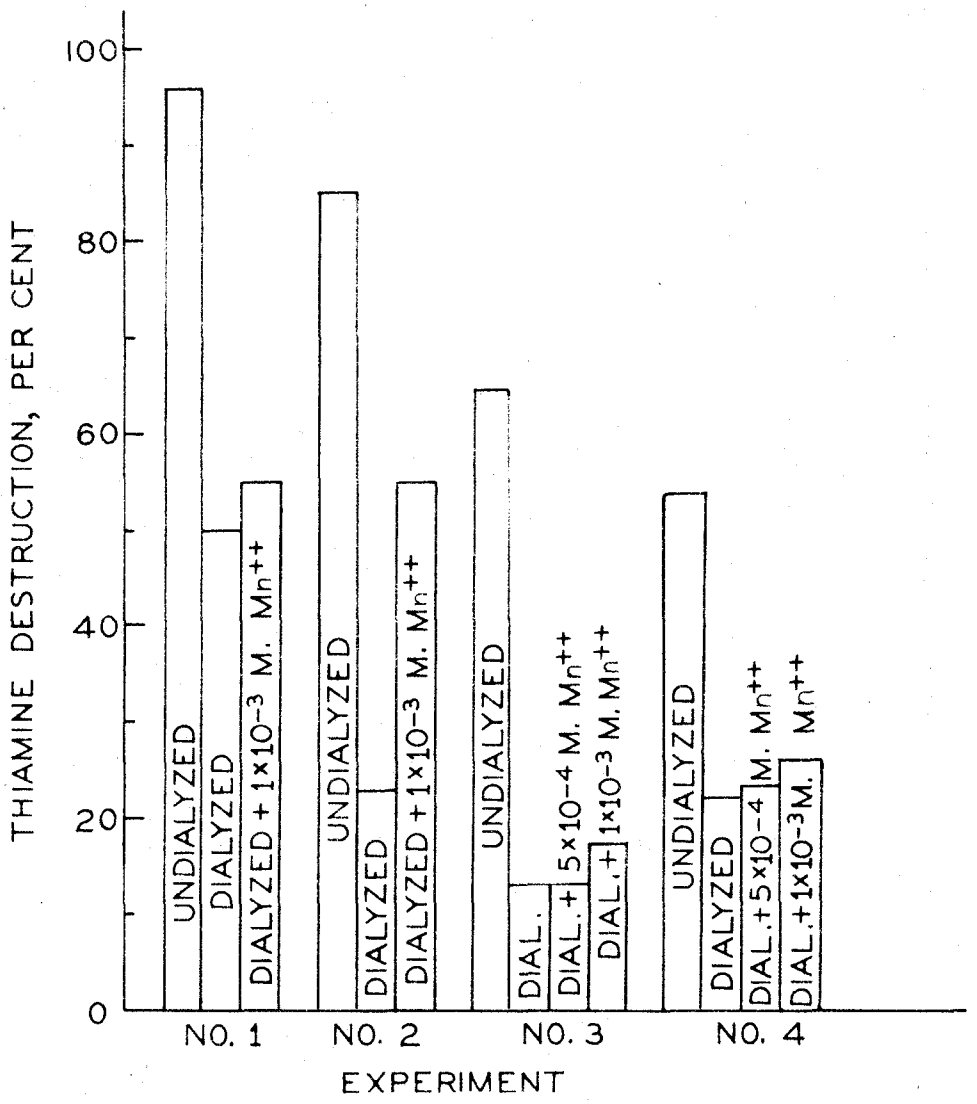


Fig. 7. **Manganese activation of dialyzed thiaminase preparations. Exp. 1. Dialyzed and assayed at 100 mg. powder level, all others dialyzed at 25 mg. and assayed at 75 mg. powder level.**

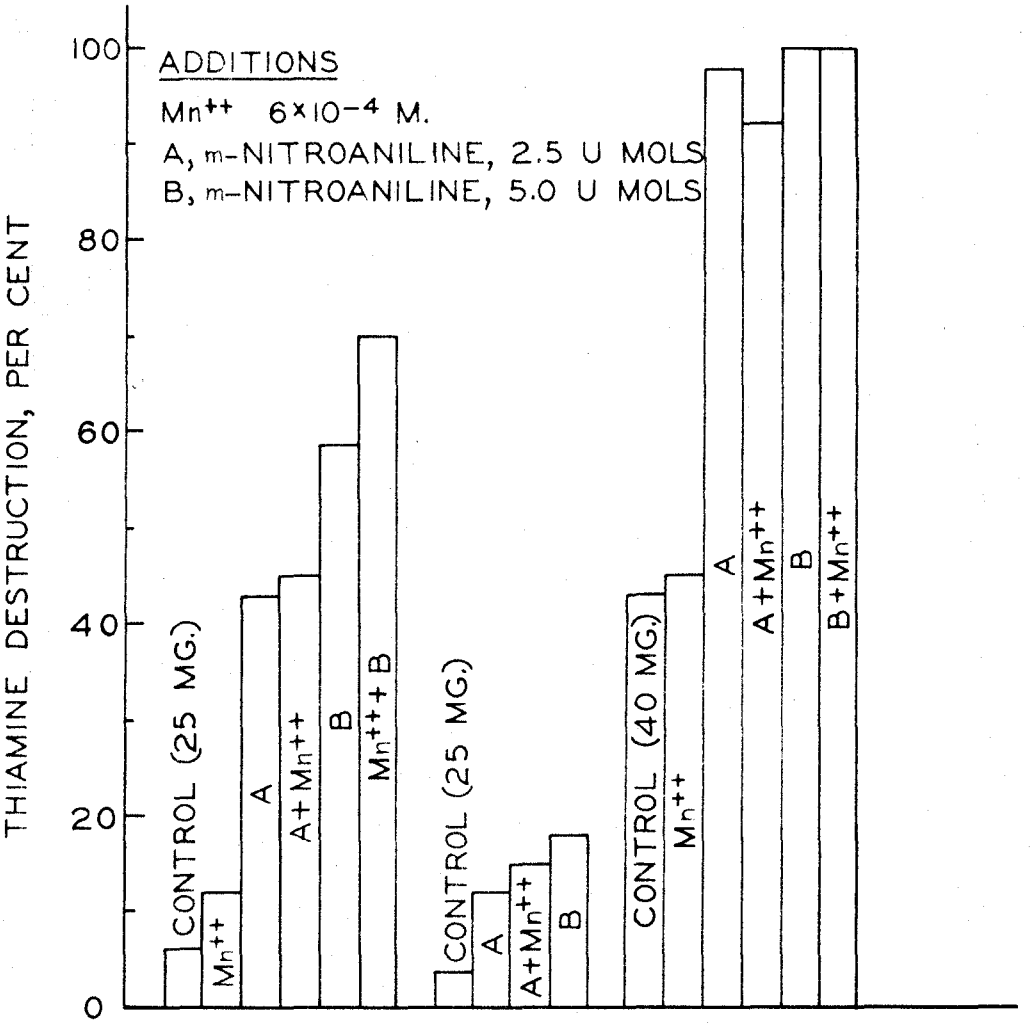


Fig. 8. Activation of undialyzed thiaminase preparations by manganese and m-nitroaniline.

are shown graphically in Figure 9.

E. Interrelation of Dialysate, Boiled Extract, and Manganese as Activators of Dialyzed Enzyme Preparations

Sealock and White[1] obtained evidence that the dialysate and boiled extract (Kochsaft), were efficient activators of the dialyzed preparations, although, like manganese, they had little or no effect on the undialyzed enzyme system. We wanted to determine, therefore, whether manganese was the essential constituent of these activating mixtures.

The enzyme extract was dialyzed in the usual manner and the dialysis-inactivated enzyme preparation tested for activity after addition of equivalents of dialysate and the ash from a portion of the dialysate. Destruction of 8.5% for the dialyzed, 12.5% for the dialysate activated system, and 16% for the ash activated preparation were measured, as compared to 18.2% for the undialyzed in one experiment and 54.5%, 68.0%, 68.0% and 88.0% respectively for another. These experimental results are shown in Table XI and XII below.

The latter experiment made it appear that manganese alone was the activating factor, and to further test this evidence two experiments were devised. In the first experiment the

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[1] Sealock, R. R., and White, H. S., Unpublished data.

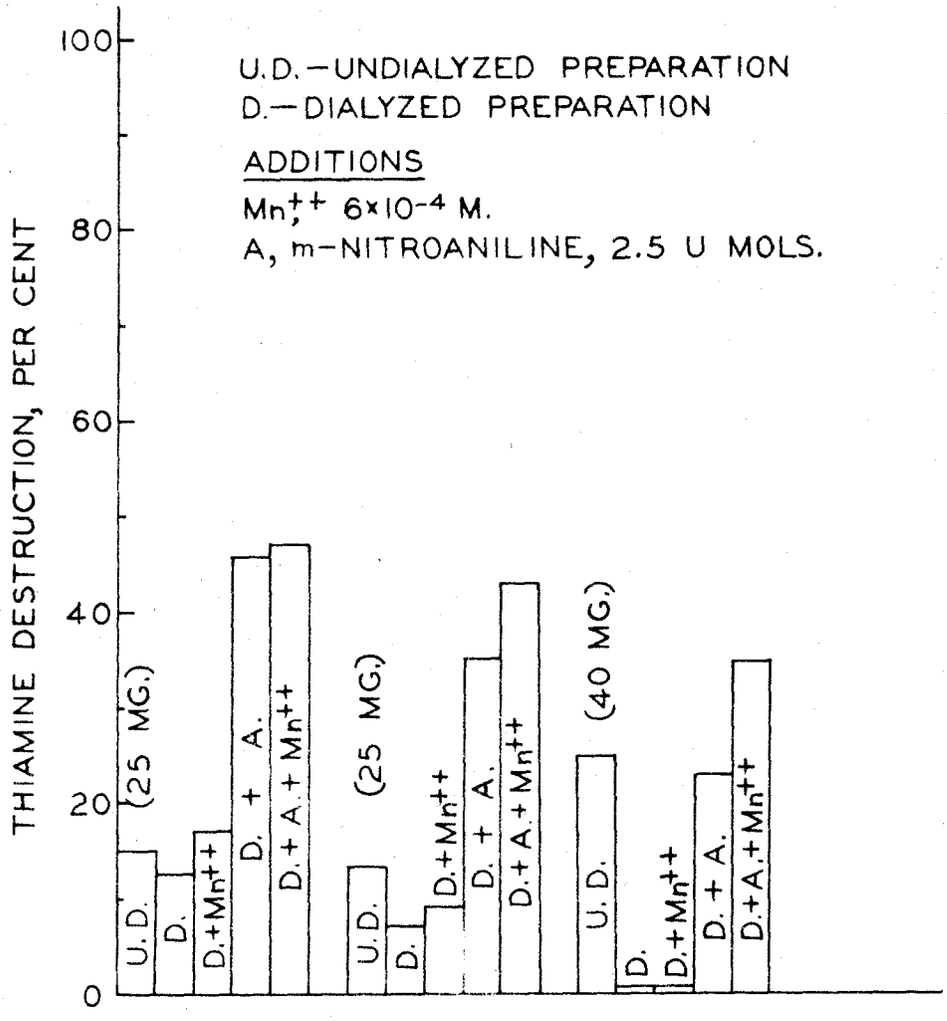


Fig. 9. Activation of dialyzed thiaminase preparations by manganese and m-nitroaniline.

Table XI

Comparison of Activating Ability of Dialysate, and Ashed Dialysate on Dialyzed Enzyme Preparation

Extract of Powder IIA at 50 mg. level. Dialyzed 6 hours, 0-5° C., pH 7.4, static condition. Assayed at 50 mg. level.

Preparation	Addition	Thiamine Destruction per cent	u moles	Activity per cent
Undialyzed	none	18.2	0.46	100.0
Dialyzed	none	8.5	0.21	46.0
"	dialysate*	2.5	0.06	13.7
"	dialysate	12.5	0.31	68.7
"	ash	16.0	0.40	88.0

*Dialyzed and dialysate added at one-third aliquots of other assays.

Table XII

Comparison of Activating Ability of Dialysate, and Ashed Dialysate on Dialyzed Enzyme Preparation

Extract of IIIA, 125 mg. equivalent/ml. Dialysis and ashing as above. Assay at 100 mg. level. Dialysate and ash added at 100 mg. equivalent.

Preparation	Addition	Thiamine Destruction per cent	u moles	Activity per cent
Undialyzed	none	88.0	2.20	100.0
Dialyzed	none	54.5	1.36	62.0
"	dialysate	68.0	1.70	77.5
"	ash	68.0	1.70	77.5

rate of loss of activity by dialysis was compared to the rate of loss of manganous ion. An extract of powder IIIA was made as usual so that 1 ml. was equivalent to 125 mg. of the dry powder. A portion of the extract was removed, and the manganese concentration determined by the combined methods of Richards and of Skinner and Peterson described above. There were 2.18 micrograms of manganese per gram of powder equivalent. The remainder of the extract was dialyzed for 24 hours at 5° C. against distilled water and the dialysate was changed every hour. Aliquots of the dialysate were removed and assayed for the time intervals indicated in Table XIII below which compares the loss of manganese to loss of activity.

Table XIII

Comparison of Loss of Manganese to Loss of Activity on Dialysis of Enzyme Preparation

Time of Dialysis hours	Loss of Manganese per cent	Loss of Activity per cent
0	---	---
4	---	4.0
8	30.0	12.5
24	71.0	70.0

While the final per cent loss of activity was equal to that of the loss of manganese, this seems merely coincidental since the rates of loss were entirely different. This point

is more evident from the graph of time vs. loss for activity and manganese illustrated in Figure 10.

Therefore, the second experiment was employed which would test the activating effect of dialysate, boiled extract, ash and manganese, each added at equal concentrations of manganous ion. An extract of IIIA-110 was divided into three portions. The first portion was dialyzed and the dialysate collected, the second portion was ashed, and the third portion was heated in a boiling water bath for ten minutes and filtered from the denatured protein to give the boiled extract or Kochsaft. Each portion was then diluted to give the same concentration of manganese (0.91 micrograms/ml) and in addition a manganese solution of this desired concentration was prepared by dissolving C. P. manganous chloride in water. These solutions were then used to activate a dialyzed preparation of IIA and their activating effect compared. Destruction of 7.5%, 8.0%, 27.5%, 25.5% and 9.0% were measured for the dialyzed preparation, ash activated, dialysate activated, boiled extract activated, and manganous chloride activated solutions respectively. The results of the experiment are listed in Table XIV.

It is apparent from these results that the manganous ion is not the only factor concerned. The dialysate and the boiled-extract or Kochsaft contain some other factor capable of causing greater destruction of thiamine when added to the

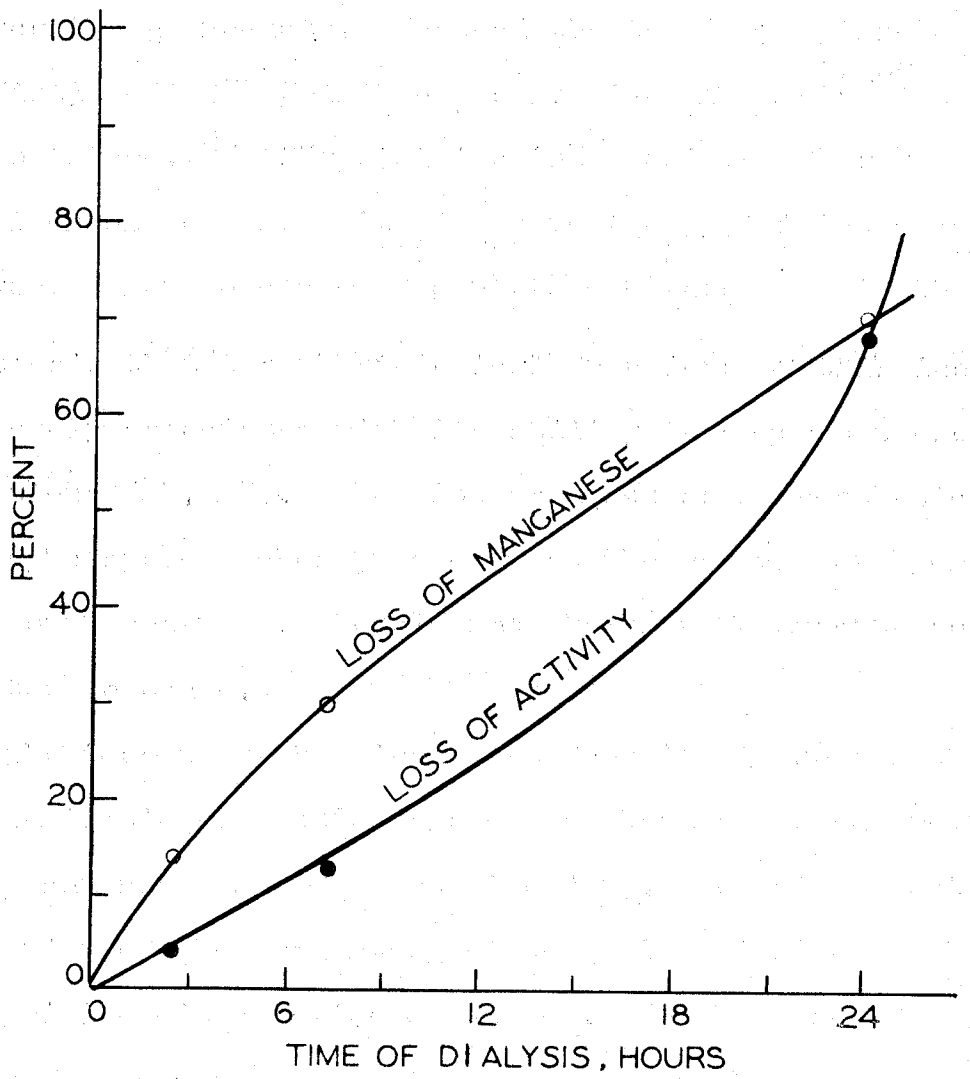


Fig. 10. A comparison of the loss of thiamine destroying activity to loss of manganese on dialysis of a thiaminase preparation; oo loss of manganese, o-o loss of activity.

Table XIV

Activation of Dialyzed Enzyme Preparation by Ash, Dialysate, Kochsaft and Manganese

Preparation	Addition	Thiamine Destruction		Activity	Activation
		per cent	micromoles	per cent	per cent
Undialyzed	None	13.0	0.33	100.0	---
Dialyzed	None	7.5	0.19	57.6	---
"	Ash	8.0	0.20	61.5	6.8
"	Dialysate	27.5	0.69	212.0	268.0
"	Kochsaft	25.5	0.63	196.0	240.0
"	Manganese	9.0	0.23	69.2	20.0

Dialysis at 25 mg. equivalents of powder, and assayed at same level.

dialyzed enzyme. In controls, run in exactly the same manner, these activating solutions showed no destruction of thiamine in the absence of the enzyme.

The evidence for other factors, coupled with the inability to achieve complete inactivation of the enzyme by dialysis, points to the presence of another factor, or factors, which might be non-dialyzable and, apparently, not heat labile. These could be two factors, one of which is heat labile and non-dialyzable and the other which is dialyzable and not heat labile.

Agren (106-109) reported that cysteine and glutathione were found to activate his dialyzed enzyme system. While both of these compounds would be dialyzable, there was also the possibility that in the enzyme mixture they were attached to a protein molecule in such a manner that they became

non-dialyzable. It seemed expedient then to study the possibility that cysteine, because of its abundant distribution, might be the other component in the dialysate or boiled extract.

First, the effect of cysteine was tested on the undialyzed enzyme preparation. An extract of IIIA was made at 20 mg. equivalent level of powder per ml. and cysteine added at the levels indicated in Table XV which shows the experimental

Table XV

Activation of Undialyzed Enzyme Preparation by Cysteine

Cysteine Concentration molarity	Klett-Summerson Readings incubated/0 time	Thiamine Destruction per cent	Activation per cent
None	55/330	83.3	---
$1x10^{-4}$	59/320	81.6	-2.0
$5x10^{-4}$	25/310	92.0	10.0
$1x10^{-3}$	06/310	98.1	18.0
$5x10^{-3}$	03/278	99.0	19.0

Assayed at 50 mg. equivalent of powder.

results. As can be seen, there was an "apparent" activating effect of the cysteine ranging from 10 to 20%, however, the color values of the zero time tubes, run as controls, showed a progressive decrease with increasing cysteine concentration, so any conclusions to be drawn from the experiment had to be withheld until this effect was considered.

This apparent activating effect was then compared with that of the boiled extract and of manganese on the dialyzed

and undialyzed enzyme preparations and also the combined effect of all three additions. Here again there was activation of the dialyzed enzyme preparation by each component. Also combination of any pair or of all three additions gave greater activity than any single addition. This again supports the idea that more than one factor is concerned with the activation of the enzyme. The results of the experiment are listed in Table XVI.

Controls for the experiment indicated an insignificant destruction of thiamine by the cysteine at either concentration. It should be noted that excess cysteine was destroyed with iodine before the color assay was made.

It was necessary to destroy the excess cysteine with iodine before assaying by the Melnick-Field method in order to remove the interference which this substance exerts on the color development. We have shown this interference to be due to the formation of a compound between the diazonium reagent and the cysteine, since it can be overcome by the addition of a higher concentration of the diazo reagent. That this is the case is shown in Table XVII. Here, by increasing the ratio of moles of reagent to moles of cysteine, the interference with the color development is completely overcome. The dye formed between the diazo reagent and cysteine is not extractable by xylene and so does not otherwise interfere with the assay method if there is sufficient reagent for

Table XVI

Activation of Dialyzed and Undialyzed Enzyme Preparations by Cysteine, Kochsaft and Manganese

Extract of powder II-236A at 40 mg. powder level. Cysteine solution added at 2 concentrations C-1 5×10^{-3} M. and C-2 2.5×10^{-3} M. Boiled extract prepared from above powder at 20 mg. equivalent level. Manganous chloride solution added at 1×10^{-3} M. level to give 2×10^{-4} M. in tube. Cysteine destroyed by iodine before Melnick-Field assay. Assayed at 40 mg. level of powder. Dialyzed preparation from same extract. Dialyzed 4 hours against phosphate buffer, 0-5° C., with stirring. Dialysate changed every half hour.*

Preparation	Addition	Thiamine Destruction per cent	micromoles	Activation per cent
Undialyzed	None	68.4	1.71	
"	Mn	72.3	1.81	
"	K	68.5	1.71	
"	C-1	89.9	2.25	
"	C-2	76.7	1.92	
"	Mn + K	73.2	1.83	
"	Mn + C-1	91.1	2.28	
"	Mn + C-2	77.5	1.94	
"	K + C-1	85.0	2.12	
"	K + C-2	76.8	1.93	
"	Mn + K + C-1	84.7	2.12	
"	Mn + K + C-2	75.8	1.90	
" (Stored)	None	52.3	1.31	
Dialyzed	None	22.4	0.56	
"	Mn	33.5	0.84	
"	K	33.5	0.84	
"	C-1	40.6	1.02	
"	C-2	37.4	0.94	
"	Mn + K	44.8	1.12	
"	Mn + C-1	52.0	1.30	
"	Mn + C-2	41.2	1.03	
"	K + C-1	46.4	1.16	
"	K + C-2	45.7	1.14	
"	Mn + K + C-1	52.8	1.32	
"	Mn + K + C-2	41.5	1.04	

*K is for boiled extract, Mn -- manganese, C-1 and C-2 -- Cysteine.

Table XVII

Reversal of Cysteine Interference in Melnick-Field Assay Method

Thiamine solution (2.5×10^{-3} M.) in a mixture of 5% trichloroacetic acid in 0.04 M. phosphate buffer. Cysteine added to give a concentration of 5×10^{-3} M. Reagent (p-acetophenyldiazonium chloride) prepared in the usual manner and assay carried out as usual.

Reagent Added micromoles	Klett-Summerson Readings Cysteine	Thiamine	Apparent Thiamine Destruction per cent
5.38	290	330	12.0
6.45	292	330	11.5
8.06	296	330	10.5
10.76	300	315	1.5

the thiamine present.

Despite some interference with the assay method, it would appear from an over all view of the cysteine experiments that the activating effect is real. Recalling the work of Barron and Singer (129) and of Nachmonsohn, et al (130-132), on sulfhydryl group dependent enzymes in which they showed inhibition by such agents as iodine, iodoacetic acid, and phenylmercuric chloride, and the reversal of this inhibition by cysteine or other sulfhydryl compounds, we thought to test if the cysteine was acting here also to prevent oxidation of the sulfhydryl groups of the enzyme. Accordingly we investigated the effect of these inhibitors on our enzyme system and the reversal of the inhibition by cysteine. As can be seen

from the results of these experiments (Tables XVIII, XIX, and XX) all three of the above reagents were effective inhibitors, and the single case tested for reversal, that of iodine inhibition, was effectively counteracted by the addition of cysteine as shown below.

Table XVIII

Inhibition of Thiaminase by Iodoacetic Acid and Phenylmercuric Chloride

Extract of II-236A at 20 mg.-equivalents of powder per ml. Iodoacetic acid and phenylmercuric chloride added at levels indicated. Assayed at 40 mg.-equivalents powder level.

Addition molarity in tube	Thiamine Destruction per cent	micromoles	Inhibition per cent
None	57.2	1.43	---
Iodine			
2×10^{-4}	56.6	1.40	1.00
1×10^{-3}	54.7	1.37	4.45
2×10^{-3}	53.8	1.34	6.00
Phenylmercuric chloride			
1.7×10^{-3}	0	0	100.0
3.4×10^{-3}	0	0	100.0

Table XIX

Inhibition of Thiaminase by Phenylmercuric Chloride and Iodine

Extract of I-142IIIA powder. Experiment exactly as in previous table, but with dialyzed enzyme.

Preparation	Addition	Thiamine Destruction		Inhibition
	ml. of 4×10^{-3} N. Iodine	per cent	micromoles	per cent
Undialyzed	None	50.8	1.27	---
Dialyzed	None	35.0	0.88	---
"	0.2	33.7	0.84	3.8
"	0.4	30.0	0.75	14.3
"	0.6	22.0	0.55	37.2
"	0.8	17.5	0.44	50.0
"	1.0	14.2	0.36	59.0
	ml. of 2.5×10 M. phenylmercuric chloride			
"	1.0	26.0	0.65	26.7

Table XX

Oxidation of Enzyme by Iodine and Reduction by Cysteine

Extract of IIIA at 50 mg. powder per ml.; dialyzed 2 hours, at 0-5° C. against 0.004 m. phosphate buffer with stirring. Dialysate changed every one-half hour. Incubated at 50 mg. powder per tube, two hours at 37.5° C. and pH 7.4.

Preparation	Addition		Thiamine Destruction		Inhibition
	Iodine ml. of 1x10 N.	Cysteine ml. of 1x10 M.	per cent	micro-moles	per cent
Undialyzed	0	0	66.2	1.65	---
Dialyzed	0	0	40.6	1.01	---
"	0.2	0	40.6	1.01	0.0
"	0.4	0	37.0	0.93	9.0
"	0.6	0	34.6	0.87	14.5
"	0.8	0	33.1	0.83	16.0
"	1.0	0	32.4	0.81	18.0
"	0.0	1.0	42.7	1.07	-5.0
	2x10 N.	2x10 M.			
"	0.5	0.1	32.4	0.81	18.0
"	"	0.2	34.0	0.85	16.0
"	"	0.3	35.9	0.90	11.9
"	"	0.4	38.3	0.96	6.8
"	"	0.5	39.7	0.99	2.0

F. Attempted Purification of Thiaminase Preparations

In the earlier stages of experimentation, when it had been established that the manganous ion had an activating effect on the dialyzed enzyme and before it had been discovered that other factors were necessary, purification of the enzyme system was undertaken to enable further work to be

carried out with a more reproducible system. These purification experiments met with little success. Two methods were utilized, the acetone precipitation at various hydrogen ion concentrations, and the ammonium sulfate precipitation method at various salt concentrations and at varying pH. The details of the experiments will not be given since they were conducted in the classical manner. The results of three such fractionation experiments will suffice to show the principles involved and these are presented in Tables XXI, XXII, and XXIII.

Table XXI

Acetone Precipitation of Thiaminase Preparation and Activation With Manganous Ion

An extract of IIIA was made at 50 mg.-equivalents of powder per ml. This was treated with 9 volumes of cold (0° C.) acetone in a 100 ml. centrifuge tube. The precipitate was centrifuged and washed with cold acetone until granular, and air dried. The dried precipitate was resuspended in a mixture of 10 parts of water and 10 parts of 0.2 M. phosphate-10% sodium chloride buffer so that 1 ml. of suspension was equivalent to 50 mg. of original powder. The precipitations were made at the pH indicated. Manganous ion was added as manganous chloride at 1×10^{-3} M. in the incubation tube.

Preparation	Thiamine Destruction per cent	Thiamine Destruction micromoles	Activity per cent
Original Extract	71.0	1.80	100.0
" " Mn	66.0	1.65	91.6
Precipitate			
At pH 7.4	13.0	0.32	17.8
" " " Mn	27.0	0.66	36.7
At pH 6.0	8.0	0.20	11.1
" " " Mn	19.0	0.47	36.1
At pH 5.0	7.0	0.17	9.4
" " " Mn	11.0	0.27	15.0

Table XXII

Ammonium Sulfate Fractionation of Thiaminase Preparation and Activation with Manganous Ion

Extract of II-196 at 100 mg.-equivalents of powder per ml. Extract made ½ saturated with ammonium sulfate by adding an equal volume of a saturated solution. Let stand 30 minutes and centrifuged. Precipitate redissolved in proper volume of buffer to give original concentration and dialyzed free of ammonium ion. Assayed for activity and total nitrogen. Centrifugate made fully saturated by addition of solid ammonium sulfate until slight excess persisted on gentle stirring. Let stand 30 minutes and filtered by suction. Precipitate treated as above. Filtrate dialyzed free of ammonium ion and assayed for activity and total nitrogen. Manganous chloride added, where indicated, at 1×10^{-3} M. concentration in assay tube. Kochsaft added at 50 mg.-equivalent of powder.

Fraction	Addition	Thiamine Destruction		Micromoles Thiamine Destroyed/Mg. N.
		per cent	micromoles	
Original Extract	None	87.0	2.18	1.01
" "	Mn	87.5	2.19	1.01
" "	K	85.3	2.13	0.99
Precipitate at ½ saturation	None	4.5	0.11	0.42
" " " "	Mn	14.0	0.35	1.35
" " " "	K	22.0	0.55	2.11
Precipitate at saturation	None	0.0	0.0	0.0
" " "	Mn	0.0	0.0	0.0
" " "	K	6.0	0.15	1.67
Filtrate from saturated ppt.	None	0.0	0.0	0.0
" " " "	Mn	0.0	0.0	0.0
" " " "	K	6.0	0.15	1.67

Mn -- Manganous Chloride; K -- Kochsaft.

Table XXIII

Ammonium Sulfate Fractionation of Thiaminase Preparation at Different Hydrogen Ion Concentrations

Experiment conducted exactly as in Table XXII with the exception that one precipiate was removed at 0.3 saturation with ammonium sulfate, and precipitates removed from 0.6 saturated solution at the pH indicated.*

Fraction	Addition	Thiamine Destruction		Micromoles Thiamine Destroyed/ Mg. N
		per cent	micromoles	
Original Extract	None	87.3	2.18	0.62
" "	Mn	88.1	2.20	0.63
" "	K	94.0	2.35	0.67
0.3 saturated precipitate	None	7.5	0.19	0.68
" " "	Mn	7.0	0.18	0.64
" " "	K	19.0	0.48	1.76
0.6 saturated pH 5.5 (1)	None	0.0	0.00	0.00
"	Mn	0.0	0.00	0.00
"	K	9.0	0.23	2.80
(2)	None	0.0	0.00	0.00
		0.0	0.00	0.00
		3.0	0.08	0.53
pH 6.5 (1)	None	0.0	0.00	0.00
"	Mn	5.0	0.13	1.60
"	K	5.0	0.13	1.60
(2)	None	0.0	0.00	0.00
"	Mn	4.0	0.10	0.38
"	K	12.0	0.13	1.15
pH 7.8 (1)	None	3.0	0.08	0.95
"	Mn	2.0	0.05	0.95
"	K	6.0	0.15	1.90
(2)	No activity			
pH 8.5 (1)	None	0.0	0.00	0.00
"	Mn	0.0	0.00	0.00
"	K	8.0	0.20	2.50
(2)	No activity			

Mn -- Manganous Chloride; K -- Kochsaft.
*(1) stands for precipitate; (2) for filtrate.

G. Experiments on Thiamine

1. Preparation of the p-aminobenzoic acid dye of thiamine and of 4-methyl-5-β-hydroxyethylthiazole methiodide

Because of the ready cleavage of thiamine by various chemical reagents mentioned earlier, we were interested in determining if the dye formed between thiamine and diazotized p-aminobenzoic acid was a compound resulting from the cleavage of thiamine, or whether it was a derivative formed by coupling of the two compounds through the pyrimidine or thiazole portion of the molecule. We also wanted to study the absorption spectrum of the dye and so developed a method of preparing it on a larger scale than that afforded by the Melnick-Field assay procedure. The following directions serve equally well for the preparation of the dye from either thiamine or the methiodide of the thiazole portion.

A solution of 4-carboxybenzene diazonium chloride was prepared by placing 4.8 gm. (0.035 M.) of p-aminobenzoic acid, 50 gm. of cracked ice, and 500 ml. of water in a 1 liter wide mouthed bottle and slowly adding, with stirring, 10 ml. of concentrated hydrochloric acid (31.5%; sp. g. 1.16) to the solution. A solution of 2.5 gm. (0.035 M.) of sodium nitrite in 100 ml. of water was cooled to 0-5° C. and run in slowly from a separatory funnel, the tip of which should dip well

below the surface of the liquid. The mixture was stirred constantly during the addition and until there was a positive test for excess nitrous acid with starch-iodide paper. This test was carried out by diluting a test-drop on a watch glass with about 1 ml. of water and then placing a drop of the diluted solution on the test paper. The sodium nitrite was added until a test for nitrous acid which was permanent for five minutes indicated the diazotization was complete.

The diazotized solution was allowed to stand for twenty minutes in the ice bath with stirring, and then 200 ml. of sodium bicarbonate buffer (20 gm. of sodium hydroxide and 28.8 gm. of sodium bicarbonate dissolved in water and diluted to 1 liter) were added.

While the diazotized solution was standing, a solution of thiamine, or the thiazole methiodide, was prepared. In a 3 liter wide mouth bottle fitted with a mechanical stirrer and a separatory funnel were placed 0.035 M. of either compound in 500 ml. of water and 100 gm. of ice. The cold diazotized mixture was then slowly added from a separatory funnel whose tip extended well below the surface of the solution. The addition took about twenty minutes and the solution was kept at 0-5° C. throughout the reaction. The mixture was stirred for one hour after the final addition, then allowed to come to room temperature. Next it was acidified to congo red with hydrochloric acid to precipitate the very water-insoluble,

micro crystalline red-violet dye. (Caution must be used in acidification because of the evolution of carbon dioxide from the buffer.) The dye was then filtered off and air dried.

In this manner was obtained 1 to 2 grams of the dye, but since the molecular weight of the dye is unknown, the yield cannot be calculated. It was low, however, since the expected yield, if the reaction was stoichiometric, would be around 25 gms. for thiamine and about 15 gms. for the thiazole dye.

The dye from thiamine and from 4-methyl-5- -hydroxyethyl-thiazole have very similar properties: both melt above 300° C. with decomposition; both are soluble in alkali and slightly soluble in glacial acetic acid, chloroform, acetone and other organic solvents, and they are insoluble in water and dilute hydrochloric acid.

The absorption spectra of the two dyes is shown below, Figure 11, in 0.1 N. sodium hydroxide at a concentration of approximately 1×10^{-5} M. calculated on the basis of a molecular weight for the dye, that being 756 gms. for thiamine and 504 gms. for the thiazole.

2. Preparation of the S-benzyl derivative of the open form of thiamine

From our work with the above dye, we had formed the opinion that the point of attachment of the diazonium compound was on the sulfhydryl group of the open form of thiamine. We

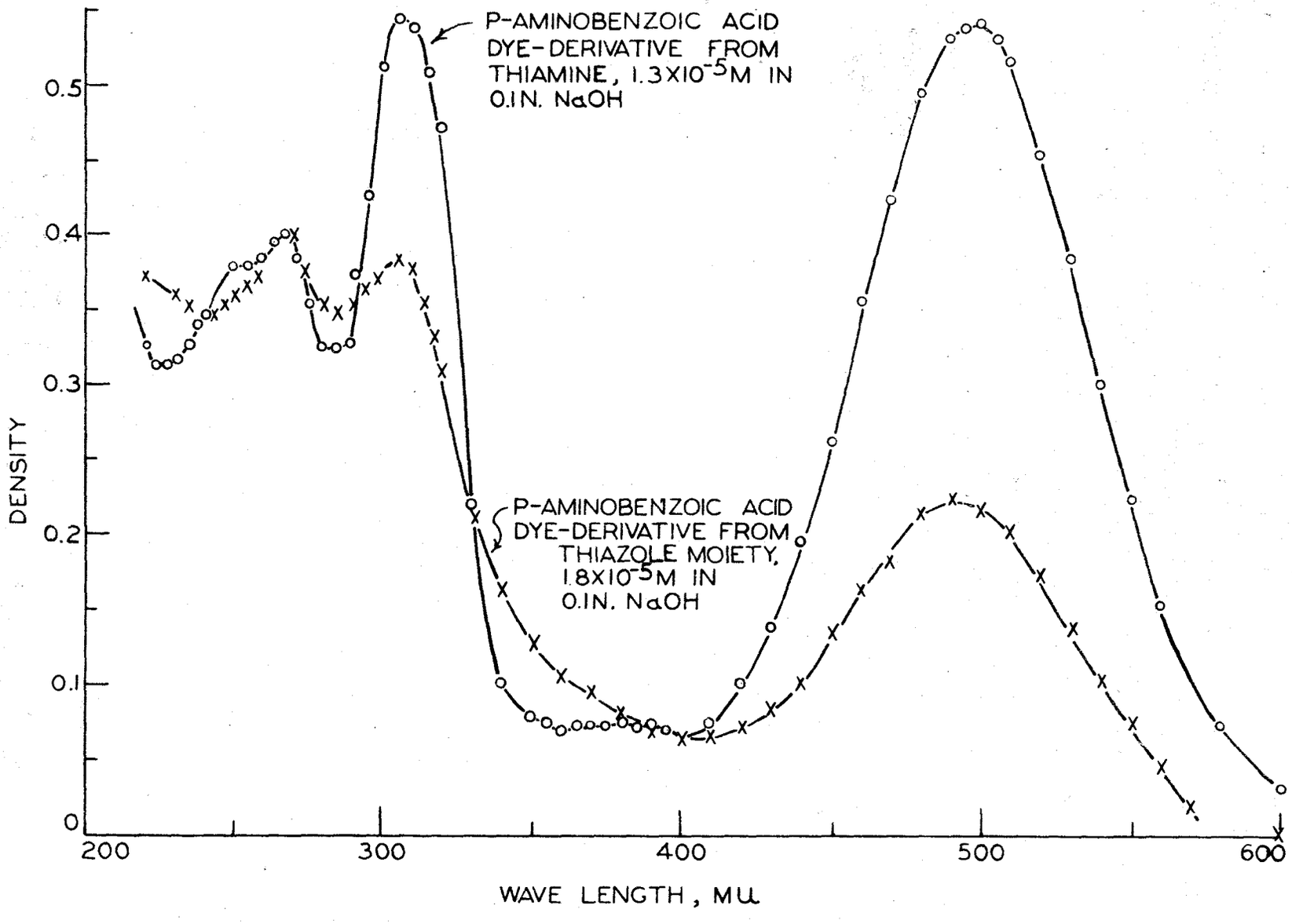


Fig. 11. Absorption Spectra of Melnick-Field Dye-Derivatives.

prepared the sodium salt of this open form of thiamine and also the disulfide form of the open compound, according to the directions of Zima and Williams (45). The latter compound did not give the Melnick Field color test, whereas the former did. We then thought to block the ring closure and the expected point of the coupling reaction to test this compound for color formation. For this purpose we prepared the S-benzyl derivative of the open form of thiamine in the following way: 3.37 gm. (0.01 M.) of thiamine chloride hydrochloride were dissolved in 10 ml. of water and treated with 35 ml. of 6 N. sodium hydroxide. An intense yellow color developed. To this solution was added, in portions, 1.28 ml. (1.27 gm.) of benzyl chloride. The cooled mixture was shaken vigorously during the addition of the benzyl chloride and for thirty minutes thereafter. The yellow color disappeared and was replaced by a white opalescense. This mixture was then extracted with ether to remove the excess benzyl chloride, and the clear, ether extracted solution was made alkaline to congo red with 6 N. sodium hydroxide, whereupon a yellow oil separated. It was again extracted with ether and the ether extracted aqueous solution placed in the ice-box. The next day the yellow oil had solidified into a white, crystalline product. This was filtered off and air dried (wt. 1.48 gms., 40% of theoretical). A further crop of 0.89 gms. was obtained from the ether extracts.

The absorption spectrum of this compound is pictured below in Figure 12 at pH 8.0.

H. Synthesis of N-(2-methyl-6-aminopyrimidyl-5-methyl)-taurine

As mentioned before, Hennessy and Warner (111) have reported isolation of a pyrimidine compound, called by them "icthiamine", from the action of the thiamine destroying enzyme of clam juice on thiamine. Its elementary composition was $C_8H_{16}N_4O_3S.2HCl$. It softened at 235° C. and melted, with decomposition, at 242° C. The picrate melted sharply at 175° C. The elementary analysis is somewhat puzzling. If it is a derivative of the pyrimidine moiety of thiamine, this leaves the formula for the derived fragment as $C_2H_9NO_3S$. From our work on the m-nitroaniline derivative and consideration of the physiological abundance of taurine, we felt this might be an N-derivative of this compound. This would be $C_2H_7NO_3S$ and the formula is in error by 2 hydrogens. In Hennessy's later papers (133, 134) he presented more information on his clam juice thiaminase and announced the isolation of a S-(2-methyl-6-aminopyrimidyl-5-methyl)-thioglycolic acid from the action of thioglycolic acid on thiamine. This seemed to us to encourage the view that "icthiamine" might be an -S derivative of cysteine, i.e.,

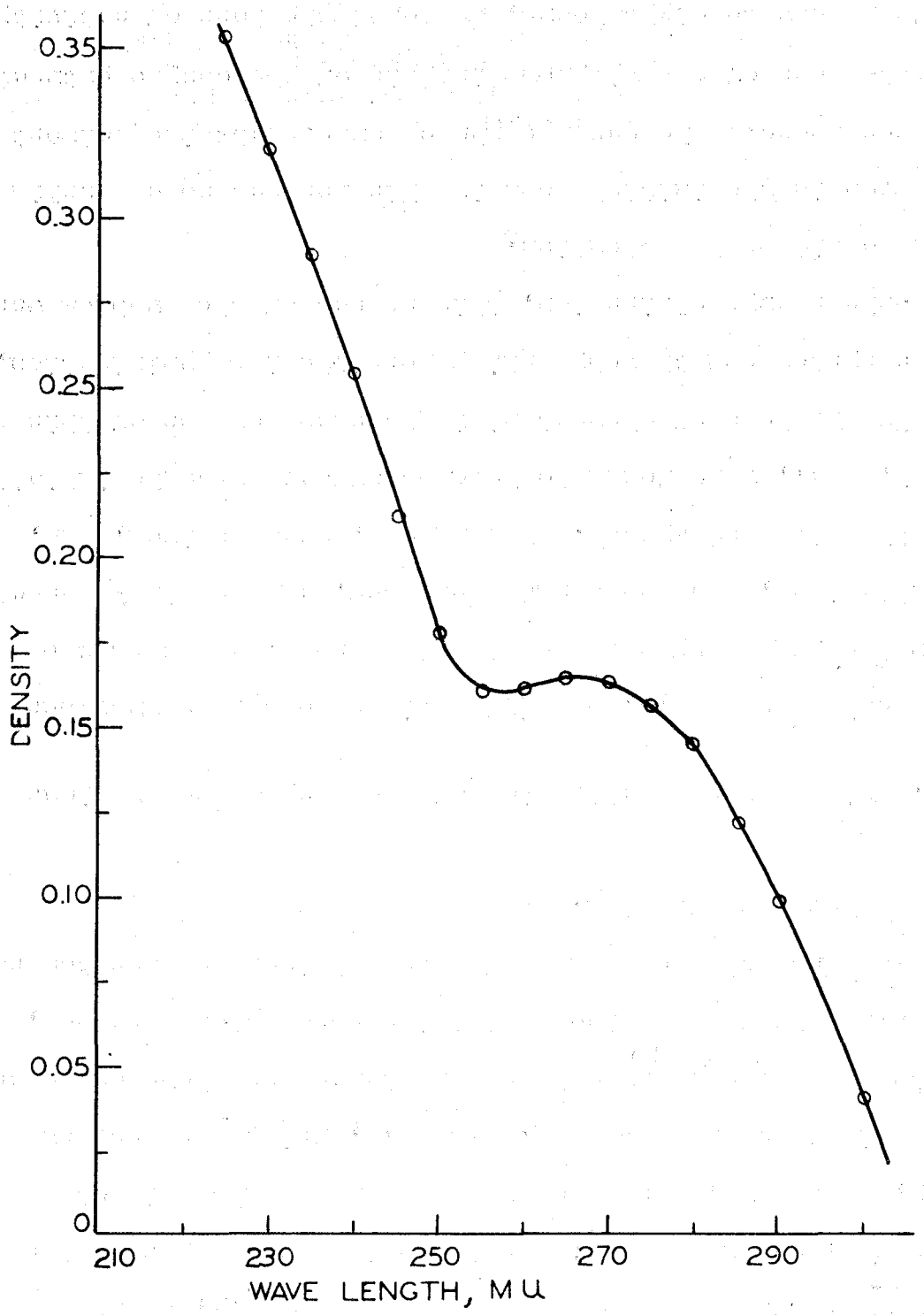
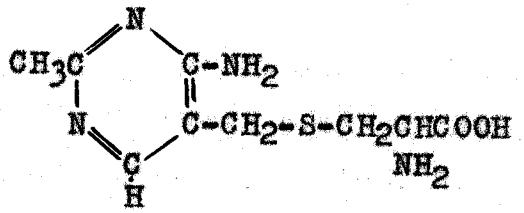


Fig. 12. Absorption spectrum of the S-benzyl derivative of the open form of thiamine. (2×10^{-6} M., pH 8.0).



and as the monohydrate-dihydrochloride, this formula $C_3H_9NO_3S.2HCl$ is in error by one carbon atom.

In hope of establishing if either of these possibilities was in truth "ichthiamine", we attempted to synthesize both compounds but so far have only been successful in preparing the taurine derivative.

For the preparation 3.64 gm. (0.01 M.) of 2-methyl-6-amino-5-bromomethylpyrimidine dihydrobromide were dissolved in 50 ml. of absolute alcohol and 1.68 gm. (0.02 M.) of sodium bicarbonate were added. The mixture was warmed and then filtered to remove the precipitated sodium bromide, the clear filtrate was then added, with stirring, to a solution of 2.5 gm. (0.02 M.) of taurine in 50 ml. of water. The final solution was refluxed for three hours on a water bath and an additional 0.84 gm. (0.01 M.) of sodium bicarbonate added. Refluxing was continued for one hour and then the solution was allowed to cool to room temperature. To the cooled, clear, orange-brown solution were added 50 ml. of absolute alcohol and the opalescent mixture which resulted was allowed to stand for one hour in an ice bath. At the end of this time the

excess taurine had precipitated and was filtered off (wt. 1.9 gms., m.p. above 300° C.). By concentration of the filtrate and the addition of more absolute alcohol and cooling, 0.18 gms. more taurine were recovered. The filtrate was then concentrated to 10 ml. under vacuo at 40-50° C. and 60 ml. of absolute alcohol added and the solution again cooled in the ice bath. At the end of one hour a small amount of inorganic material which had separated was filtered off and the filtrate concentrated to dryness under an air stream at room temperature. A residue remained consisting of a white, amorphous material colored with an orange oil. This was dissolved in the minimum amount of hot 1% hydrochloric acid (3 ml.) and filtered. To the filtrate were added 24 ml. of absolute ethanol and the mixture was cooled in the ice box overnight. The next day a white, needle-like crystalline precipitate had formed and was filtered off (wt. 0.143 gms., m.p. 205-206° C.). By concentration and addition of more absolute alcohol, a second and third crop of these crystals were obtained (wt. 0.45 gms., m.p. 206-207° C.). This gave a total of 0.59 gms. of the material which corresponds to an 80% yield of theoretical based on the amount of taurine used up in the reaction and assuming the molecular weight of the compound to be 246.2 gms.

The picrate was prepared from an aqueous solution by the addition of a saturated, aqueous solution of picric acid. It

shrinks at 175° C. with darkening and melts at 195-6° C.

An electrometric titration of 32.7 mg. of the material in 25 ml. of water required 13.40 ml. of 0.01056 N. sodium hydroxide for neutralization. Calculated 0.408% H^+ ion; found 0.412%. There was a single inflection in the titration curve which corresponds to the one replaceable hydrogen of the sulfonic acid group. A test for chloride was negative and all attempts to form a hydrochloride of the compound were unsuccessful, therefore it can not be "icthiamine".

IV. DISCUSSION

The existence of a thiamine destroying enzymatic system in various forms of marine life such as fish, clams, mussels, etc., seems something of a paradox, inasmuch as this vitamin appears to be a universally required body constituent for maintenance of healthy physiological processes. True, the question as to whether the function of this system *in vivio* is anabolic or catabolic has not been answered. At the present time, however, no reversibility of the destructive reaction has been demonstrated to substantiate an anabolic role. Perhaps in the organisms possessing this system there is no need for thiamine, its action having been successfully taken over by some other body constituent. This idea is entertaining in view of the reported absence of thiamine in carp (135, 136) and in common suckers and smelt (136). It will be interesting to compare the absence of thiamine in these fish with similar assays for other forms known to contain a thiaminase system.

Whatever the ultimate answer to this problem may be, it is apparent that a better understanding of the mechanism of thiamine destruction by thiaminase is essential. With this view in mind we have endeavored to gain a clearer insight into the *in vitro* reaction in which thiamine is destroyed by that

part of the carp thiaminase system represented by the acetone dessicated powder preparations of Sealock, et al.

From the accumulated evidence of our experiments it is apparent that the manganous ion is a part of the enzyme concerned with the cleavage of thiamine to give the thiazole moiety and some pyrimidine intermediate of as yet undetermined nature. This conclusion is well supported by the activating effect of this ion on dialyzed enzyme preparations under varying conditions, and even more significantly by the experiment of Sealock and Livermore (116) in which the manganous ion was shown to increase the level of inhibition of o-aminobenzylthiazolium salts in the thiaminase reaction.

Since this inhibition is competitive and argues for a similar mode of attachment of the substrate and inhibitor to the enzyme, it also argues that the manganous ion is in some way involved in the formation of the enzyme-substrate or enzyme-inhibitor complex.

The measured increase of inhibition in the presence of this metallic ion for a given level of inhibitor is at first inspection rather startling, and indeed seems to be theoretically impossible according to the Michaelis-Menton (117) theory of enzyme action. If, however, we stop to realize that the so-called Michaelis constants (K_s and K_i) for the dissociation of the enzyme-substrate complex and that of the enzyme-inhibitor are measured indirectly by a measurement of reaction velocity

the results are more understandable. That is, since the velocity of the reaction, and even the calculated maximal velocity, are both functions of the manganous ion concentration, then the two dissociation constants are also functions of the metallic ion concentration and are subject to change even as the concentration of the manganous ion. Thus in analyzing the expression for the measured velocity of a reaction in the presence of a competitive inhibitor, i.e.,

$$\frac{1}{v} = \frac{1}{V}\left[K_s + \frac{K_s(I)}{K_i}\right]\frac{1}{(S)} + \frac{1}{V}$$

It can be seen that the ultimate, measured velocity, or rather its reciprocal value, can either increase or decrease according to whether K_s and K_i increase or decrease, and furthermore, according to the ratio of change of K_s to K_i.

If the manganous ion functions in some way to form the respective complex between the enzyme and substrate or inhibitor, then increasing the metallic ion concentration would cause a decreased dissociation of the complex and a resultant decrease in K_s and K_i. We can see qualitatively that since both of the constants are small numbers, in the order of 10^{-5}, their ratio is of a much higher order than that of either constant. Thus the term $(K_s + K_s(I)/K_i)$ can represent an increased value even as K_s and K_i are decreasing.

We have also shown that the manganous ion is not the only

non-protein factor in the system. This is evident from the inability of this ion to completely activate the dialyzed preparations to the activity of the undialyzed material and is also apparent from the fact that we have been unable to completely inactivate the enzyme system by dialysis even under conditions of temperature and hydrogen ion concentration which should normally lead to complete loss of a metallic activator. Thus it was readily seen that there was at least one more component which seems to be relatively stable to heat, oxidation, and which was slowly dialyzable or nondialyzable. These same conclusions have been reached by Hennessy and his group in their work on the thiamine destroying system of clams.

This second factor, necessary for high activity of our thiaminase system, has resisted all our attempts at isolation. Regarding Krampitz and Wooley's (104) suggestion that there was a pyrimidine intermediate of unknown composition released by the initial cleavage of thiamine, and the evidence of Sealock and Livermore (116) that m-aminobenzylthiazolium salts caused increased destruction of thiamine by the thiaminase system, we reasoned that a logical approach to the second factor might be made through a study of this pyrimidine intermediate. It might well be that the pyrimidine intermediate sought was a derivative of the activating factor. We were able to show that both m-aminobenzoic acid and m-nitroaniline, as well as the above mentioned compound, were extremely

effective activators of the enzyme action and furthermore, that they disappeared during the course of the reaction. In later experiments the pyrimidine intermediate formed in the presence of m-nitroaniline was isolated and shown to be N-(2-methyl-6-aminopyrimidyl-5-methyl)-m-nitroaniline. These activators should then more correctly be termed "reactors" since they seem to couple with the pyrimidine moiety as it is split from thiamine.

The report of Hennessy, et al (111), in which they announced the isolation of a compound "icthiamine" from the action of their clam-thiaminase system on thiamine, attracted our interest. The analysis of the compound gave its composition as $C_8H_{16}N_4O_3 \cdot S$ 2HCl, and as yet the structure has not been elucidated although it is known to be a derivative of the 6-aminopyrimidine portion of thiamine. In this laboratory we felt it plausible from evidence at hand that it might be either the N-taurine derivative or S-cysteine derivative of the pyrimidine moiety. As reported, the taurine derivative was synthesized and found to possess entirely different properties from Hennessy's compound. The S-cysteine derivative has not been successfully prepared to date and so its identity or non-identity with "icthiamine" can only be conjectured. Both Hennessy (111) and Agren (107) have reported that cysteine has an activating effect on thiaminase. Our results support these findings but in view of the rather low activation with

cysteine and our demonstrated inhibition of the enzyme with compounds that are specific for sulfhydryl groups and reversal of the inhibition by cysteine, we feel that its action is more probably exerted by maintenance of the oxidation-reduction state of thiaminase. This action of cysteine has been encountered elsewhere by Barron and Singer (129), Nachmansohn (130-132), and others.

On the other hand, the ready cleavage of thiamine by low valence sulfur compounds discovered by Williams (18) and the cleavage of analagous benzylammonium salts demonstrated by Snyder and Speck (21, 22) to give pyrimidine or benzyl derivatives attached to the sulfur atom certainly promote cysteine to a logical position as a "reactor" in the thiaminase system. If this viewpoint is taken then at least two such compounds are known, and it is logical to assume that in the enzyme preparation there would be a large number of different reactors capable of so combining with the pyrimidine moiety to furnish not one but many pyrimidine intermediates. Indeed, a protein molecule could afford many such points of attachment as could the protein breakdown products.

This picture is in agreement with the nondialyzable nature of the enzyme extract. It also fits in with the low order of activity of such preparations if it is assumed that smaller reactor molecules are lost from the system which would normally function as the preferred ones, both because of

concentration and mobility. This latter factor may also serve to explain the relative heat-stability of the second factor; a fact which would not be consistent with the protein as the only reactor.

There is still a large question here as to whether the pyrimidine moiety reacts with the reactor molecule under the influence of the enzyme as it is split from thiamine, or whether it is first split and then combines with the reactor in a non-enzymatic fashion. Our experiment in which the m-nitroaniline derivative was isolated is of little help here since the concentration of the "artificial-reactor" was so high that it could well replace all natural reactors whether protein or non-protein. We feel, however, that our attempts at isolation of a natural intermediate derivative would not have been unsuccessful if such a compound were present. The only other conclusion then is that the pyrimidine was attached to the protein and was precipitated and discarded in the early steps of the experiment. We have not had sufficient time to determine if such is the case, but intend to do so in subsequent experiments.

With the available knowledge of the thiaminase system two equally important methods of attack suggest themselves for the pursuance of further characterization of this system in respect to nature and mechanism. The first of these has already been mentioned. That is the investigation of the pyrimidine

intermediate in the protein precipitate from the enzymatic reaction. The second is the purification of the enzyme by fractionation procedures using the manganous ion and a reactor such as m-nitroaniline as an activating mixture to assay for activity. In this way it might be possible to obtain a purified enzyme system which would seem to be necessary before the final answer can be given as to the nature of the second factor.

Although our work on the Melnick-Field dye formed with thiamine and diazotized p-aminobenzoic acid or p-aminoacetophenone is admittedly incomplete, we have nevertheless accumulated a few facts[1] which might be of interest to anyone reviewing this subject.

(1) As stated before and corroborated elsewhere (68) the essential requirements for the formation of the dye seem to be a thiazolium salt having a free 2-position and a -hydroxyethyl group at the 5 carbon. The necessity of the latter is not clear, but the former requirement seems to us to fit in nicely with an earlier observation of Williams (43) that if the 2-position is substituted there can be no ring opening.

(2) The S-benzyl compound made by us, as well as the disulfide of the open thiol form of thiamine, will not take part in the diazo coupling.

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[1] Sealock, R. R., and Davis, N. C., Unpublished work.

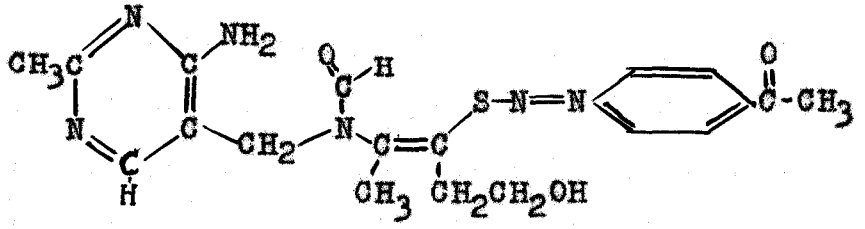
(3) Heavy metal salts which are known to combine with free sulfhydryl groups inhibit the reaction.

(4) The presence in the reaction mixture of other sulfhydryl-containing molecules such as cysteine or glutathione inhibit the coupling. However, this inhibition can be overcome by the addition of an excess of the diazonium reagent. The relation is not stochiometric.

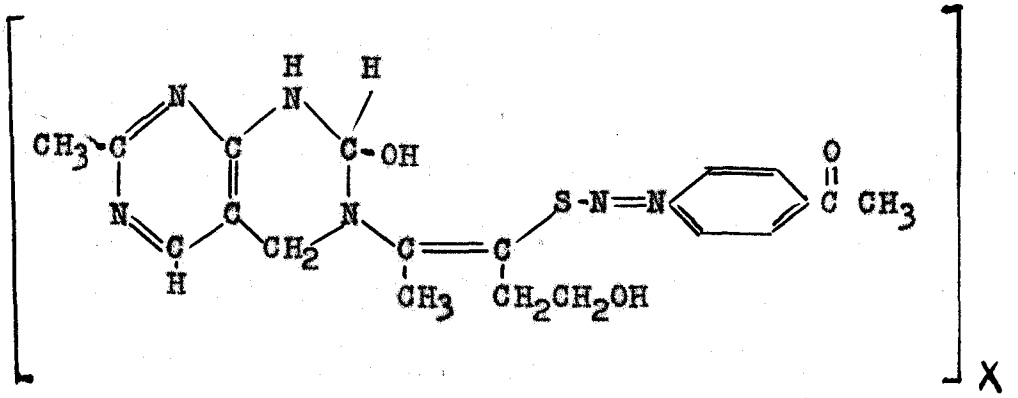
(5) Thiochrome, in which the thiazole ring is intact but in which the position of the pyrimidine indicated by Runti as the point of coupling is free, does not give a positive test.

(6) In the dye the sulfur atom and the diazo nitrogen atoms have an unusual stability. The sulfur is not removed by alkaline plumbite as would certainly be expected in the compound pictured by Runti. The nitrogen is not removed by boiling in phosphorbronz, again an unexpected fact if Runti's formula is correct.

It is our belief, although we admittedly do not have conclusive proof, that at least the first step in the reaction is the coupling of the thiol group of the open ring form of thiamine with the diazotized amine.



This molecule then must undergo some polymerization, or rearrangement which involves the -hydroxyethyl side chain and which accounts for the stability of the -S-N=N- grouping. It is furthermore suggested that the absence of characteristic absorption spectra of the 6-aminopyrimidine group might be due to its inability to resonate in the usual manner due to its combination with the formyl amino group of the open form of the thiamine, i.e.,



Whatever the true structure of the dye, it is patently evident that there is need for further experimentation along this line before a conclusive answer can be given to this problem.

V. SUMMARY

M-nitroaniline and m-aminobenzoic acid in concentrations as low as 5×10^{-4} M. have been shown to activate undialyzed thiaminase preparations from 100 to 200% and to activate dialyzed preparations to an even greater extent. The activation of undialyzed extracts was not enhanced by manganous ion, but it was increased in the case of dialyzed extracts. In experiments in which the thiamine concentration was held constant at 5×10^{-4} M. and the m-nitroaniline concentration was increased from 5×10^{-5} M. to 1×10^{-3} M., the ratio of micromoles activator lost to micromoles thiamine destroyed increased from 0.21 to 0.80. We believe that these activators combine with the pyrimidine moiety of thiamine as it is split from the vitamin molecule, and the latter experiment demonstrates that they compete with such natural activators as are normally present in the enzyme preparations.

The pyrimidine derivative from a m-nitroaniline activated enzyme reaction has been isolated in a 74.5% yield as a crystalline dihydrochloride melting with decomposition at 211-212° C. (corrected). The free base, which crystallized on addition of alkali to the acid solution, was yellow and melted at 227.5-228.5° C. (corrected). These melting points are

identical to those obtained with the secondary amine, N-(2-methyl-6-aminopyrimidyl-5-methyl)-m-nitroaniline, synthesized from the corresponding pyrimidylmethyl bromide and m-nitroaniline by warming the alcoholic solution in the presence of sodium bicarbonate. The mixed melting point showed no depression, and identity was further established by comparison of ultraviolet absorption spectra in aqueous solutions at pH 5.0, 7.0 and in 0.1 N. sodium hydroxide (maximum at 2420-2430 A.).

Several attempts were made to isolate the natural pyrimidine intermediate product using both a silver salt precipitation method and the Craig counter current distribution technique but without success in isolating such a compound. The uncertainty as to the nature of the compound, or compounds, somewhat handicapped the isolation procedures, and we now believe that the pyrimidine moiety in the unactivated enzyme mixture might have been coupled with the protein or protein break-down products in the mixture and was, therefore, precipitated and discarded with this fraction in the initial steps of the isolation procedures.

Analyses of the optimum conditions for dialysis of the enzyme preparations were made. It was concluded that the most satisfactory dialysis was accomplished with a low concentration of the enzyme at pH 6.5-7.5 and for a time period of six to eight hours of dialyzing against distilled water. Under

these conditions losses of 50-60% of initial activity resulted. Static dialysis was apparently as effective as a dialysis with constant stirring if the dialysate was changed every thirty minutes.

Activation experiments on dialyzed and undialyzed enzyme preparations indicated that the manganous ion was intimately connected with thiaminase activity. It was not, however, the only factor. Another factor, or factors, was necessary for optimum activity of the enzyme and appeared to be non-dialyzable and not heat-labile. The second factor could be replaced by m-nitroaniline or Kochsaft, but not by cysteine.

Cysteine in concentrations of 1×10^{-3} M. or higher interfered with the Melnick-Field colorimetric test for thiamine. The interference could be removed by destroying the excess cysteine with iodine prior to the addition of the diazotized p-aminoacetophenone reagent or by adding a higher concentration of this reagent. The reaction is not stochiometric and in order to completely remove the interference of 10 micromoles of cysteine in the presence of 0.5 micromoles of thiamine, 10.76 micromoles of reagent were required.

Specific sulfhydryl inhibitors such as iodoacetic acid and iodine had little inhibitory action on thiaminase. Phenyl mercuric chloride at a concentration of 1×10^{-3} M., however, gave 100% inhibition. In the case of the iodine inhibition (18% at a concentration of 2×10^{-4} M.), addition of cysteine

($1x10^{-3}$ M.) completely reversed the inhibition. It is suggested that thiaminase is a sulfhydryl-dependent enzyme and that the small activating effect of cysteine on dialyzed enzyme preparations may be due to its action of maintaining such groups in a favorable oxidation-reduction state.

Several attempts have been made to purify thiaminase by fractional precipitation of the enzyme preparations with acetone or ammonium sulfate. The purification was not successful, the activity being either partly or entirely destroyed by the purification procedures. Perhaps this type of investigation should now be renewed with the addition of the manganous ion and the newly discovered activators such as m-nitroaniline to restore the activity.

A preparative method has been developed for the Melnick-Field dye from diazotized p-aminobenzoic acid and either thiamine or 4-methyl-5-β-hydroxyethylthiazole methiodide. Both dyes are dense, red-violet, microcrystalline compounds with melting points above 300° C. They are insoluble in water, slightly soluble in organic solvents and soluble in alkali. Absorption spectra of both compounds in 0.1 N. alkali showed maxima at 268, 310 and 500 A. for thiamine dye and 266, 306 and 490 A. for the thiazole dye. From the similarity of the properties of the 2 dyes and from experiments in this laboratory, combined with reports by other investigators, we have concluded that the formation of the dye proceeds by a

coupling of the diazo reagent at the sulfur atom of the open form of thiamine and that this molecule undergoes further rearrangement, possibly polymerization, to give the final product.

The S-benzyl derivative of the open form of thiamine was prepared by the action of benzyl chloride on thiamine in aqueous alkali. It is a white crystalline compound completely lacking the characteristic thiazole odor of thiamine. Its absorption spectrum in aqueous solution at pH 8.0 shows a single, broad maximum around 270 A. It was demonstrated that this compound in which the sulfhydryl group of the open form of thiamine is blocked, would not give a positive Melnick-Field test.

N-(2-methyl-6-aminopyrimidyl-5-methyl)-taurine was synthesized by the action of the corresponding pyrimidinemethyl bromide on taurine by warming an alcoholic solution in the presence of sodium bicarbonate. It is a white crystalline compound melting with decomposition at 211-212° C. (uncorrected), and its picrate melts at 178-179° C. (uncorrected). It does not form a hydrochloride. Its absorption spectrum in aqueous solution exhibits 2 maxima (235 and 272 A.) at pH 7.4 and a single maximum (245 A.) at pH 4.9. Comparison of these properties with Hennessy's ichthiamine shows that the 2 compounds are not identical. It is suggested that ichthiamine might be S-(2-methyl-6-aminopyrimidyl-5-methyl)-cysteine, but all

attempts at preparing the compound have been unsuccessful and no definite conclusions can be drawn.

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