STUDIES ON THE BIOSYNTHESIS OF THIAMINE

by

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ABSTRACT

Studies on the Biosynthesis of Thiamine

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The following enzymatic reactions were found to be the steps involved in the biosynthesis of thiamine and its phosphate esters from 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-(2-hydroxyethyl)-thiazole: (a) the formation of a mixture of mono- and pyrophosphate esters of the 5-hydroxymethylpyrimidine by reaction of the pyrimidine compound with adenosine-5'-triphosphate; (b) the reaction of the thiazole compound with adenosine-5'-triphosphate to yield a monophosphate ester; (c) the reaction of the pyrophosphate ester of the pyrimidine compound with the monophosphate ester of the thiazole compound to yield thiamine monophosphate; (d) the hydrolysis of thiamine monophosphate to thiamine; and (e) the formation of thiamine pyrophosphate (cocarboxylase) from thiamine and adenosine-5'-triphosphate. Reactions (a), (b), (c), and (e) were activated by magnesium ion. The products of these reactions were identified with the use of chromatographic, microbiological, chemical, and spectrophotometric techniques. The enzymes which catalyze the reactions were partially purified from extracts prepared from an autolysate of baker's yeast.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Beriberi (or polyneuritis), a disease of humans resulting from a deficiency of thiamine, occurs endemically in those geographical areas in which unsupplemented cereal grain products or polished rice constitute the bulk of the dietary ration. In many of these areas, notably the Far East, the disease problem can be further aggravated by the additional dietary consumption of raw, fresh-water fish, mollusks and some arthropods since these food-stuffs contain an enzyme, thiaminase, which acts to destroy the thiamine molecule (1). Although this disease has been recognized for over 4,000 years (2), it was not until Eijkman (3), in 1897, demonstrated that a paralytic condition, avian polyneuritis, could be induced in birds by feeding them polished rice that the first major breakthrough in the study of this disease was obtained. The importance of this discovery cannot be overstated since it not only swayed many competent investigators to the idea of a deficiency disease, but also provided these workers with a biological assay system for subsequent attempts to isolate the antineuritic factor from natural materials. The thiamine isolation work finally culminated in 1926 when Jansen and Donath obtained a small amount of crystalline thiamine which was subsequently shown by Eijkman to be antineuritic in activity (4). Eight years later in 1934, Williams, et. al., (5) were able to isolate comparatively large amounts of thiamine using certain modifications of Jansen and Donath's original

isolation procedure, and subsequently, in a brilliant series of studies, were, by degradative procedures (6-12), able to assign a structure to the vitamin (13). Shortly thereafter, Williams and Cline (14) (and Finkelstein) (15) in collaboration with the Merck Laboratories confirmed the structure by synthesis.

The synthesis procedure used to prove the structure of thiamine involved the separate syntheses of the thiazole and pyrimidine components followed by their condensation to yield thiamine. More specifically, the thiazole moiety was formed by condensing thioformamide with bromacetopropanol, and the pyrimidine was synthesized by the following series of reactions: ethyl formate and ethyl β-ethoxyproprionate were condensed with sodium to yield ethyl α-sodioformyl-β-ethoxyproprionate which was subsequently reacted with acetamidine to yield 2-methyl-4-oxy-5-ethoxymethylpyrimidine. The 4-oxypyrimidine derivative was reacted with $POCl_3$ to give the 4-chloropyrimidine which was then treated with alcoholic ammonia to yield the 4-aminopyrimidine. The latter compound was converted to 2-methyl-4-amino-5-bromomethylpyrimidine hydrobromide by treatment with hydrobromic acid. Thiamine bromide hydrobromide was then synthesized by heating this pyrimidine with the thiazole compound.

Since the original synthesis of thiamine, many other chemical syntheses have been devised. These syntheses essentially involve one of three possible approaches: (a) by separately forming the two rings and condensing them; (b) by forming the pyrimidine ring with an elongated side chain at the 5 position followed by a ring closure to give the thiazolium ring; and (c) by forming the thiazolium ring with an elongated

side chain attached to the quaternary nitrogen, followed by a ring closure to give the pyrimidine ring. A very excellent review of these syntheses can be found in an article by Wuest (16).

The stability of thiamine in solution is in large measure a function of both the pH of the solution and the presence or absence of oxidizing agents. At low pH values, the compound appears to be very stable and the addition of hydroxide results mainly in the removal of a proton from the protonated pyrimidine ring (17). At pHs greater than 7, the thiamine molecule becomes less stable and can undergo several reversible and irreversible chemical changes. Under mildly alkaline conditions (pH 8.5 - 10), a hydroxyl ion attack at the 2 position of the thiazolium ring will initiate a series of displacements resulting in the reversible opening of the thiazolium ring between positions 1 and 2 (9). In this opened-ring compound, an ionized thiol is obtained in place of the original ring-sulfur and a formyl group is obtained from the ring-carbon at position 2. In the presence of oxidizing agents such as I_2 or H_2O_2, this open-ringed compound is reversibly oxidized to the familiar disulfide form of thiamine (9, 18). The pyrophosphate derivative of thiamine, cocarboxylase, will similarly undergo this same series of reactions to yield thiamine pyrophosphate disulfide (19). These two types of disulfides are frequently found in nature (19-21). In addition, the open-ringed form of thiamine can also react with several disulfides to yield mixed disulfides of thiamine (22, 23). One of the most commonly occurring of these mixed disulfides is allithiamine which can be formed by a reaction of thiamine with the disulfide of 1-mercapto-2-propene.

Under strongly alkaline conditions (at pHs greater than 10) and in the absence of oxidizing agents, the thiamine molecule is rearranged to yield a yellowish-colored compound. Maier and Metzler (24) in studying the spectrum of thiamine in alkaline methanol have proposed that the formation of the yellow thiamine compound procedes through a tricyclic intermediate which is formed by an intramolecular interaction of the pyrimidine 4-amino group and the 2 position of the thiazolium ring with the subsequent loss of a proton. The loss of a proton from the tricyclic intermediate presumably then results in (a) the formation of a double bond between the pyrimidine amino group and the 2 position of the thiazoline ring, and (b) the opening of the thiazoline ring between the 1 and 2 positions (yielding an ionized thiol at the ring-sulfur atom), to yield the yellow thiamine compound as proposed by Zima and Williams (18). The inclusion of an oxidizing agent such as potassium ferricyanide in these alkaline thiamine solutions will result in the formation of the well-known fluorescent compound, thiochrome, which conceivably is formed via Maier and Metzler's tricyclic intermediate (24). Thus, it would appear that the yellow compound was formed by a non-oxidative loss of a proton from the pyrimidine amino group of the tricyclic intermediate while thiochrome was formed by an oxidative removal of a proton from the 2 position of the thiazoline ring.

The susceptibility of thiamine to cleavage by various nucleophilic bases such as bisulfite ion has been known since the original determination of the thiamine structure by Williams, et. al. (7). The cleavage of thiamine by bisulfite appears to be quantitative

and is dependent upon temperature, time and pH (25). In these cleavage reactions thiazole is liberated by displacement with a nucleophilic base. One of the more interesting observations which has been made in recent times is that of Matsukawa and Yurugi (26) in which they noted that bisulfite apparently can catalytically act to displace the thiazole moiety with another base such as pyridine or nicotinic acid, and vice versa.

The group of enzymes called thiaminases are in general characterized by their destructive action towards thiamine and are supposedly differentiated from one another primarily on the basis of their pH optima of activity. The deleterious effects of these enzymes were first observed in 1932 on the farm of a Mr. Chastek when it was noted that foxes, fed a diet containing raw, fresh-water fish, developed a paralysis (subsequently called the Chastek paralysis) and died. Since the initial investigations by Green, et. al. (27, 28) and Sealock, et. al. (29), these enzymes have been extensively studied and very excellent reviews are available (30, 31). The mechanism by which the thiaminases act on thiamine is to catalytically displace the thiazole moiety with either another nucleophilic base or hydroxyl ion. Although in vitro studies have shown that various compounds such as m-nitroaniline, m-aminobenzoic acid and pyridine can effectively serve as displacing bases, the only in vivo displacing base which has been completely characterized to date is hypotaurine, which yields icthiamine, 2-methyl-4-amino-5-(2-aminoethanesulfonyl)methylpyrimidine, and the thiazole moiety (32). Hydrogen ion is liberated in this displacement.

In addition to the thiaminases described above, there are many naturally-occurring antithiamine materials which have been erroneously designated as thiaminases in the past. However, since these compounds have been inadequately characterized both as to composition and to their mechanism of inhibition, and since they have been discussed in the review articles cited above, they will not be discussed at this time.

Although many early workers suspected that vitamins served as catalysts in biochemical reactions by virtue of their profound biological activity when used in small amounts, it was not until the early 1930s that the first major breakthrough occurred. In the classic experiments of Auhagen (33, 34), it was demonstrated that the activity of a carboxylase preparation, which had been inactivated by washing with phosphate buffer at approximately pH 8, could be restored by the addition of a boiled yeast extract, and this observation led Auhagen to postulate the existence of a coenzyme in yeast extract which he named cocarboxylase. Four years later, Lohmann and Schuster (35) were able to isolate this cofactor in a crystalline form from yeast and subsequently showed it to be the pyrophosphate ester of thiamine. Banga, Ochoa and Peters (36, 37) as well as Lohmann and Schuster were also able to demonstrate that this thiamine phosphate derivative was the active form of the vitamin. However, it is not entirely clear even at this time as to whether or not thiamine pyrophosphate is the only active form of thiamine. It has been suggested by some workers (38, 39) that thiamine triphosphate might also have coenzymic functions, but in most cases it has been found

that this compound is considerably less active than cocarboxylase (40-42). The outstanding exception to these above findings is the discovery by Plotka, et. al. (38) that thiamine triphosphate is more effective than thiamine pyrophosphate in maintaining normal heart action.

It does not appear likely that free thiamine has any coenzymic function. In those few cases when an enzymatic activity of a cocarboxylase-requiring enzyme system is stimulated by the addition of thiamine, it has been shown that the thiamine effect is indirect and probably due to both an inhibition of a powerful phosphatase which acts to degrade cocarboxylase (43-45) and a thiamine saturation of extraneous cocarboxylase-adsorbing protein which would have otherwise complexed with the free thiamine pyrophosphate (46).

Examination of the specificity requirements of in vivo and in vitro biological systems requiring thiamine (or cocarboxylase) has shown that only a very few thiamine analogues can substitute for the intact thiamine molecule. The only substitutions which apparently can be tolerated are the substitutions of an ethyl, propyl, or isopropyl group for the pyrimidine-2-methyl group, or the substitution of an ethyl group for the thiazolium-4-methyl group. Alteration of the pyrimidine ring, the thiazolium ring, the pyrimidine-4-amino group, the thiazolium-2-methylyne ring-carbon, or the thiazolium-5-(2-hydroxyethyl) group results in a complete loss of biological activity. A very excellent tabular review of these thiamine analogues may be found in a recent A.C.S. monograph (47).

Some thiamine anologues, in addition to being biologically

inactive, are also competitive inhibitors of cocarboxylase. Some of the more widely studied and potent antagonists are: (a) oxythiamine, the pyrimidine-4-oxy analogue of thiamine; (b) butylthiamine, the pyrimidine-2-n-butyl analogue, and (c) pyrithiamine (neopyrithiamine), the pyridinium analogue. These and other thiamine antagonists have also been reviewed by the above-cited authors (48).

The many reactions which are catalyzed by cocarboxylase-requiring enzymes basically involve either the cleavage or the formation of carbon bonds adjacent to carbonyl groups. For the sake of discussion, these reactions have been divided into two groups consisting of those enzymes which either act upon (a) α-keto acids (either oxidatively or non-oxidatively) or (b) α-ketols and α-diketones.

Although reactions involving α-keto acids are fairly common in biological systems, the mechanisms by which all of these reactions occur are analogous to the many reactions which pyruvic acid undergoes, and for this reason, only the reactions of pyruvate will be discussed below. In all of the pyruvate decarboxylation reactions, pyruvate is presumably first decarboxylated in the presence of a divalent metal such as Mg^{++} to yield carbon dioxide and an intermediate, enzyme-bound acetol group (to be discussed shortly) which can then undergo several possible reactions. In simple, non-oxidative decarboxylations, the enzyme-bound acetol group can either be liberated as acetaldehyde or it can be condensed with an appropriate acceptor aldehyde or ketone to yield compounds such as acetylmethylcarbinol (acetoin), acetylethylcarbinol, and α-acetolactate (49-51). In the oxidative decarboxylation of pyruvate (52-53), the enzyme-bound acetol group is

generally oxidized by- and transferred to lipoic acid at the acetyl level of oxidation. The acetyl group is then transferred from acetyl lipoic acid to reduced coenzyme A by lipoic transacetylase and the reduced lipoic acid is reoxidized back to the disulfide by lipoic dehydrogenase and DPN^+. The acetyl coenzyme A thus formed can then react with any one of several substrates to yield products such as acetyl phosphate, citrate, fatty acids and acetylated amines.

However, the enzyme-bound acetol group is not always oxidized by lipoic acid. In the phosphoroclastic cleavage of pyruvate by Clostridium butylicum, both coenzyme A and inorganic phosphate are required, but it is doubtful as to whether lipoic acid is involved (54). The final products of this reaction are acetyl phosphate, carbon dioxide and molecular hydrogen (55). In addition, there is also some question as to whether lipoic acid is involved in the E. coli phosphoroclastic cleavage of pyruvate yielding acetyl phosphate and formate (56). Lastly, it should be mentioned, in passing, that the only α-keto (aldehyde) acid decarboxylation reaction in which the involvement of cocarboxylase is uncertain is the conversion of glyoxylate to carbon dioxide (from the carboxyl group) and formate (from the aldehyde group) (57).

The second group of reactions catalyzed by cocarboxylase-requiring enzymes are the transketolase reactions (58) in which various donor, trans α-ketol compounds are cleaved, in the presence of Mg^{++}, to yield aldehydes (or carbon dioxide from β-hydroxypyruvate) and presumably an enzyme-bound "active glycoaldehyde" unit. However, in order to obtain this cleavage, an appropriate acceptor aldehyde must be

simultaneously present as a substrate for condensation with the "active glycoaldehyde" to yield a trans, α-ketol carbohydrate. A typical reaction catalyzed by these enzymes is the conversion of fructose-6-phosphate and ribose-5-phosphate to erythrose-4-phosphate and seduheptulose-7-phosphate. The only known exceptions to the general transketolase reactions described above are the reactions catalyzed by two cocarboxylase-Mg^{++}-requiring enzymes of bacterial origin (59, 60), in which acetyl phosphate and aldehyde phosphates are liberated from the reaction of phosphate and either fructose-6-phosphate or xyulose-5-phosphate (trans α-ketol carbohydrates). In all these cases, presumably, the "active glycoaldehyde" is bound to cocarboxylase in a manner analogous to the bound "acetol" derived from pyruvate.

The precise molecular mechanism by which thiamine is able to decarboxylate α-keto acids and cleave α-ketols is even at this late date, still imperfectly known. Of the many mechanistic theories in which it has been postulated that thiamine interacted with the substrate at the level of the pyrimidine-4-amino group (61, 62), the methylene-bridge carbon (63), the ionized thiol of the open-ringed form of thiamine (64), or the reversibly reduced thiazolium ring (65), only Breslow's most recent theory (66) of an interaction at the thiazolium-2-position appears to be adequate on both an empirical and theoretical basis. The other theories, for one reason or another (66-71), have been discarded. Breslow, in his theory, postulates that the loss of a proton from the thiazolium-2-position of thiamine (or cocarboxylase) would result in the formation of a thiazolium zwitter ion which could then interact with the α-carbon of pyruvate

to yield a thiazolium substituted lactate intermediate. Decarboxylation of the substituted lactate could then occur by a reversible shift of electrons to form a double bond between the acetol substitution and the now thiazoline ring. Reformation of the thiazolium ring with the concurrent addition of a proton to the acetol substitution would result in the formation of an acetol analogue of cocarboxylase. Presumably, this acetol derivative could then react with any one of several possible compounds to yield the characteristic products of pyruvate reactions as discussed earlier. The observations which tend to support Breslow's theory include: (a) the rapid exchange of deuterium at the thiazolium-2-position at almost neutral pH values (72); (b) the complete loss of thiamine activity when the thiazolium-2-position is altered (47), and (c) the non-enzymatic, catalytic activity of 3-benzyl-4-methyl-thiazolium chloride (63). As further support for Breslow's theory, Krampitz, et. al. (73) have recently reported that the chemically prepared thiazolium-2-(1-hydroxyethyl) (acetol) analogue of thiamine was able to enzymatically substitute for pyruvate and thiamine for the production of acetaldehyde, and G. Carlson (of this laboratory -- personal communication) has partially purified a cocarboxylase analogue from biological materials which, on the basis of preliminary information, appears to be the postulated acetol compound.

At the present time, very little information is available concerning the biosynthetic formation of thiamine. Part of the difficulty is traceable to the very small amounts of thiamine which are produced biologically and to a lack of rapid, reproducible, and sensitive assay procedures for either thiamine or the pyrimidine and/or thiazole

moieties. Of the many chemical assays which have been developed for thiamine, only the thiochrome and the diazotized p-aminoacetophenone procedures (74) are specific enough to be generally used for assay purposes. However, these procedures are too cumbersome and too insensitive to be widely used in studying biosynthetic pathways. Macrobiological assay procedures for thiamine (74) have also, in general, been discarded because of reproducibility difficulties, and because long periods of time are required to obtain thiamine deficient organisms. Microbiological assays for either thiamine or the pyrimidine and/or thiazole moieties (74) are usually sensitive enough, but, especially with some fungii, these assays are often time tedious to perform and insufficiently specific. The success of the studies reported in the Experimental section of this paper was, in large measure, made possible by the development of three highly specific, sensitive and rapidly performed microbiological assays for thiamine and its pyrimidine and thiazole moieties.

The de novo biosynthetic formation of the pyrimidine moiety of thiamine is completely obscure. It is not even known whether this biologically unique compound is biosynthetically related in any way to normal nucleotide pyrimidines. On the basis of studies on Salmonella typhimurium ATh (adenine-thiamine) mutants (75) where it was found that these single-gene purine mutants also exhibited a dietary requirement for the pyrimidine moiety of thiamine, it is conceivable that the formation of this pyrimidine moiety is in some manner related to the biosynthesis of purines.

As with the pyrimidine moiety, the biosynthetic origin of the

thiazole moiety is also obscure. The extent of our knowledge in this area is limited to three seemingly unrelated observations: (a) that possibly methionine, acetaldehyde, and ammonia are, in some manner, thiazole precursors (76) since the product of their condensation, 2-amino-3-(4-methylthiazole-5)-proprionic acid can be degraded to thiazole by yeast (77); (b) that pea roots can synthesize the thiazole moiety from thioformamide and either 3-chloro-4-ketopentanol or 4-ketopentanol (78); and (c) that some thiazole-requiring Escherichia coli and Neurospora crassa mutants respond to either cystine or thiazolidinecarboxylic acid (79).

Examination of the thiamine requirements of many organisms has provided the bulk of the information known about the biosynthesis of thiamine. In general, these requirements can be summarized by noting that an organism can either synthesize its own thiamine requirement de novo,or it must be supplied with either thiamine itself or at least with the pyrimidine and/or thiazole moieties. Only freshly isolated Neisseria gonorrhoeae is known to require cocarboxylase rather than thiamine (80). Presumably, all other organisms can synthesize their cocarboxylase requirements from thiamine. A tabular review of the minimal thiamine requirements of a large number of organisms can be found in the previously cited A.C.S. monograph (81).

It would thus appear, on the basis of these thiamine requirements, that the thiazole and pyrimidine moieties are separately synthesized and are then condensed to yield thiamine. Tatum and Bell (82) and Harris (83) in studying this sequence of reactions in Neurospora mutants blocked at various points in the synthesis of

thiamine, find that this sequence of steps is involved in the formation of thiamine. However, Harris also suggests the possibility of an alternate pathway of thiamine biosynthesis in which the pyrimidine moiety condenses with a thiazole precursor to yield a thiamine precursor.

Recently, Harris (84) has also reported that the enzymatic coupling of the pyrimidine and thiazole moieties can occur in cell-free extracts of baker's yeast in the presence of ATP and Mg^{++}. He has further suggested on the basis of this preliminary information that this coupling could occur without ATP if the pyrimidine monophosphate ester were substituted for the pyrimidine moiety. However, it was doubtful, even at that time, whether a simple phosphate ester was energetically active enough to condense with the thiazole moiety. Subsequent work performed in this laboratory (to be presented in this dissertation) has shown, contrary to Harris' preliminary findings, that the pyrophosphate ester of the pyrimidine moiety rather than the monophosphate ester is the most direct precursor of thiamine, and that in order to obtain the pyrophosphate from the monophosphate, ATP is required. Both pyrimidine phosphates have been isolated from enzymatic reaction mixtures. More recently, Leder (85) has confirmed the ATP requirement for the conversion of the pyrimidine monophosphate to the pyrimidine precursor and has further suggested that the product was a phosphorylated form of thiamine. The data to be presented in this paper shows that: (a) thiazole monophosphate is an obligate intermediate in the coupling reaction; (b) the enzymatic condensation of thiazole phosphate and pyrimidine pyrophosphate did not require ATP,

and (c) the sole product of the coupling reaction was thiamine monophosphate. Very recently, while this dissertation was being prepared, Nose, et. al. (86) have shown that chemically prepared pyrimidine pyrophosphate and thiazole phosphate could enzymatically condense without ATP. However, as these workers were using a crude enzyme preparation, they were not able to identify the product of this reaction, although they do suggest that the product was probably a phosphorylated form of thiamine.

Several workers (87-89), in studying the enzymatic formation of cocarboxylase, have suggested that thiamine is a more direct precursor of cocarboxylase than is thiamine monophosphate. The strongest evidence which supports this conclusion was provided independently by Leuthardt, et. al. (90) and Mano, et. al. (91). Leuthardt, et. al. using an enzyme purified from rat liver have found that thiamine was converted to thiamine pyrophosphate about 4 to 5 times more effectively than was thiamine monophosphate. Mano and collaborators have reported that a rat liver preparation used thiamine, but not thiamine monophosphate, as a substrate for the formation of cocarboxylase. Work to be described in this dissertation shows that a partially purified enzyme from baker's yeast can form cocarboxylase only from thiamine, and that in order for thiamine monophosphate to serve as a precursor, it must first be degraded to thiamine.

The present investigations were undertaken to establish the reaction sequence and the identity of the intermediates in the biosynthesis of thiamine and its phosphates from 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-(2-hydroxyethyl)-thiazole. The

results of these investigations have shown that a pyrophosphoryl ester of the pyrimidine compound and a monophosphoryl ester of the thiazole compound are intermediates in the reaction sequence and that these two intermediates react to yield thiamine monophosphate as a product. Furthermore, it has been established that thiamine monophosphate can be converted to thiamine pyrophosphate (cocarboxylase) only by prior degradation to thiamine, which serves as the most direct precursor of thiamine pyrophosphate.

METHODS

Abbreviations. -- The abbreviations used in this dissertation are:

AMP, adenosine-5'-monophosphate

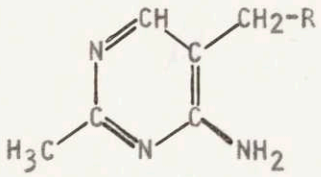
ADP, adenosine-5'-diphosphate

ATP, adenosine-5'-triphosphate

CMP, cytidine-5'-monophosphate

CDP, cytidine-5'-diphosphate

DEAE, N,N-diethylaminoethylcellulose



2-methyl-4-amino-5-R methylpyrimidine

where R = -OH, pyrimidine
-Br, bromomethylpyrimidine
-NH_2, aminomethylpyrimidine
-OCH_3, methoxymethylpyrimidine
-thiazole, thiazolemethylpyrimidine (thiamine itself)
-SO_2OH, pyrimidine sulfonate
-P, pyrimidine phosphate
-PP, pyrimidine pyrophosphate

thiamine-P, thiamine monophosphate

thiamine-PP, thiamine pyrophosphate, cocarboxylase

thiazole, 4-methyl-5-(2-hydroxyethyl)-thiazole

thiazole-P, phosphate ester of thiazole

Tris, tris-(hydroxymethyl)-aminomethane

versene, ethylenediaminetetraacetic acid

Reagents. -- All the reagents used in the Experimental section, except those listed below were readily obtained from various commercial sources and were either C.P. or Reagent grades. In addition, all of the organic chemicals which were tested were chromatographically pure. Pyrimidine, bromomethylpyrimidine, methoxymethylpyrimidine and thiazole were donated by Merck Pharmaceutical Company. Pyrimidine sulfonate was prepared by the method of Williams, et. al. (7). Pyrimidine-P, pyrimidine-PP and thiazole-P were prepared as indicated in the Experimental section.

Microbiological assay of thiamine. -- Lactobacillus viridescens strain S38A, A.T.C.C. #12706, a heterofermentative bacterium, was the test organism used for the microbiological assay of thiamine. This organism also responds to thiamine-P and thiamine-PP and has been used extensively to assay for both of these compounds. Pyrimidine and/or thiazole do not substitute for thiamine in this assay.

The L. viridescens stock cultures were maintained in Nutrient Agar (Difco) stabs which had been incubated at 38° for 12 to 24 hours and stored at 4°. Transfers were made at 4 to 6 week intervals.

In order to obtain rapid and reproducible assays with this organism, it was necessary to keep the "working" cultures at a high level of metabolic activity. This was accomplished by transferring inoculum-broth cultures at 2 to 7 day intervals. These subcultures were incubated at 38° for 7 to 8 hours and were stored at 4° until used.

The inoculum tubes contained 6 to 8 ml. of single-strength broth (Table I) to which thiamine was added in amounts of 50 to 80 μμmoles per ml. The tubes were sterilized by autoclaving them for 8

minutes at 15 lb. pressure and were stored at 4°. One to 4 drops of a frequently transferred subculture (above) were aseptically added to each inoculum tube. The inoculum was then incubated either at 38° for 4 to 7 hours or at 30° for 12 to 16 hours and the fully-grown inoculum was stored in a refrigerator for no more than 10 to 12 hours. The inoculum was washed two times and finally resuspended in 8 to 12 ml. of sterile distilled water. One drop of this suspension was used to inoculate each assay tube.

To the assay tubes, appropriate thiamine dilutions or dilutions of the samples being assayed were added, the volumes brought to 5 ml. with distilled water and 5 ml. of double-strength medium (Table I) were added. The tubes were covered with aluminum caps and sterilized by autoclaving for 8 minutes at 15 lb. pressure. The tubes were then cooled, inoculated, warmed to 38 to 40° in a water bath and incubated either at 38° for 5 to 7 hours or at 30° for 10 to 14 hours.

Growth was measured turbidimetrically at 660 mμ in a Coleman Junior Spectrophotometer. The thiamine standard curve covered a range of 0 to 280 μμmoles of thiamine per assay tube (10 ml.) and the total thiamine activity of a sample, irrespective of whether it was thiamine or one of its phosphate derivatives, was expressed in terms of a certain number of moles of thiamine.

Bioautographic plates were prepared as follows: 200 ml. of single-strength L. viridescens medium containing 2% Difco Agar-Agar were autoclaved for 8 minutes at 15 lb. pressure. After the medium had cooled to 47 to 48° in a water bath, 1 ml. of washed inoculum was added and the medium was poured into a uniform layer in the bottom of

a sterile, shallow Pyrex baking dish. The dish was covered with a sheet of aluminum and the agar was allowed to solidify for at least one hour. Bioautograms were then made by placing a dried, developed paper chromatogram in contact with this solidified seeded agar plate for 8 to 10 minutes, removing the chromatogram and incubating the plate at 38° for 3 to 6 hours. Examination of the plates showed zones of opaque growth located in the agar at those places which corresponded to the location on the paper chromatogram of thiamine or one of its phosphate derivatives. In this manner, it was possible to identify the different forms of thiamine on the basis of their R_f values and also to semi-quantitatively measure the amounts of each from the size of the growth zones.

The L. viridescens medium which was used was that of Deibel, Evans, and Niven (92) which was modified by the addition of asparagine and a vitamin supplement. The thiamine-free yeast extract used in the medium was also prepared in a somewhat different manner than that recommended by Deibel, et. al.

Preparation of thiamine-free yeast extract. -- Eighty gm. of Difco Yeast Extract were dissolved in distilled water and the volume was adjusted to 1 liter. The solution was then autoclaved for 15 minutes (30 minutes for 4 liters) at 15 lb. pressure, cooled and adjusted to pH 3 with concentrated hydrochloric acid. One hundred thirty gm. of Superfiltrol-Grade 19 (Filtrol Corporation, Los Angeles, California) were added and the resulting suspension was vigorously shaken for 30 minutes on a rotary shaker. After centrifugation, the clear supernatant solution was adjusted to pH 1 with concentrated hydrochloric

TABLE I

Basal Growth Medium Used for the Assay of Thiamine by *Lactobacillus viridescens*[a]

Component	Amount/liter Double Strength
Bacto-tryptone (Difco)	20 gm.
Thiamine-free yeast extract[b]	10 gm.
NaCl	10 gm.
K_2HPO_4	10 gm.
Sodium citrate	10 gm.
Glucose	20 gm.
L-asparagine	2 gm.
$MgSO_4 \cdot 7H_2O$	1.6 gm.
$FeSO_4 \cdot 7H_2O$	0.08 gm.
$MnCl_2 \cdot 4H_2O$	0.288 gm.
Tween-80[c]	2 ml.
Vitamins[d]	10 ml.
Distilled water	to 1 liter

[a]The medium was adjusted to pH 6.0 and was frozen and stored at -20° in polyethylene bottles. The use of benzene and toluene as preservatives was avoided as it was found that these compounds interfered with the assay.

[b]The preparation of thiamine-free yeast extract is discussed below.

[c]Tween-80, polyoxyethylene sorbitan monooleate.

[d]See Table II.

TABLE II

Vitamin Solution Used in the Basal Medium for the Thiamine Assay with *L. viridescens*[a]

Component	Amount
Pantothenic acid	10 mg.
Nicotinamide	10 mg.
Riboflavin	10 mg.
p-Aminobenzoic acid	20 mg.
Pyridoxine[b]	40 mg.
Folic acid	2 mg.
Biotin	0.2 mg.
Distilled water	to 100 ml.

[a]This vitamin solution is part of a medium used by Macias R (93) to assay for thiamine with *Lactobacillus fermenti*. The solution was stored at 4° for no more than 2 to 3 months.

[b]Experimentally, pyridoxal can be substituted for pyridoxine

acid, autoclaved for an additional 15 minutes (30 minutes for 4 liters) at 15 lb. pressure and cooled. One hundred thirty gms. of Superfiltrol Grade 19 were again added and the suspension was vigorously shaken on a rotary shaker overnight. The next morning the suspension was centrifuged and 19.5 gm. of dipotassium hydrogen phosphate were added to the clear solution. The solution was adjusted to pH 7.0 with 10 M sodium hydroxide and was then autoclaved for 15 minutes (30 minutes for 4 liters). After cooling, the suspension was centrifuged for the last time and the clarified solution was brought up to a final volume of one liter. This thiamine-free yeast extract was frozen and stored at -20°.

Microbiological assay of pyrimidine. -- The micro-organism which was used to assay for the pyrimidine portion of thiamine was one of several Salmonella typhimurium ATh (adenine-thiamine) mutant strains which were made available by Dr. Haruo Ozeki of the Department of Genetics at Cold Spring Harbor. These mutants are characterized by having a single gene mutation which is biochemically reflected in a specific nutritional requirement for one of several purine bases. In addition, for reasons which remain unknown, these mutants also exhibit a second requirement for either thiamine or its pyrimidine moiety. The thiamine requirement of some of the mutants can be spared by the addition of pantothenate, and still others by the addition of either pantothenate or methionine. An excellent genetic and biochemical review of these mutants can be found in a recent Carnegie Institute publication (75).

Preliminary experiments with these mutants (numbers 4, 5, 6,

8, 10, 11, 17 and 20) showed that ATh mutant 4 was the most suitable mutant for pyrimidine assay determinations. This conclusion was based on the following considerations: (a) either thiamine or pyrimidine were absolutely required by this mutant for growth; (b) pantothenate, methionine or thiamine-free yeast extract did not spare the thiamine or pyrimidine requirement, nor did they synergistically stimulate growth; and (c) ATh mutant 4 had the broadest standard curve of all the mutants, (i.e., the largest difference between the amount of pyrimidine required for maximal and minimal growth).

In addition to thiamine and pyrimidine, ATh mutant 4 also responds to bromomethylpyrimidine and the phosphorylated forms of thiamine. The aminomethyl- and methoxymethylpyrimidines and the phosphorylated derivatives of pyrimidine are not active in sparing the pyrimidine requirement of this organism. Pyrimidine sulfonate is only active at high concentrations and low oxygen tensions.

A single-cell isolate of ATh mutant 4 served as the source for the first stock culture of this organism and was designated as ATh mutant 4-1 to differentiate it from Dr. Ozeki's ATh mutant 4. The stock cultures were maintained on Nutrient Agar (Difco) slants which had been incubated at 38° for 18 to 24 hours and stored at 4°. Transfers were made at 4 to 6 week intervals.

As with L. viridescens, it was necessary to keep the "working" cultures of S. typhimurium at a high level of metabolic activity in order to obtain as rapid and reproducible assays as possible. This was accomplished by frequently transferring this mutant to fresh Nutrient Agar slants.

The inoculum was prepared by aseptically suspending one loopful of bacteria obtained from a fully-grown agar slant in approximately 10 ml. of sterile distilled water, centrifuging this suspension, resuspending the bacteria in sterile distilled water and diluting out the inoculum until only a very faint haze was discernible. One drop of this suspension was used to inoculate each assay tube.

The microbiological assay was prepared in the same manner as was the L. viridescens assay except that pyrimidine was substituted for thiamine in the standard curve tubes. The basal medium which was used is given in Table III. The assay tubes were sterilized by autoclaving for 10 to 12 minutes at 15 lb. pressure. Incubation was at 38° for 12 to 16 hours and growth was again measured turbidimetrically. The standard curve covered a range of 0 to 10 μμmoles of pyrimidine per assay tube (10 ml.) although the usable portion of this curve usually lay within the range of approximately 2 to 5 μμmoles.

Bioautographic techniques were as described in a previous section except that S. typhimurium medium was used and the autoclaving time was increased to 12 minutes. The inoculum which was used was much heavier than that which was used in the tube assays and had a light transmission of approximately 80%. It is interesting to note that when a smaller inoculum was used, the growth zones were very diffuse in size and difficult to distinguish against the slight opacity of the agar. The plates were incubated for 7 to 12 hours at 38°.

The S. typhimurium ATh 4-1 growth medium is that of Dr. Ozeki in which mannitol at the concentration shown below was substituted for glucose and to which thiamine-free yeast extract and a vitamin

TABLE III

Basal Growth Medium Used for the Assay of Pyrimidine by *Salmonella typhimurium* ATh 4-1[a]

Component	Amount/Liter Double Strength
K_2HPO_4	21 gm.
KH_2PO_4	9 gm.
Sodium citrate·$2H_2O$	0.94 gm.
$MgSO_4 \cdot 7H_2O$	0.10 gm.
$(NH_4)_2SO_4$	2.0 gm.
Adenine sulfate	68 mg.
d-Mannitol	20 gm.
Thiamine-free yeast extract	10 gm.
Vitamins[b]	10 ml.
Distilled water	to 1 liter

[a]The medium was adjusted to pH 6.9, a small amount of toluene was added and the medium was stored at 4°.

[b]See Table II.

supplement were added.

Microbiological assay of thiazole. -- The test organism used to assay for the thiazole portion of thiamine was a thiazole-requiring mutant of Escherichia coli obtained from Dr. Bernard Davis of the Harvard Medical School. This organism can also utilize either thiazole-P or thiamine in place of thiazole, although thiamine is required in considerably larger amounts than thiazole on a molar basis. The phosphorylated derivatives of thiamine have not been tested for biological activity.

The E. coli mutant stock cultures were maintained on glucose-yeast-extract agar slants which had been incubated at 38° for 12 to 24 hours and stored at 4°. Transfers were made at 4 to 6 week intervals. The "working" cultures of this thiazole-less mutant were kept at a high level of metabolic activity in order to obtain as rapid and reproducible assays as possible. This was accomplished by the daily transfer of this mutant to fresh glucose-yeast-extract agar slants for several days followed by less frequent transfers at 2 to 7 day intervals.

The inoculum was prepared in the same manner as was the S. typhimurium inoculum except that the washed cells were diluted to a much greater extent resulting in a suspension which was so dilute that it could only be seen by holding it up to a light source and comparing it with a tube of distilled water. The reproducibility of this assay and the amount of growth in the inoculated blanks are related to the size of the inoculum. For bioautographic studies, however, it was necessary to increase considerably the size of the inoculum until its

light transmission was approximately 70 to 80%. In this way, sharp and clearly defined zones of growth were obtained.

Tube assays were prepared in 25 mm. x 200 mm. pyrex culture tubes to which appropriate thiazole dilutions or dilutions of the samples being assayed were added. The volume of each tube was brought up to 5 ml. with distilled water and 5 ml. of double-strength medium (Table IV) were added. The tubes were covered with aluminum caps and sterilized by autoclaving them for 12 minutes at 15 lb. pressure. The tubes were then cooled, inoculated with one drop of inoculum, inclined at approximately a 45° angle on a reciprocal shaker at 38°, and shaken for 16 to 20 hours. When thiamine-free yeast extract was added to the medium the incubation time was decreased to approximately 10 to 12 hours. After the assay was fully grown, the samples were individually transferred to a smaller tube and growth was measured turbidimetrically as in the L. viridescens assay. The standard curve covered a range of approximately 0 to 180 μμmoles of thiazole per assay tube (10 ml.).

Bioautograms were prepared in the same manner as were those of S. typhimurium except that E. coli medium was used. The agar was seeded with the heavy inoculum discussed above and the plates were incubated at 38° for at least 16 hours. When thiamine-free yeast extract was added to this medium, the incubation time was reduced to approximately 10 hours, but unfortunately, the yeast extract permitted the background amounts of growth to increase to such a level that it was practically impossible to see the zones of growth.

The E. coli thiazole-less medium shown in Table IV is the

TABLE IV

Basal Medium Used for Assay of Thiazole with the Thiazole-Requiring Mutant of *E. Coli*[a]

Component	Amount/liter Double Strength
$Na_2HPO_4 \cdot 7H_2O$	12 gm.
KCl	8 gm.
K_2SO_4	2 gm.
Ammonium lactate	4 gm.
$MgSO_4 \cdot 7H_2O$	0.05 gm.
Glucose	4 gm.
(thiamine-free yeast extract)[b]	(10 gm.)
(Vitamins)[b]	(10 ml.)
Distilled water	to 1 liter

[a]The medium was adjusted to pH 7.6, a small amount of toluene was added and the medium was stored at 4°.

[b]Thiamine-free yeast extract and vitamins were sometimes added to the minimal medium in order to decrease the incubation time of the assays. See Table II for the composition of the vitamin solution.

minimal medium which Dr. Davis recommended for use with this organism. The additions of thiamine-free yeast extract and vitamins to this medium were occasionally made in order to decrease the incubation time of the assay. These additions however, were never made to the medium used for bioautographic studies (see discussion above).

Paper Chromatography. -- Ascending paper chromatograms were prepared in the customary manner by spotting appropriate solutions approximately $\frac{3}{4}$ to $1\frac{1}{4}$ inches apart and $1\frac{1}{2}$ inches from the bottom of large sheets of Whatman #1 paper (unless stated otherwise) and developing these chromatograms in an ascending fashion using one of a number of solvent systems. After development, the chromatograms were thoroughly air-dried and the different compounds on the paper were located either by noting zones of quenching of ultraviolet light or by bioautographic techniques.

Chromatograms were developed in the solvent systems listed below. These solvents are referred to in the Experimental section by the numerical designations shown.

Solvent 1. -- isobutyric acid-NH_4OH-H_2O -- Pabst (94)

99 ml. distilled water
3 ml. concentrated ammonium hydroxide (28%)
mix well
198 ml. isobutyric acid

Solvent 2. -- n-butanol-H_2O -- Wyatt (95)

258 ml. n-butanol
42 ml. distilled water

Solvent 3. -- 2-propanol-NH_4OH-H_2O -- Wyatt (95)

255 ml. 2-propanol
45 ml. distilled water
3.9 ml. concentrated ammonium hydroxide (28%)

Solvent 4. -- 2-propanol-HCl-H_2O -- Wyatt (95)

170 ml. 2-propanol
41 ml. concentrated hydrochloric acid (sp. gr. 1.19)
brought to a final volume of 250 ml. with distilled water

Solvent 5. -- n-propanol-H_2O-pH 5.0 acetate buffer -- Siliprandi et. al. (96)

210 ml. n-propanol
60 ml. distilled water
30 ml. 1 M acetate buffer, pH 5.0

Solvent 6. -- EtOH-pH 7.5 NH_4OAc -- Pabst (94)

7.7 gm. ammonium acetate
75 ml. distilled water
adjust to pH 7.5 with ammonium hydroxide and dilute to a final volume of 100 ml. with distilled water

90 ml. of the above ammonium acetate solution
210 ml. 95% ethanol

Solvent 7. -- (Na^+) phosphate-$(NH_4)_2SO_4$-n-PrOH -- Pabst (94)

120 gm. ammonium sulfate
200 ml. 0.1 (Na^+) phosphate buffer, pH 6.8
4 ml. n-propanol

METHODS OF PREPARING YEAST EXTRACTS

Cell-free yeast extract prepared in a Hughes Press. -- Several grams of fresh Anheuser-Busch baker's yeast were crumbled into liquid nitrogen and the frozen yeast was then processed in a Hughes press. The resulting broken-cell preparation was diluted to approximately twice its volume with cold 0.02 M (K^+) phosphate buffer, pH 6.9, and was thoroughly suspended using a chilled Potter-Elvehjem homogenizer. The cold suspension was centrifuged at 150,000 xg for 6 hours in a Spinco preparative ultracentrifuge at 4°. The resulting cloudy supernatant solution was filtered through coarse filter paper to remove some of the suspended lipid material. The filtrate was

divided into small batches, frozen and stored at -20°.

Acetone-powder preparation. -- Fifty gms. of fresh Anheuser-Busch baker's yeast were suspended in a minimal amount of distilled water and the thick slurry was slowly added with continuous stirring to 10 volumes of acetone at -20°. The suspension was stirred for an additional 5 to 10 minutes and then allowed to settle. The bulk of the supernatant acetone was discarded and the remaining acetone-yeast suspension was filtered at 4° on a Büchner funnel under a water-pump vacuum. The precipitate was washed with 2 to 5 volumes of fresh anhydrous acetone at -20°, and finally with 2 to 3 volumes of peroxide-free, anhydrous diethyl ether at -20°. After the filter-cake was reasonably dry, it was transferred to a piece of paper at room temperature where it was crushed, spread and mixed with a spatula until the ether odor was no longer detectable. Yield: 14.9 gm.

The acetone powder was then suspended in 140 ml. of 0.02 M (K^+) phosphate buffer, pH 6.9 (100 mg. of powder per ml.) and extracted overnight at 4°. The next morning, the suspension was centrifuged at 105,000 xg for 2 hours in a Spinco ultracentrifuge and the resulting clear supernatant solution was assayed for thiamine synthesizing activity and stored at -20°. Yield: 12.4 mg. of protein per ml.

Autolysis of yeast with toluene. -- (This procedure is a modification of the method devised by Meyerhof in 1927 which may be found in Methods in Enzymology (97)). Forty-five gm. of fresh Anheuser-Busch baker's yeast were thoroughly mixed with 24 ml. of toluene. The mixture was placed in a 45° water bath and continuously stirred until it had reached a temperature of 37 to 38°. The suspension was

then allowed to stand at room temperature (20 to 25°) for $3\frac{1}{2}$ hours with periodic stirring and was then chilled in an ice bath. Forty-eight ml. of cold 0.02 M (K^+) phosphate buffer, pH 6.9, were added to the chilled preparation and the resulting suspension was continuously stirred for 15 to 30 minutes. After storage overnight at 4°, the bottom aqueous layer was carefully siphoned off and was centrifuged at 105,000 xg for 2 hours in a Spinco ultracentrifuge. The resulting cloudy supernatant solution was assayed for thiamine synthesizing activity and stored at -20°. Yield: 26 mg. of protein per ml.

In subsequent large scale preparations, a mixture of 5 lbs. of yeast and 1,200 ml. of toluene was brought to a temperature of 37 to 38° as discussed above and was periodically stirred at room temperature for only $2\frac{1}{2}$ hours. The mixture was then immersed in an ice bath until it had attained a temperature of 5 to 8° (approximately 30 minutes) at which time 2,400 ml. of cold 0.04 M (K^+) phosphate buffer, pH 7.0 were added to the mixture and the suspension was adjusted to pH 7.0. Following this pH adjustment, the suspension was thoroughly mixed for 15 to 30 minutes and was stored at 4° overnight. In a repeat of this procedure, 0.04 M (Na^+) phosphate-0.02 M versene-0.02 M cysteine buffer, pH 7.0 was substituted for the above buffer as a result of finding that versene and cysteine markedly protect the enzyme(s) under investigation.

The cloudy extracts were clarified by filtration with the aid of Johns-Manville Hyflo-Super-Cel. For this purpose, the Hyflo-Super-Cel was added to the extract in amounts of 100 gm. per liter. The suspension was then thoroughly mixed for 5 to 10 minutes and filtered

at 4° on a large Büchner funnel under a water-pump vacuum. The filtrate was frozen and stored at -20° in small batches.

Autolysis of yeast with bicarbonate. -- (This procedure is a modification of a method which may be found in Methods in Enzymology (98)). Fifty gm. of fresh Anheuser-Busch baker's yeast were suspended in 40 ml. of 0.1 M sodium bicarbonate-0.02 M (K^+) phosphate buffer, pH 6.9. The suspension was then incubated in a water bath at 37° for $4\frac{1}{2}$ hours and dialyzed overnight in versene-treated cellophane dialysis tubing against 0.02 M (K^+) phosphate buffer, pH 6.9.

The dialyzed material was centrifuged at 105,000 xg for 2 hours in a Spinco ultracentrifuge. The cloudy supernatant solution was assayed for thiamine synthesizing activity and stored at -20°. Yield: 3.8 mg. of protein per ml.

Sonic extracts. -- Forty gm. of fresh Anheuser-Busch baker's yeast were suspended in distilled water and the volume was brought up to a final volume of 100 ml. The suspension was then treated for 60 minutes in a properly tuned, water cooled Raytheon 250W-10Kc sonic oscillator and centrifuged at 105,000 xg for 2 hours in a Spinco ultracentrifuge. The clear supernatant solution was assayed for thiamine synthesizing activity and stored at -20°. Yield: 22 mg. of protein per ml.

Treatment of dialysis tubing with versene. -- Prior to its use in dialysis procedures, all of the cellophane dialysis tubing was treated with versene to remove any heavy metal contaminants which might have been present in the tubing. This versene treatment was inaugurated at the suggestion of Dr. Edward Herbert of the Massachusetts Institute

of Technology. In the washing procedure, lengths of unwashed dialysis tubing were filled with distilled water, tied off at both ends, and dialyzed against a 0.025 M versene solution, pH 7-8, for a few days at 4°. The tubing was then extensively dialyzed against many changes of cold, deionized water and was stored in deionized water at 4° until it was used.

Preparation of DEAE columns. -- Eighteen gm. of DEAE (Eastman, #7392) were mixed with 500 ml. of 0.1 M versene and the suspension was adjusted to approximately pH 10 with 10 M sodium hydroxide. The suspension was stirred for 20 to 25 minutes, filtered through a glass wool plug in the bottom of a column and washed with 1,500 ml. of distilled water. The DEAE was resuspended in 500 ml. of 1 N sodium hydroxide, stirred for 15 to 20 minutes and allowed to settle for 10 minutes. The semi-clear supernatant suspension was filtered through a glass wool plug in the bottom of a column and then the remaining thick DEAE-slurry was added to the column and filtered. The column was washed with distilled water until the effluent was approximately pH 6-7. The DEAE was then resuspended in 500 ml. of distilled water, titrated to pH 7.0 with a saturated solution of sodium dihydrogen phosphate, cooled to 0 to 5° in an ice bath and vigorously stirred for one hour at 4° to break up any lumps which resulted from the previous filtration steps. The suspension was then allowed to settle for 10 minutes at 4°. The fine, slowly-settling material was decanted off and discarded, and the remaining DEAE-slurry was resuspended in 500 ml. of cold 0.005 M (Na^+) phosphate-0.005 M versene buffer, pH 7.0. This procedure was repeated until practically all the fine material had

been discarded.

Columns having sintered-glass bottoms were packed by successively pouring aliquots of a buffer-DEAE-slurry into the column and allowing the DEAE to settle under an air pressure of no more than that exerted by a water column 5 to 6 inches in height. Larger air pressures only serve to compress the DEAE into a smaller volume without any appreciable increase in the flow rate.

After the columns were packed, they were washed with 1,000 ml. of the above phosphate-versene buffer, closed off at the bottom and stored at 4° for no more than 24 hours before use.

Protein Determinations. -- Optical density readings at 260 and 280 mμ were most frequently used to calculate the amounts of protein present in the various samples. An excellent discussion of this method can be found in Methods in Enzymology (99). The formula which was used to calculate these protein concentrations is shown below:

$$\text{protein concentration (mg./ml.)} = 1.55(\text{O.D.}_{280m\mu}) - 0.76(\text{O.D.}_{260m\mu}).$$

Biuret protein determinations were made according to the method of Mokrasch and McGilvery(100). A fresh Bovine serum albumin (Pentex Corp.) solution was used as the standard.

Modified biuret protein determinations were performed according to the method of Lowry, et. al. (101). Bovine serum albumin was used as the protein standard.

The TCA protein determinations were carried out according to the method of Stadtman, Novelli, and Lipmann (102). The turbidity

measurements were made at 660 mμ in a Coleman Junior Spectrophotometer against a reagent blank. Bovine serum albumin was used as the protein standard.

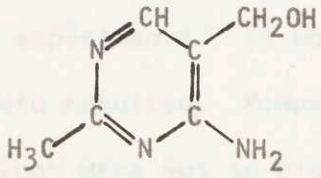
Phosphate determinations. -- Inorganic phosphate determinations were performed according to the procedure of Lowry and Lopez (103).

Total phosphate determinations were performed according to the procedure of Koerner and Sinsheimer (104).

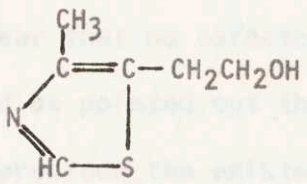
EXPERIMENTAL

PROPERTIES OF THE ENZYME SYSTEM

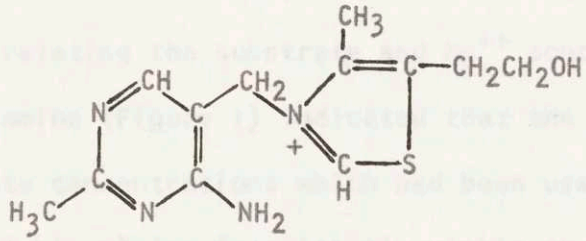
The enzyme system under investigation is the one which forms thiamine from its pyrimidine and thiazole moieties. The structural formulae for these compounds are shown below.



2-methyl-4-amino-5-hydroxymethyl-pyrimidine



4-methyl-5-(2-hydroxyethyl)-thiazole



thiamine

In the preliminary experiments reported in the first part of this section, an extract prepared from cells of baker's yeast which had been disrupted in a Hughes press was used as a source of enzymes. For later studies on the fractionation of these enzymes, extracts prepared from toluene autolyzed yeast preparations were used.

Substrate and cofactor requirements. -- In order to investi-

gate more completely the enzymatic formation of thiamine, it was necessary as a first step to define the minimum substrate and cofactor requirements of the enzyme system. The necessity for supplying ATP, Mg^{++}, pyrimidine and thiazole in order to achieve thiamine synthesis by an undialyzed Hughes press extract is shown in Table V. No increase in the production of thiamine was observed when additions of commercial yeast extracts or "boiled juice" were made to the reaction mixture. A dialyzed extract was used as a source of enzymes in these latter experiments. It would thus appear that no cofactors other than Mg^{++} were required. However, it should be pointed out that dialysis conditions were not so rigorous as to preclude the existence of a tightly bound cofactor. Future work on the purification of the individual enzymes will be necessary to settle this question.

Studies relating the substrate and Mg^{++} concentrations to the synthesis of thiamine (Figure 1) indicated that the arbitrarily selected substrate concentrations which had been used before this study (given in Table V) lay fortuitously within the range of good enzyme activity. As a result, these substrate and Mg^{++} concentrations have been retained in most of the subsequent work.

On the basis of the data presented in Figure 1, several interesting observations can be made: (a) that the approximate 1/1 correspondence of (ATP)/(Mg^{++}) tends to support current theories which postulate that an initial (Mg^{++}-ATP-enzyme) complex is a necessary prerequisite for ATP reactions; (b) that at ATP concentrations of 30 µmoles per ml., the formation of thiamine is still increasing linearly, while in many other ATP-requiring enzyme systems, concentrations in

TABLE V

Substrate and Cofactor Requirements for Thiamine Synthesis by Enzymes Prepared from Yeast

System	mμmoles of thiamine synthesized[a]
Experiment 1.	
Complete[b]	17.1
Omit ATP	0
Omit Mg^{++}	0
Omit pyrimidine	0
Omit thiazole	0
Experiment 2.	
Complete[c]	14.0
Omit Mg^{++}	0
Plus A.B.[d] yeast extract[e]	13.4
Plus Difco yeast extract[e]	12.9
Plus "boiled juice"[f]	10.2

[a] In Experiment 1, the undialyzed enzyme contained 0.8 to 1.0 mμmoles of thiamine/0.1 ml., and each value in this experiment has been corrected for this enzyme blank. In Experiment 2, thiamine blanks were determined for each sample. These blanks ranged from 0 mμmoles for the control sample to 1.7 mμmoles for the "boiled juice" sample.

[b] The incubation mixtures in Experiment 1 contained 0.1 ml. of undialyzed extract, 58 mμmoles of pyrimidine, 38 mμmoles of thiazole, 10 μmoles of neutralized ATP and 10 μmoles of $MgCl_2$ in a final volume of 1.0 ml. of 0.1 M phosphate buffer, pH 6.9. Incubations were at 37-38° for 3 hours. The reactions were stopped by immersing the tubes in a boiling water bath for a few minutes. Thiamine was measured by a microbiological assay using L. viridescens.

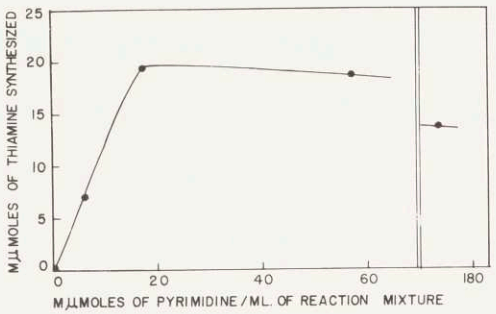
[c] Procedures, incubations and assay were as above using an extract which had been dialyzed overnight in versene-treated cellophane dialysis tubing against 500 volumes of 0.02 M phosphate buffer, pH 6.9.

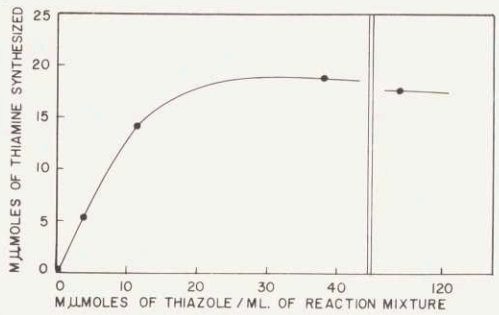
[d] A.B., Anheuser-Busch.

[e]1.0 mg. of yeast extract was added/ml. of reaction mixture.

[f]"Boiled juice" was prepared by boiling an undialyzed extract for 5 minutes followed by centrifugation to remove denatured protein. The "boiled juice" was used in amounts of 0.1 ml./ml. of reaction mixture.

Figure 1. Thiamine synthesis as a function of pyrimidine, thiazole, ATP and Mg^{++} concentrations. Procedures, incubations and assay were as in Table V varying the substrate and Mg^{++} concentrations one at a time as indicated in the figures. The thiamine values have been corrected for the enzyme blanks shown in Table V.


MμMOLES OF THIAMINE SYNTHESIZED
0
5
10
15
20
25
0
20
40
60
180
MμMOLES OF PYRIMIDINE / ML. OF REACTION MIXTURE


MμMOLES OF THIAMINE SYNTHESIZED
0
5
10
15
20
25
0
10
20
30
40
120
MμMOLES OF THIAZOLE / ML. OF REACTION MIXTURE

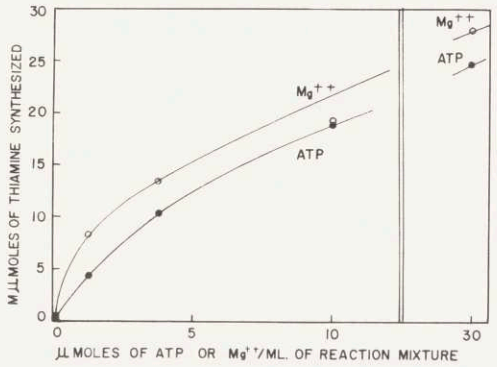

MμMOLES OF THIAMINE SYNTHESIZED
0
5
10
15
20
25
30
0
5
10
30
μMOLES OF ATP OR Mg++/ML. OF REACTION MIXTURE
Mg++
ATP
Mg++
ATP

FIGURE 1.

excess of 5 to 10 μmoles of ATP per ml. strongly inhibit enzyme activity; and (c) that the ATP and Mg^{++} requirements of the system were roughly 1,000 times larger than those of the pyrimidine and thiazole substrates (on a molar basis). Later work with purified enzyme preparations similarly showed the need for these relatively high ATP and Mg^{++} levels.

Temperature stability of the enzyme system. -- Studies on the stability of the enzyme system responsible for the synthesis of thiamine indicated that frozen (-20°) preparations were completely stable as determined (a) over a ten day period in the preliminary experiments using a Hughes press extract and (b) over a time period in excess of three months in later experiments using a toluene autolyzed preparation. Similarly, storage of the enzyme for 20 to 24 hours at 4° or for several hours at temperatures as high as 38° did not affect enzyme activity.

Thawing and refreezing of a Hughes press extract followed by storage at -20° detrimentally affected the enzyme and if repeated several times actually resulted in a complete loss of activity. Later work on purified enzyme preparations corroborated this finding.

Pretreatment of the extract at a temperature of 45° for 15 minutes resulted in an increased amount of thiamine synthesis, while exposure to temperatures of 60° or higher for short periods of time resulted in a rapid destruction of enzymatic activity. A possible explanation for the enhanced thiamine synthesis resulting from the 45° pretreatment might be that some inhibitory enzyme(s) (such as ATPase) had been inactivated during this procedure.

Dialysis and cysteine effects. -- Dialysis of the enzyme preparation for 16 hours at 4° against phosphate buffer (0.02M, pH 6.9) resulted in no loss of enzymatic activity. Dialysis against phosphate buffer containing 0.02 M cysteine not only resulted in no loss of activity, but actually an increase in activity of 50% was observed. This marked stimulation by cysteine was not observed when cysteine was merely added to the reaction mixture, nor was it as great when the enzyme was pretreated with cysteine for a short period of time. This has led to the conclusion that several hours contact with cysteine is required to stabilize one or more sulfhydryl groups on the enzyme(s) by keeping the sulfhydryl group(s) reduced and/or by removing an inhibiting heavy metal. The activating properties of cysteine were also observed in later work with purified enzyme fractions.

Effect of pH. -- Although it was realized that the extract was probably a mixture of several enzymes, each with its own pH optimum, it was nonetheless desirable to study the effect of pH on thiamine synthesis in order to choose a pH value which permitted maximal synthesis. Figure 2 is a plot of thiamine produced versus pH and indicates that a pH optimum probably exists at a pH higher than 6.8. Since good synthesis occurs at pH 7.4 and since any intermediate which might be formed during the biosynthesis of thiamine might be expected to be most stable at neutral pH values, pH 7.4 was selected for future work.

Effect of various buffers. -- A comparison of the results achieved by carrying out the thiamine synthesizing reaction in

Figure 2. Effect of pH on the enzymatic synthesis of thiamine. Incubations, procedures and assay as in Table V using an undialyzed Hughes press preparation in a reaction mixture 1/30 M with respect to sodium acetate, potassium phosphate and Tris at the pH's indicated in Figure 2. In these studies, the maximum amount of thiamine which could be formed was 38 mμmoles since only 38 mμmoles of thiazole were present in the reaction mixtures. The thiamine values have been corrected for the enzyme blanks shown in Table V.

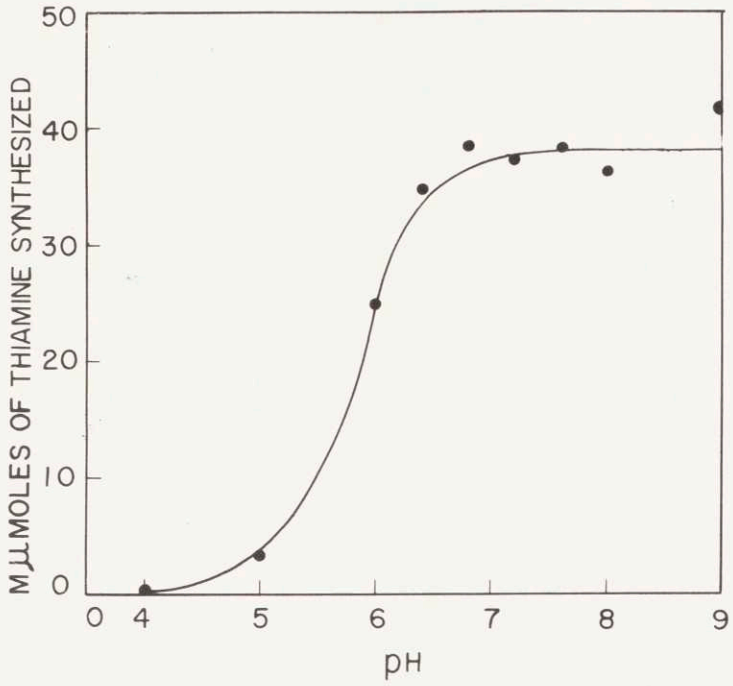


FIGURE 2.

several different buffers is shown in Table VI. The use of either Tris or imidazole buffer resulted in a better thiamine production than did the use of phosphate. The use of glycylglycine buffer, however, resulted in the best synthesis, the amount of thiamine produced being twice that produced in the presence of phosphate.

Three possible explanations for this enhanced thiamine synthesis were that there had been either (a) a stimulation of the assay organism; (b) a synergistic stimulation of the assay organism; or (c) a chelation of one or more heavy metal inhibitors. Additional investigations have indicated that the last possibility is the one which caused the effect. This was indicated when it was found that dialysis of the extract against versene eliminated the apparent buffer stimulation of thiamine synthesis. In addition, the assay organism was in no way stimulated by glycylglycine in experiments designed to check this possibility.

The rate of thiamine synthesis as a function of preincubation of substrates with the enzyme preparation. -- When pyrimidine was preincubated with the enzyme in the manner described in Figure 3, the rate of thiamine synthesis was increased from 50 to 100% over that noted in the control in which no preincubation was performed (Figure 3). Preincubation of thiazole with the enzyme in a similar manner resulted in no increased rate of thiamine synthesis. These results can be interpreted to indicate that an intermediate is formed from pyrimidine and ATP which is a more direct precursor of thiamine than is pyrimidine itself.

Although the preincubation studies described above did not

TABLE VI

Enzymatic Synthesis of Thiamine as a Function of Using Different Buffers in the Reaction Mixture[a]

Buffer Used	mμmoles of thiamine synthesized[b]	
	Experiment 1	Experiment 2
Phosphate	18.7	12.8
Imidazole	24.2	16.5
Tris	35.2	16.6
Glycylglycine	39.0	25.3

[a]Procedures, incubations and assay were as in Table V with reaction mixtures made up in the 0.1 M buffer indicated in the table at pH 7.4.

[b]The thiamine values have been corrected for the enzyme blanks shown in Table V.

Figure 3. Rate of thiamine synthesis as a function of preincubation of substrates with an enzyme preparation. Pyrimidine was preincubated for 1 hour at 37° in a complete reaction mixture lacking thiazole. At time "0", thiazole was added and the reaction mixtures were incubated at 37° for the times indicated in the figure. The composition of these reaction mixtures is described in Table V. Thiazole was similarly preincubated for 1 hour in the absence of pyrimidine and then pyrimidine was added at time "0". The control samples were complete reaction mixtures in which these preincubations had been omitted. The thiamine values have been corrected for the enzyme blanks shown in Table V.

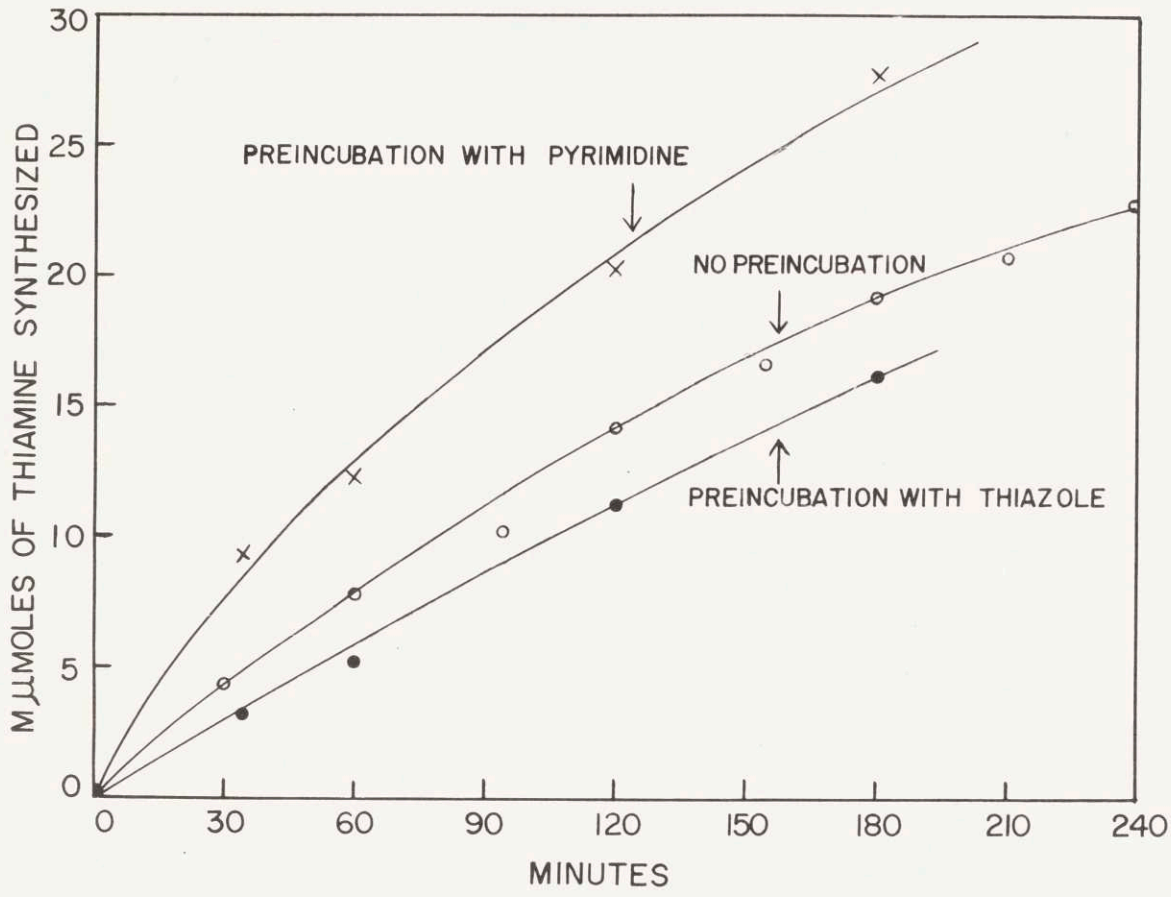

30
25
20
15
10
5
0
M μMOLES OF THIAMINE SYNTHESIZED
PREINCUBATION WITH PYRIMIDINE
NO PREINCUBATION
PREINCUBATION WITH THIAZOLE
0
30
60
90
120
150
180
210
240
MINUTES

FIGURE 3.

indicate the presence of any intermediate formed from thiazole, they likewise did not exclude the possibility that such an intermediate might be formed since in experiments of this type, an intermediate would not be expected to be detected unless its formation were a rate-limiting step in the sequence.

These preliminary findings were in agreement with the previously cited note by Harris, et. al. (84) who postulated that a single pyrimidine intermediate, the pyrimidine monophosphate, coupled directly with the thiazole moiety to yield thiamine and inorganic phosphate. Later work, however, showed that the coupling system was more complex than Harris had visualized. Pyrimidine-P is only one of several intermediates which can be demonstrated to exist.

FRACTIONATION OF THE ENZYME SYSTEM

Once some important and basic information about the enzyme system which forms thiamine had been obtained, it was possible to attempt the separation and purification of the enzymes. To this end a cell-free yeast preparation was separated into two enzyme fractions, both of which were necessary for the coupling of pyrimidine and thiazole to yield a compound with thiamine activity as measured microbiologically by L. viridescens. Data presented in this section describe how this fractionation was effected.

Thiamine synthesis as a function of enzyme concentration. -- A study relating thiamine synthesis to enzyme concentration was undertaken. This was performed in order to determine whether or not, during enzyme fractionation and purification, a correction factor would have to be employed in calculating purification and yields of enzyme activity. The results of this study (Figure 4) established the existence of such a linear relationship and eliminated the necessity for applying any correction factors.

Selection of a method for obtaining the enzyme system from yeast. -- The Hughes press procedure which was used to furnish enzyme for the preliminary experiments, yielded fairly active enzyme preparations. However, this method suffered from the serious drawback of being limited with respect to the amount of yeast which can be processed in a given time. As a result, several alternate methods of obtaining cell-free yeast preparations were investigated. The results of this study are summarized in Table VII.

The data presented in Table VII show that the extract prepared from the toluene autolyzed yeast had the highest specific activity of

Figure 4. Thiamine synthesis as a function of enzyme concentration. Incubations, procedures and assay were as in Table V using the above-indicated amounts of enzyme/ml. of reaction mixture. The thiamine values have been corrected for the enzyme blanks shown in Table V.

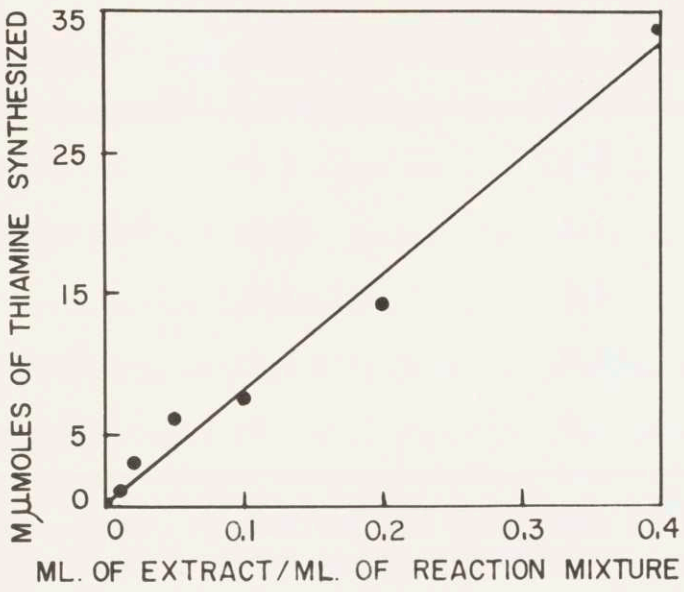

35
25
15
5
0
0
0.1
0.2
0.3
0.4
MμMOLES OF THIAMINE SYNTHESIZED
ML. OF EXTRACT/ML. OF REACTION MIXTURE

FIGURE 4.

TABLE VII

Selection of a Method for Obtaining the Coupling Enzymes from Yeast[a]

Extract prepared from	mμmoles of thiamine synthesized/ ml.of enzyme	Specific Activity (mμmoles of thiamine synthesized/mg. of protein[b])	Ratio of Specific Activities x 100
Hughes press[c]	15.1	3.8	100%
Toluene autolysate[c]	10.7	4.1	110%
Sonicate[c]	4.7	2.1	55%
Acetone powder[c]	0.3	0.25	7%
HCO_3^- autolysate[c]	0	0	--

[a]Procedures, incubations and assay were as in Table V using 0.1 ml. of each of the above enzyme preparations.

[b]The protein concentrations were determined using O.D.$_{260,280m\mu}$ measurements as discussed in the Methods section. Toluene dissolved in the toluene autolyzed extract was removed by dialysis prior to the protein determination.

[c]The extracts were prepared as indicated in the Methods section.

all the preparations which were tested. In addition, by the use of the toluene autolysis method, it is possible to process practically any amount of yeast in a relatively short period of time, thus overcoming the disadvantages of the Hughes press method. For these reasons, the toluene autolysis procedure was selected as the method for obtaining crude extracts of yeast for use in fractionation studies.

Ammonium sulfate fractionation studies. -- Several identical ammonium sulfate fractionation experiments were performed and the results obtained from these studies were in very close agreement one with the other. It is for this reason that the data from only one experiment is presented in this section.

The following general fractionation procedure was performed at 4° using cold solutions: a saturated ammonium sulfate solution was slowly added drop-wise (from a burette) with continuous stirring to 24 ml. of the filtered toluene autolyzed extract. After a certain volume of the ammonium sulfate solution had been added to the enzyme extract, the burette was closed off and the mixture was stirred for 5 to 10 minutes more to insure that the "salting out" equilibrium had been reached. The suspension was then centrifuged and the resultant clear supernatant solution was decanted into a beaker. The precipitate was dissolved in 6 mls. of 0.02 M phosphate buffer, pH 7.0, and temporarily stored in an ice bath. The procedure was then repeated until all of the desired fractions had been collected. These dissolved fractions were then placed in versene-treated cellophane dialysis tubing and dialyzed against first one liter and then two liters of

0.02 M phosphate-0.02 M cysteine buffer, pH 7.0 for a total of 12 hours at 4°.

The six fractions listed in Table VIII were collected using the procedure described above. After dialysis, the volume of each fraction was measured, protein concentrations were determined using O.D.260, 280mμ measurements (see Methods section) and enzymatic activities were assayed both individually and in combinations one with the other. This recombination method of assay was employed in order to ascertain whether any enzymatic separation had been achieved during the fractionation procedure. Such a separation would be reflected in higher amounts of thiamine synthesis than would be predicted on the basis of the sum of the individual fractions.

Recombination of the fractions listed in Table VIII did not result in any appreciable synergistic effect. In retrospect, this is rather surprising when one realizes that at least four enzymes are required for the coupling reaction to occur. Even more surprising and interesting is the fact that six enzymes (as shown in later work) were fractionated within the narrow 57.9 to 65% ammonium sulfate fraction.

Refractionation of a 57 to 67% ammonium sulfate fraction with ammonium sulfate did not result in any additional purification.

Because of the satisfactory results obtained with ammonium sulfate fractionation, this procedure was selected to serve as the first step in the purification scheme.

In subsequent large-scale ammonium sulfate fractionation procedures, a 57.9 to 65% ammonium sulfate fraction was obtained directly

TABLE VIII

Ammonium Sulfate Fractionation of Yeast Autolysate[a]

Fraction Number	Percent of saturation with $(NH_4)_2SO_4$	mg. protein[b]/ ml. of enzyme	mg. protein/ enzyme fraction	mμmoles of thiamine synthesized[c]/ ml. of enzyme	mμmoles of thiamine synthesized[c]/ enzyme fraction	Specific Activity (mμmoles of thiamine synthesized/mg.protein
Yeast Autolysate	-	-	490	-	2560	5.1
1	0 - 45	6.4	37	2	12	0.3
2	45 - 53.8	5.3	35	7.5	50	1.4
3	53.8 - 57.9	4.0	26	27	180	6.8
4	57.9 - 61.9	3.6	24	76	510	21
5	61.9 - 65	5.5	37	107	720	19
6	65 - 70	8.9	63	67	480	7.5
7	70% supernatant solution	1.5	230	0	0	-
			452 mg.(total) 92% recovery		1952 (total) 76% recovery	

[a]The ammonium sulfate fractionation procedure is indicated in the text.

[b]The protein concentrations were determined using $O.D._{260,\ 280m\mu}$ measurements (see Methods section).

[c]Incubations, procedures and assay were as in Table V using 0.2 ml. of each of the protein fractions indicated above. 0.1 M glycylglycine buffer, pH 7.4 was substituted for the phosphate buffer.

without taking any intermediate fractions, although the general procedure which was followed was essentially a scaled-up version of the procedure discussed above. For this purpose, 289 ml. of a cold, saturated ammonium sulfate solution were added at a rate of approximately 3 ml. per minute with continuous stirring at 4°, to 210 ml. of filtered toluene autolysate (see Methods section). The suspension was then stirred for an additional 5 to 10 minutes and centrifuged. To the resulting clear supernatant solution, 101 additional ml. of the cold saturated ammonium sulfate solution were added in the same manner as above and the suspension was centrifuged. The precipitate was dissolved in either 0.02 M phosphate buffer, pH 7.0, or 0.02 M phosphate-0.02 M versene-0.02 M cysteine buffer, pH 7.0. This 57.9 to 65% ammonium sulfate fraction was then dialyzed and used for subsequent purification studies.

This large-scale ammonium sulfate fractionation procedure which has been performed many times has proved to be very reproducible, yielding results very similar to those presented in Table VIII, with the exception that only a 2.5 to 3 fold purification of the enzyme system was obtained.

Calcium phosphate gel fractionation studies. -- Several experiments were performed using calcium phosphate gel in conjunction with an extensively dialyzed 57.9 to 65% ammonium sulfate fraction in both differential adsorption and elution procedures. The results obtained from this series of experiments were completely unsatisfactory. Although both enzyme-gel adsorption and elution had been obtained, neither purification nor separation of the coupling enzyme

system resulted from the use of these procedures.

Fractionation on DEAE columns. -- Fifty ml. of the 57.9 to 65% ammonium sulfate fraction (obtained from 420 ml. of autolysate) were dialyzed in thin-walled versene-treated cellophane dialysis tubing against several changes of buffer at 4°. The first dialysis was against 40 volumes of 0.005 M phosphate-0.005 M versene-0.020 M cysteine buffer, pH 7.0 for $5\frac{1}{2}$ hours. The tubing was then rinsed in distilled water and the enzyme was dialyzed against 40 volumes of fresh phosphate-versene-cysteine buffer (at the same concentration as was used in the first dialysis) for 12 more hours. Lastly, the tubing was again rinsed in distilled water and the enzyme was further dialyzed for 12 additional hours against 0.0044 M phosphate-0.0044 M versene-0.0044 M cysteine buffer, pH 7.0. The resulting 61 ml. of enzyme were centrifuged to remove a small amount of precipitated material, and 44 ml. of this centrifuged enzyme (containing 34.1 mg. of protein per ml.) were applied to the DEAE column (see below). The remainder of this centrifuged enzyme was stored at -20° for subsequent assay and future use.

The DEAE column was prepared according to the general directions given in the Methods section. Specifically, 15 to 18 gm. of DEAE were packed into a 2.2 cm. diameter column to a height of 35 cm. The column was operated at a temperature of 4° using cold, freshly-prepared reagents and at a flow rate of 2.5 ml. per minute. At no time during the overall procedure was the liquid level above the column allowed to fall below the glass wool layer in the top of the column.

Just before use the column was given a final wash with 500 ml. of 0.005 M phosphate-0.005 M versene-0.005 cysteine buffer, pH 7.0, as indicated in the Methods section and the eluate was discarded. Forty-four ml. of the dialyzed, centrifuged enzyme containing a total of 1.5 gm. of protein were then put on the column and the eluate was collected in 10 ml. fractions. Following this enzyme adsorption step, the column was washed with 200 ml. of the phosphate-versene-cysteine buffer described above. The eluate was again collected in 10 ml. fractions, as were all subsequent eluates.

At this point in the procedure, a sodium chloride-cysteine logarithmic gradient elution of the column was begun. In this type of elution a more concentrated solution contained in a reservoir is slowly and continuously added to a less-concentrated solution which is being mixed by a magnetic stirring bar in a mixing chamber. From the mixing chamber the resulting solution which becomes increasingly more concentrated is continuously fed into the column. The critical invariable in this procedure is the maintenance of a constant liquid volume in the mixing chamber throughout the entire elution. Experimentally, this is accomplished by using a closed system in which air tight connections are made between the reservoir and the mixer, and between the mixer and the column. In this manner the inflow from the reservoir is kept equal to the outflow to the to the column.

Mathematically, the elution is described by the equation:

$$\frac{V_{out}}{V_{mixer}} = -\ln \left[1 - \frac{C_{out}}{C_{reservoir}} \right]$$

where "V" refers to volume, "C" refers to concentration, and "out" refers to that liquid which leaves the mixer. A semi-log paper plot of

$$\left[1 - \frac{C_{out}}{C_{reservoir}}\right] \text{ vs. } \frac{V_{out}}{V_{mixer}}$$

results in a straight line curve which can be used to calculate and predict concentration-volume changes which occur during the logarithmic elution.

Experimentally, the mixing chamber initially contained 1,000 ml. of 0.005 M (Na^+) phosphate-0.005 M versene-0.005 M cysteine buffer, pH 7.0. This buffer was also 0.005 M with respect to sodium chloride which resulted from the neutralization of the cysteine·HCl. The reservoir initially contained 1,500 ml. of 0.005 M (Na^+) phosphate-0.005 M versene-0.040 M cysteine-0.130 M sodium chloride buffer, pH 7.0. The final sodium chloride molarity of the reservoir buffer included the cysteine·HCl contribution.

After the reservoir buffer had been depleted, the gradient elution was stopped and the column was given a final wash with 100 ml. of 0.005 M (Na^+) phosphate-0.005 M versene-0.020 M cysteine-0.12 M sodium chloride buffer, pH 7.0. Again, this sodium chloride molarity included the contribution from the cysteine·HCl.

As the elution fractions were collected, 0.2 ml. of a 1.0 M cysteine solution, pH 7.0 was added to each fraction to give a final cysteine molarity of 0.020 M plus whatever came through the column. The fractions were then stored at 4° for several days until all the necessary enzymatic assays had been completed and until the fractions had been pooled into several subgroups.

Preliminary experiments have established the necessity of taking certain precautionary measures in the operation of this column procedure. These measures which have been incorporated into the procedure presented above, include (a) the initial washing of the DEAE with versene to remove any heavy metal contaminants, (b) the extensive dialysis of the ammonium sulfate fractionated enzyme preparation against a dilute buffer prior to its adsorption on the DEAE, (c) the incorporation of both cysteine and versene into the elution buffers, and (d) the periodic addition of cysteine to the eluted enzyme fractions during storage at 4°. Failure to observe one or more precautionary measures will result in the rapid loss of enzymatic activity and/or the incomplete adsorption of the enzyme to the DEAE.

The initial enzymatic assays of the eluted fractions were primarily concerned with establishing that a separation of the enzyme system had been realized. Such a separation would be reflected in the finding of (a) little or no thiamine synthesizing activity in any one single fraction and (b) appreciable thiamine synthesizing activity in fractions which had been assayed in combination with other appropriate fractions. When these assays were performed, it was found that separation had been obtained. Subsequent assays were mainly concerned with repeating the above experiments and with locating more precisely the fractions which contained the enzymatic activity. The results of these many assays have been summarized in Figure 5.

The first recombination experiments were designed to show in a qualitative manner whether an enzymatic separation had been obtained

and if so, what eluate fractions contained the separated enzymes. This was accomplished by assaying the fractions obtained from this DEAE column in the presence of appropriate fractions obtained from a previous smaller scale experimental column. More specifically, 0.2 ml. of every third fraction from number 3 through number 90 were assayed in combination with a 0.1 ml. aliquot of an enzyme fraction from the smaller column which contained the second eluting fraction. Also, 0.2 ml. of every third fraction from number 63 through number 175 were assayed in combination with a 0.2 ml. aliquot of an enzyme fraction from the small column which contained the first eluting enzyme or enzymes. In other words, the first or second eluting enzyme fraction required for the synthesis of thiamine was located by assaying suspected fractions in combination with the second or first eluting enzyme, respectively, which were obtained from a previous experimental column. Subsequent recombination experiments were performed in exactly the same manner except that enzyme fractions obtained from this DEAE column were used in place of the first and second eluting enzyme fractions obtained earlier.

In addition to the recombination experiments, the amounts of unseparated thiamine synthesizing activity present in the various eluate fractions was determined. These assays were performed by separately incubating 0.2 ml. of every third eluate fraction, and the results of this experiment are shown in the figure as Curve 3.

It can be noted from Figure 5 that the thiamine synthesizing enzyme system had been separated into two fractions, both of which were required for the synthesis of thiamine. As a consequence, these

Figure 5. The elution pattern of the enzyme fractions separated on the DEAE column. Incubations, procedures and assay were performed as indicated below using 0.2 ml. of the various eluted fractions either singly or in combination with other fractions as discussed in the text.

Curve 1 = reaction mixtures which contained a constant amount of enzyme Fraction 2. Thus, Curve 1 represents the distribution of enzyme Fraction 1 in the column eluate. The first enzyme peak in Curve 1 represents that portion of enzyme Fraction 1 which was not initially adsorbed to the DEAE and which immediately eluted from the column with the first buffer washings. The second enzyme peak in Curve 1 represents enzyme Fraction 1 which was adsorbed and chromatographed.

Curve 2 = reaction mixtures which contained a constant amount of enzyme Fraction 1. Thus, Curve 2 represents the distribution of enzyme Fraction 2 in the column eluate.

Curve 3 = reaction mixtures which contained only a single enzyme eluate fraction, thus measuring the amounts of unseparated thiamine synthesizing activity present in the eluate fractions.

The incubation mixtures contained an amount of enzyme specified in each experiment, 58 mμmoles of pyrimidine, 38 mμmoles of thiazole, 10 μmoles of neutralized ATP, 100 μmoles of $MgCl_2$ in a final volume of 1 ml. of 0.1 M glycylglycine buffer, pH 7.4. Incubations were at 37 to 38° for three hours. The reactions were stopped by immersing the tubes in a boiling water bath for a few minutes. Thiamine was measured microbiologically with L. viridescens. In later experiments, 58 mμmoles of various pyrimidine or thiamine compounds were substituted for pyrimidine.

The $MgCl_2$ concentration was arbitrarily increased 10-fold in an attempt to overcome the expected increased chelation properties of the incubation mixture -- brought on by the change to glycylglycine buffer and by the inclusion of versene in the enzyme preparations. Later work showed that this concentration increase of $MgCl_2$ was not only unnecessary but was actually somewhat inhibitory to thiamine synthesis. However, it was not possible to check this point for some time.

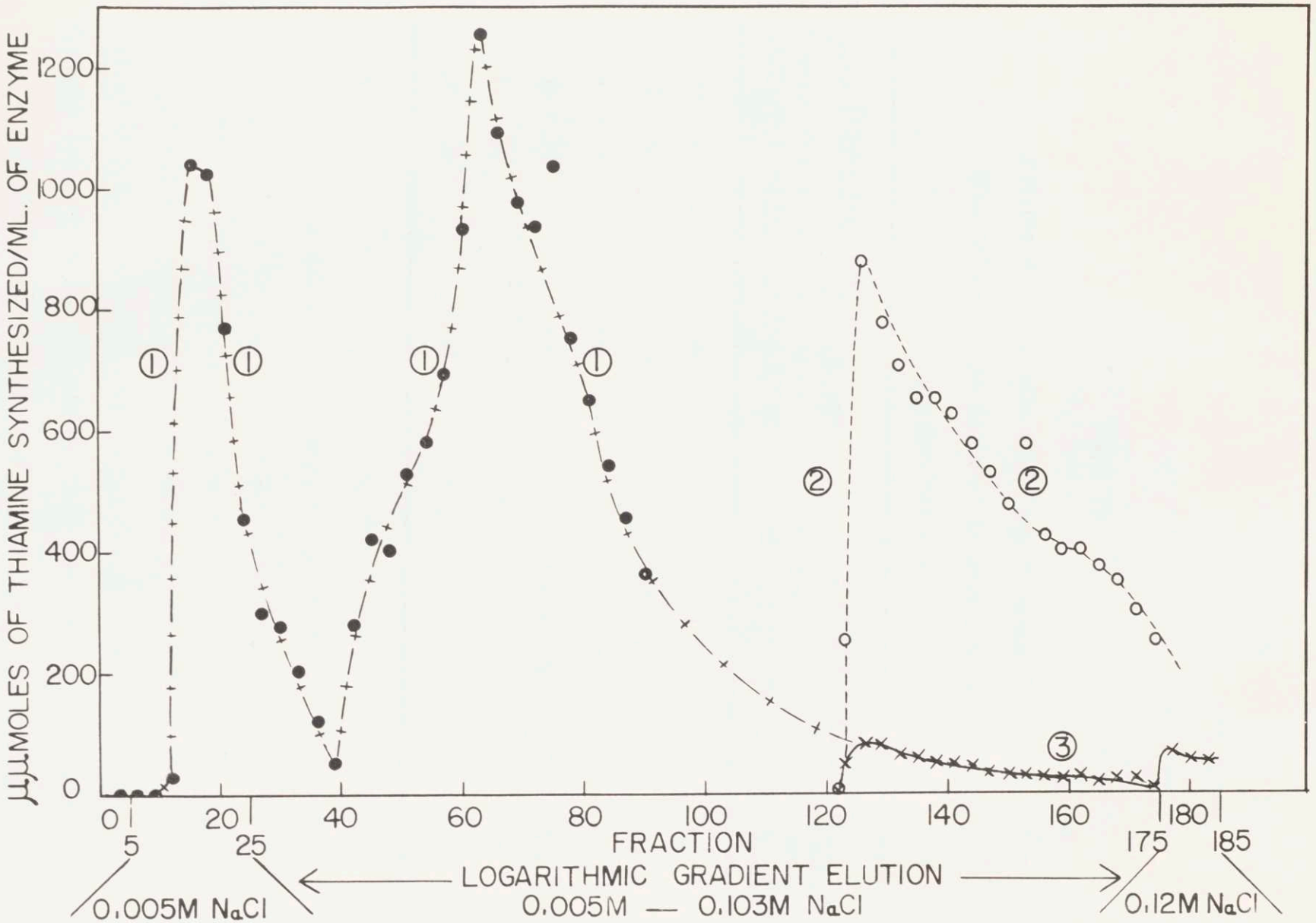

μμMOLES OF THIAMINE SYNTHESIZED/ML. OF ENZYME
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
160
180
5
25
175
185
FRACTION
LOGARITHMIC GRADIENT ELUTION
0.005M NaCl
0.005M — 0.103M NaCl
0.12M NaCl
①
②
③

FIGURE 5.

TABLE IX

Pooling of the Eluate Fractions Obtained from the DEAE Column on the Basis of Enzymatic Activity and Elution Pattern

Enzyme Fraction 1		Enzyme Fraction 2	
Elution fractions which were pooled Number	Approximate volume of pooled group ml.	Elution fractions which were pooled Number	Approximate volume of pooled group ml.
14 - 27	140	124 - 140	160
28 - 40	130	141 - 156	150
41 - 58	180	157 - 175	190
59 - 76	180	176 - 185	100
77 - 90	140		
90 -120	300		

two separated fractions were, for the sake of convenience, designated as Fraction 1 and Fraction 2 based on their relative order of elution from the column. This numerical designation later proved to be very fortuitous as coincidentally, it was found that these enzymes sequentially catalyzed the formation of thiamine in the same numerical order. However, later investigations also revealed that more than two enzymes are involved in the thiamine synthesizing system and that Fraction 1 and Fraction 2 as described above, each actually contain more than one enzyme. Nevertheless, these enzymatic notations have been retained throughout the remaining investigations.

Because of serious difficulties encountered in the many attempts to measure the protein concentrations of the separated enzyme fractions (to be discussed shortly) it was impossible to calculate the enzyme purification. As a result, the fractions were pooled into several groups only on the basis of their relative activities and their relative positions in the elution scheme. The reason for pooling only consecutive fractions into any one group was that in the eventuality that it was found that more than two enzymes were involved in the synthesis of thiamine, there would be a good chance of finding these enzymes partially separated in one of the groups. This pooling operation is summarized in Table IX.

After pooling, a sufficient amount of a 1.0 M cysteine solution at pH 7.0 was added to each pooled group to increase their cysteine concentrations by 0.020 M. This addition was intended to be an added safeguard against enzymatic inactivation, and to compensate

for subsequent losses of cysteine which was oxidized to cystine. The resulting pooled enzyme groups were frozen and stored in small batches to prevent excessive thawing and refreezing of any one sample, and served as a source of the coupling enzymes throughout the remaining investigation.

Recovery and purification determinations. -- After the DEAE fractions had been pooled, enzyme recovery experiments were performed by assaying one enzyme fraction, in the presence of an excess of the second enzyme fraction, for thiamine synthesizing activity. However, later experiments showed that several enzymes were involved in the overall biosynthesis of thiamine and since it was not clear whether each enzyme had really been assayed in the presence of an excess of each of the other enzymes, these recovery values were meaningless.

Attempts to measure the protein concentrations of the pooled samples also gave unsatisfactory results. The protein concentrations of the samples were too low to be measured by either the Biuret, optical, or TCA methods, or by the contemplated use of microbiological and chemical assays for specific amino acids. Furthermore, both the cysteine and versene present in the samples interfered with the Biuret and Lowry protein determinations and cysteine would also be expected to interfere with a ninhydrin assay.

No further attempts were made to measure the protein concentrations or enzymatic recoveries since the main object of these investigations was to define in a qualitative manner, the biosynthetic pathway involved in the formation of thiamine.

Stability of the enzymes to storage, lyophilization and temperature. -- Studies on the stability of a toluene autolyzed yeast preparation, a 57.9 to 65% ammonium sulfate fraction, and the fractions from DEAE, indicated that the thiamine synthesizing enzymes were completely stable at a temperature of -20° as determined over 1 to 3 month time intervals. As was observed with the Hughes press preparation, repeated thawing and refreezing of these enzyme fractions resulted in a marked loss of enzymatic activity.

Lyophilization of Fractions 1 and 2 did not affect their enzymatic activities, and this procedure was frequently used in later experiments to concentrate these enzymes.

Incubation of Fractions 1 and 2 at the various temperatures shown in Table X followed by testing for residual thiamine synthesizing activity of each, showed that activity present in Fraction 1 was rapidly destroyed at 50° while activity in Fraction 2 was able to withstand 55° temperatures for 5 minutes with no effect. As a result of this observation, it was possible to destroy the trace of Fraction 1 present as a contaminant in Fraction 2. This was accomplished by heating contaminated Fraction 2 to 55° for 5 minutes followed by rapid cooling in an ice bath.

Influence of ATP and Mg^{++} concentrations on thiamine synthesis using partially purified enzymes. -- Studies relating the ATP and Mg^{++} concentrations to the synthesis of thiamine using the DEAE-separated enzyme fractions showed that the conclusions obtained in the preliminary enzyme studies using a Hughes press extract were applicable to the partially purified DEAE-separated enzyme fractions. The only

TABLE X

Temperature Stability of Enzymatic Activities in Fractions 1 and 2[a]

Enzyme treatment		mμmoles of thiamine synthesized after treatment	
Temp.	Minutes	Fraction 1	Fraction 2
--	--	6.0	6.0
50°	5	0	6.0
50°	15	0	6.0
55°	5	0	5.9
55°	15	0	4.5
60°	5	0	0

[a]0.2 ml. aliquots of Fractions 1 and 2 were separately incubated for the times and at the temperatures indicated in the table and were chilled in an ice bath. 0.2 ml. aliquots of untreated Fraction 1 were added to the tubes containing treated Fraction 2 and vice versa. Pyrimidine, thiazole, ATP, magnesium and buffer were added to the tubes and the incubations and assay were performed as described in Figure 5.

new information which was gained from this experiment was that Mg^{++} concentrations of 100 μmoles per ml. caused approximately a 20 to 25% decrease in thiamine synthesis as compared to Mg^{++} concentrations of 10 μmoles per ml.

REACTIONS AND INTERMEDIATES IN THE BIOSYNTHESIS OF THIAMINE AND ITS PHOSPHATE ESTERS

Having obtained a separation of the thiamine synthesizing enzyme system into two fractions both of which were necessary for the biosynthesis of thiamine, it was now possible to attempt to define the intermediates and enzymes involved in the conversion of pyrimidine and thiazole to thiamine and its phosphate esters.

Pyrimidine compounds used as substrate for thiamine synthesis. -- The effectiveness with which various pyrimidine compounds can be used for the synthesis of thiamine was tested. The results, given in Table XI, show (a) that bromomethylpyrimidine was as good a substrate as pyrimidine itself, (b) that methoxymethyl- and aminomethylpyrimidines were almost as good as pyrimidine, and (c) that pyrimidine sulfonate was a very poor substrate. When thiazolemethylpyrimidine (thiamine itself) was incubated with only Fraction 1 under the same experimental conditions as above, a 30% decrease in thiamine activity was observed. Later work showed that this decrease was partially due to the formation of pyrimidine compounds from thiamine.

The most logical explanation for these results was that a hydrolase type of enzyme had acted on the different pyrimidine derivatives to yield free pyrimidine which could then be used in the normal manner for thiamine synthesis.

To test this hypothesis, aminomethyl- and methoxymethylpyrimidines were incubated for 3 hours at 37° with Fraction 1, magnesium and glycylglycine buffer, pH 7.4. ATP was omitted from the reaction mixtures. The incubated samples were then spotted on chromatographic

TABLE XI

Thiamine Synthesis as a Function of Using Different Pyrimidine Compounds as Substrates

Pyrimidine substrate	mμmoles of thiamine synthesized	
	Experiment 1[a]	Experiment 2[b]
Pyrimidine	3.6	3.2
Bromomethylpyrimidine	3.9	3.3
Methoxymethylpyrimidine	3.9	2.3
Aminomethylpyrimidine	2.6	- [c]
Pyrimidine sulfonate	0.2	- [c]

[a] In Experiment 1, incubations, procedures and assays were performed as in Figure 5 using 0.2 ml. aliquots of enzyme Fraction 1 and 0.3 ml. aliquots of Fraction 2. The amount of pyrimidine derivative used in each vessel was the same as the amount of pyrimidine which was used, on a molar basis.

[b] In Experiment 2, pyrimidine or the pyrimidine derivatives were preincubated for 2 hours and 15 minutes at 37° with 0.3 ml. aliquots of enzyme Fraction 1 and with Mg^{++}, ATP and buffer in the amounts described in Figure 5 in a volume of 0.6 ml. The reaction was stopped by immersing the tubes in a boiling water bath for a few minutes and the tubes were cooled. Thiazole (concentration given in Figure 5) and 0.3 ml. aliquots of Fraction 2, which had been pretreated at 55° for 5 minutes to destroy Fraction 1 activity present as a contaminant, were added to the cooled samples and these complete reaction mixtures were incubated at 37° for 3 hours. The reactions were stopped and assays were performed as described in Figure 5. This experiment was performed sometime after Experiment 1 and after preincubation studies (to be presented shortly) were made.

[c] Aminomethylpyrimidine and pyrimidine sulfonate were not tested in Experiment 2.

paper, the chromatograms were developed, and bioautograms were prepared using S. typhimurium ATh mutant 4-1 as the test organism.

Aminomethyl- and methoxymethylpyrimidine were chosen to serve as substrates in these studies because these compounds are not active in replacing pyrimidine as a growth factor for the mutant, thus making it much easier to demonstrate the conversion of these non-active pyrimidine derivatives to the active pyrimidine. It was not as feasible to use bromomethylpyrimidine or thiamine in these studies as both are active for the test organism and the R_f values of these compounds in the solvents used are so close to that of pyrimidine that it would have been difficult to decide whether any conversion had taken place.

The results of these studies (Figure 6) showed that an enzyme was present in Fraction I which had, in effect, acted to remove by hydrolysis the amino and methoxy groups from the pyrimidines used, to yield a compound which microbiologically and chromatographically was identical with pyrimidine. Although the precise mechanism by which this enzyme acts is unknown, it has been tentatively named "pyrimidine hydrolase" because of its apparent action.

The activity of this apparent hydrolase towards thiamine and the pyrimidine derivatives raises several interesting questions: (a) is this enzyme specific for the pyrimidine molecule or is it merely a cellular hydrolase with a broad substrate specificity; (b) is the pyrimidine moiety of thiamine biosynthesized as a pyrimidine derivative and, if so, is such a hydrolase required to liberate free pyrimidine so that thiamine may be formed; and (c) how closely does this

Figure 6. The bioautographic detection of a pyrimidine hydrolase. The figure is a drawing of one of the bioautograms obtained from a chromatogram spotted with 0.002 ml. of the reaction mixtures described in the text as well as the reference pyrimidines indicated in the figure. The chromatogram was developed for 13 hours at room temperature in Solvent 1 (see Methods section). The spot sizes shown in the figure have been proportionately scaled down to the size of the figure.

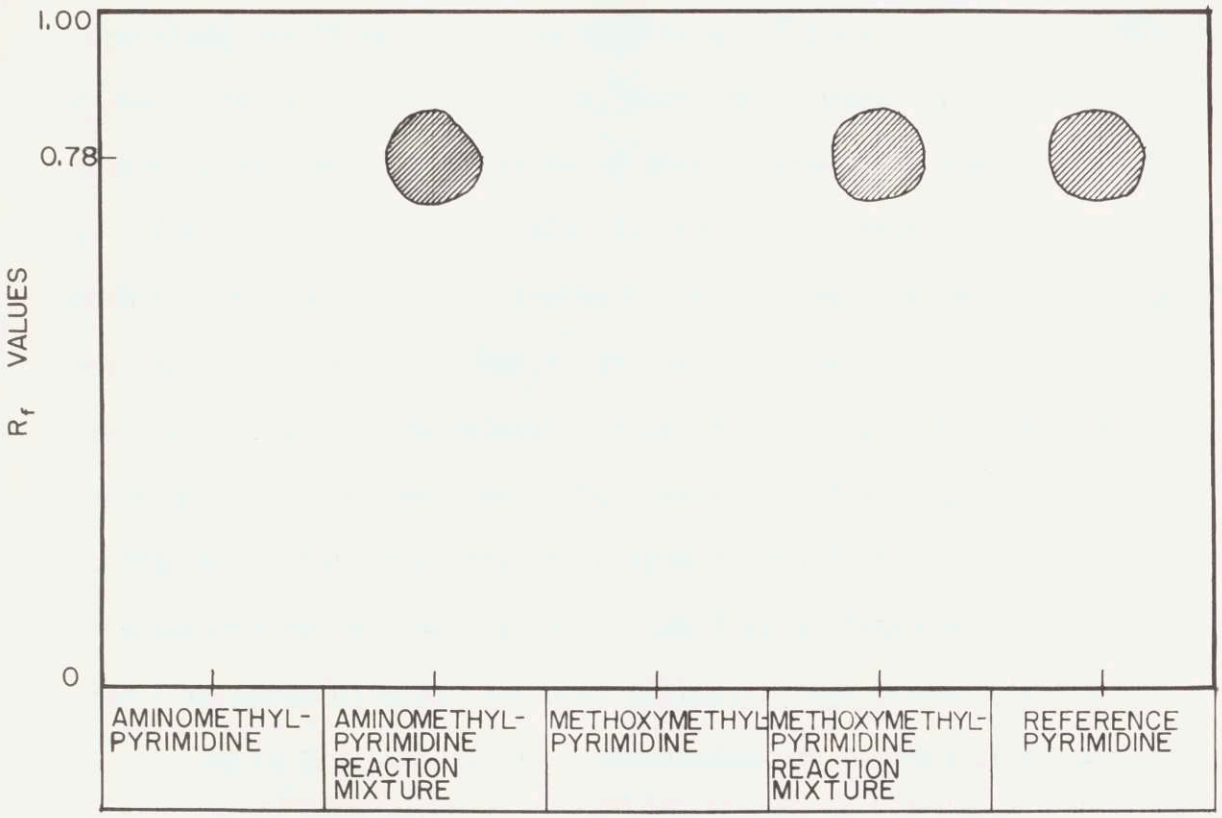

1.00
0.78
0
R_f VALUES
AMINOMETHYL-PYRIMIDINE
AMINOMETHYL-PYRIMIDINE REACTION MIXTURE
METHOXYMETHYL-PYRIMIDINE
METHOXYMETHYL-PYRIMIDINE REACTION MIXTURE
REFERENCE PYRIMIDINE

FIGURE 6.

enzyme resemble the thiaminases described in the Introduction and do the thiaminases also have as wide a range of substrate specificity as does this enzyme? Further work is needed to answer these questions.

Preincubation studies with Fractions 1 and 2. -- Results presented earlier indicated that in thiamine synthesis, a pyrimidine intermediate was formed from the incubation of pyrimidine and ATP with a crude extract. As a consequence, experiments were designed to determine whether the formation of this intermediate was catalyzed by enzyme Fraction 1 or Fraction 2. For this purpose, either one or both of the fractions were preincubated with pyrimidine, ATP and Mg^{++}, and then thiazole and either of the omitted fractions were added. The mixtures were then reincubated and thiamine synthesis was followed as a function of time. The results of these studies, presented in Figure 7, indicated that the enzyme responsible for the formation of a pyrimidine intermediate,which could be utilized more effectively than free pyrimidine for thiamine synthesis, was present in Fraction 1, but not in Fraction 2. This conclusion was reached after noting that in those pyrimidine reaction mixtures which had been preincubated with either Fraction 1 or Fractions 1 and 2, the rate of thiamine synthesis was greater than the rate of synthesis obtained in the control samples in which this preincubation had been omitted.

Similar experiments were performed by preincubating either one or both fractions with thiazole, ATP and Mg^{++} followed by the addition of pyrimidine and either of the omitted enzyme fractions. Under these conditions, no evidence was obtained for the formation of a thiazole intermediate which, in the system used would be utilized

Figure 7. Rate of thiamine synthesis as a function of preincubation of substrates with DEAE Fractions 1 and 2. The composition of the samples is shown below. The components enclosed in the parentheses (below) were preincubated for 1 hour at 37° in complete reaction mixtures (described in Figure 5) which lacked the components shown outside of the parentheses. After the preincubation, at time "zero," the lacking components were added to the preincubation reaction mixtures, the complete reaction mixtures were incubated at 37°, and the amounts of synthesized thiamine were determined at the time intervals shown in the figure. 0.2 ml. aliquots of Fraction 1 and Fraction 2 were used in the reaction mixtures. Thiamine was assayed microbiologically with L. viridescens.

Sample No.	Preincubation and incubation sequence (see above)
1	pyrimidine + thiazole + Fraction 1 + Fraction 2 -- control
2	(pyrimidine + Fraction 2) + thiazole + Fraction 1
3	(pyrimidine + thiazole + Fraction 2)+ Fraction 1
4	(pyrimidine + Fraction 2) + (thiazole + Fraction 1)
5	(pyrimidine + Fraction 1) + thiazole + Fraction 2
6	(pyrimidine + thiazole + Fraction 1) + Fraction 2
7	(thiazole + Fraction 1) + pyrimidine + Fraction 2
8	(thiazole + Fraction 2) + pyrimidine + Fraction 1
9	(pyrimidine + Fraction 1) + (thiazole + Fraction 2)

Curve 1 = a control with no preincubation as described by Sample 1 above. Samples 2, 3, and 4 also yielded the same curve.

Curve 2 = preincubation of pyrimidine with Fraction 1 followed by incubation with thiazole and Fraction 2 as shown in Sample 5 above. Sample 6 also yielded the same curve.

Curve 3 = preincubation of thiazole with either Fraction 1 or 2, followed by incubation with pyrimidine and either Fraction 2 or 1, respectively, as shown in Samples 7 and 8 above.

Curve 4 = separate preincubations of pyrimidine with Fraction 1 and thiazole with Fraction 2 followed by the incubation of preincubated pyrimidine with preincubated thiazole as described in Sample 9 above.

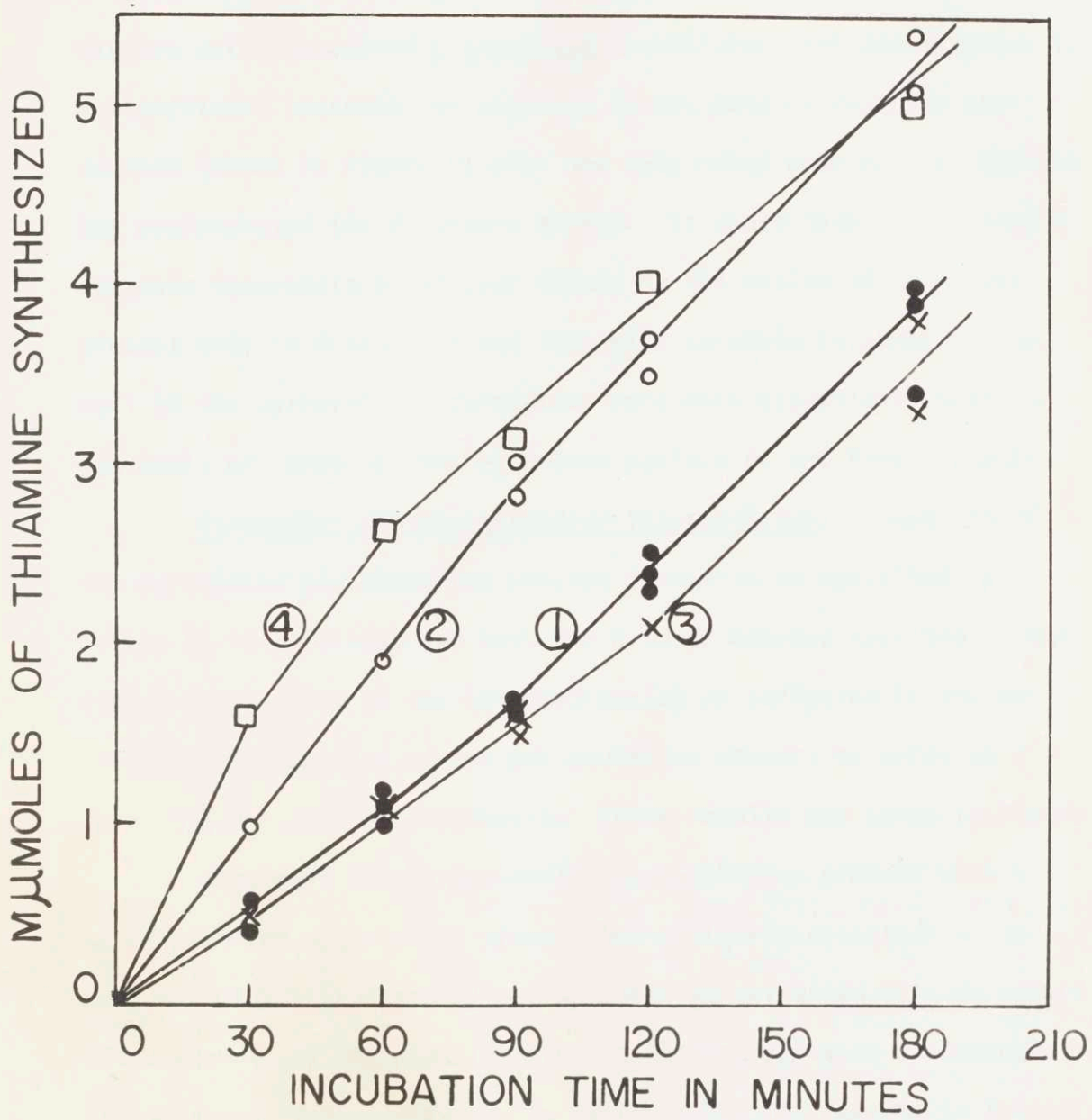

MμMOLES OF THIAMINE SYNTHESIZED
0
1
2
3
4
5
0
30
60
90
120
150
180
210
INCUBATION TIME IN MINUTES
④
②
①
③

FIGURE 7.

more effectively than free thiazole. However, when thiazole was preincubated with ATP, Mg^{++}, and Fraction 2, and then added to a mixture which contained preincubated pyrimidine, ATP and Fraction 1, a significant increase was observed in the rate of thiamine production (shown in Figure 7) over the rate noted when only pyrimidine was preincubated (as described above). It would thus appear that a thiazole intermediate had been formed by the action of an enzyme present only in Fraction 2 and that this thiazole intermediate as well as the pyrimidine intermediate were more effectively used for synthesis of thiamine than were free pyrimidine and free thiazole.

Properties of the pyrimidine intermediates. -- Heating of the pyrimidine preincubation product (prepared as described in Figure 8) in a boiling H_2O bath for 0 to 30 minutes resulted in the linear destruction of the intermediate(s) as reflected in the decreased effectiveness of the preincubation product to serve as a substrate for thiamine synthesis. These results are shown in Figure 8.

Treatment of the pyrimidine preincubation product with a crude alkaline phosphatase preparation similarly resulted in the destruction of the intermediate(s) again as reflected in a decreased effectiveness of the preincubation product to serve as a substrate for thiamine synthesis (shown in Table XII). The results in Table XII also show that, apparently, only about 50% of the intermediate was destroyed by the phosphatase treatment. This is probably the result of an insufficient amount of phosphatase having been used in the experiment.

Figure 8. Destruction of the pyrimidine preincubation product by heat. Pyrimidine was incubated for three hours at 37° with Fraction 1, $MgCl_2$, ATP, and buffer as in Figure 5. Protein was denatured by heating the reaction mixtures to 55° for 10 minutes. The mixtures were then immediately cooled in an ice bath. The reaction mixtures were then heated in a boiling water bath for the times indicated in the figure and again cooled in an ice bath. Thiazole (concentration given in Figure 5) and 0.5 ml. of Fraction 2 which had been pretreated at 55° for 5 minutes to destroy Fraction 1 activity present as contaminant, were added to the cooled samples and these complete reaction mixtures were incubated at 37° for 3 hours. Assays were performed as described in Figure 5.

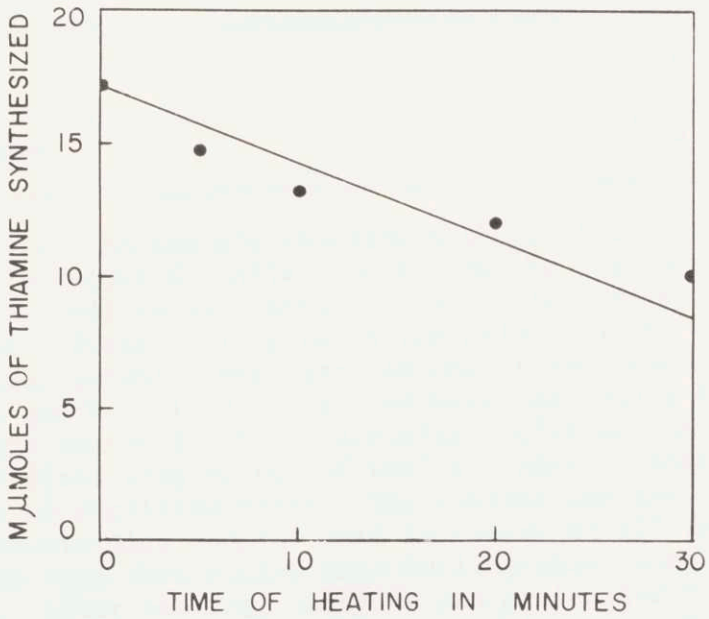

M μMOLES OF THIAMINE SYNTHESIZED
20
15
10
5
0
0
10
20
30
TIME OF HEATING IN MINUTES

FIGURE 8.

TABLE XII

Destruction of the Pyrimidine Preincubation Product by Phosphatase[a]

Treatment	mμmoles of thiamine synthesized
None	4.6
Phosphatase	2.3

[a]Pyrimidine was preincubated with Fraction 1 as indicated in Figure 8. After incubation, the enzyme was destroyed by heating the samples in a boiling water bath for 5 minutes. After cooling in an ice bath, 0.1 ml. of a phosphatase solution was added to one of the reaction mixtures and 0.1 ml. of distilled water was added to the other (the control). The phosphatase solution was prepared by dissolving 40 mg. of Bovine alkaline phosphatase in 1 ml. of distilled water. The control and the phosphatase-containing samples were incubated at 37° for 2 hours and were then heated in a boiling water bath for 5 minutes. After cooling, thiazole and pretreated Fraction 2 were added to the samples as described in Figure 8. In addition, 10 μmoles of neutralized ATP were also added to the samples to compensate for any destruction of ATP by the phosphatase, and the complete reaction mixtures were incubated at 37° for 3 more hours. Assays were performed as described in Figure 5.

Detection of phosphorylated pyrimidine derivatives on paper chromatograms. -- If a phosphorylated pyrimidine is an intermediate, it should be possible to detect its presence in enzymatic reaction mixtures by paper chromatographic methods. Accordingly, suitable aliquots of a pyrimidine preincubation reaction mixture (prepared as in Figure 8) were chromatographed and the zones of migration were determined by bioautographic techniques using S. typhimurium ATh mutant 4-1 as the test organism. When this procedure was followed, no zones of growth appeared in the bioautogram. As a consequence, it seemed likely that the test organism might not be able to use a phosphorylated pyrimidine in place of the free pyrimidine, and if such is the case, then in order to bioautographically detect the phosphorylated pyrimidine, it would be necessary to treat developed paper chromatograms in such a way that free pyrimidine would be liberated from the intermediate before preparing the bioautograms. For this purpose, dried developed paper chromatograms were sprayed with either 1 N hydrochloric acid or a solution of one of three different phosphatases, and the wetted chromatograms were incubated for 1 to $1\frac{1}{2}$ hours at 38° in closed containers saturated with either 1 N hydrochloric acid vapors or water vapor, respectively. After incubation, the chromatograms were again thoroughly air-dried and S. typhimurium mutant 4-1 bioautograms were prepared.

As can be seen in Figure 9, phosphatase treatment of the chromatograms liberated microbiologically active pyrimidine from presumed pyrimidine phosphates at two places on the bioautograms.

After hydrochloric acid treatment, however, only the slower moving spot was observed. In order to determine why the faster moving spot was not found after the hydrochloric acid treatment, some chromatograms were successively treated with 1 N HCl and then phosphatase, and others with phosphatase and then 1 N HCl. In between the two treatments, the chromatograms were thoroughly air dried. In bioautograms prepared with these doubly-treated chromatograms, both of the growth zones were found. This suggested that the top spot was probably not affected by the HCl treatment and that a phosphatase treatment was required to liberate pyrimidine from this fast moving compound.

As mentioned above, three different phosphatases were experimentally used to spray the chromatograms. The first was a "purified" Bovine intestinal alkaline phosphatase, commercially available from the Pentex Corporation, having diesterase, and ortho- and pyrophosphatase activities. The phosphatase solution was freshly prepared in 0.1 M Tris buffer, pH 8.5, at a concentration of 10 mg. per ml. The second phosphatase which was used was an E. coli phosphomonoesterase having some pyrophosphatase activity. This enzyme was obtained from Drs. Cyrus Levinthal and Frank Rothman of the Massachusetts Institute of Technology, and was freshly prepared in 0.1 Tris buffer, pH 8.5, at concentrations of 2-10 μgm. per ml. The last phosphatase which was used was an acid prostate phosphomonoesterase which had only a very small amount of pyrophosphatase activity. This enzyme was kindly donated by Dr. Gerhard Schmidt of Tufts University and was freshly prepared in 0.1 M acetate buffer, pH 5.6, at the various concentrations listed in a later section.

Figure 9. The detection of the phosphorylated pyrimidine intermediates on paper chromatograms. The figure is a drawing of the results obtained when developed paper chromatograms were treated as indicated in the figure and described in the text, prior to the preparation of bioautograms. The R_f values shown in the figure were obtained from chromatograms which had been spotted with 0.002-0.003 ml. of the pyrimidine preincubation product described in Figure 8 and which had been developed at room temperature in Solvent 1 (see Methods section). Average R_f values for all of the pyrimidine derivatives in several solvent systems can be found in Table XIII.

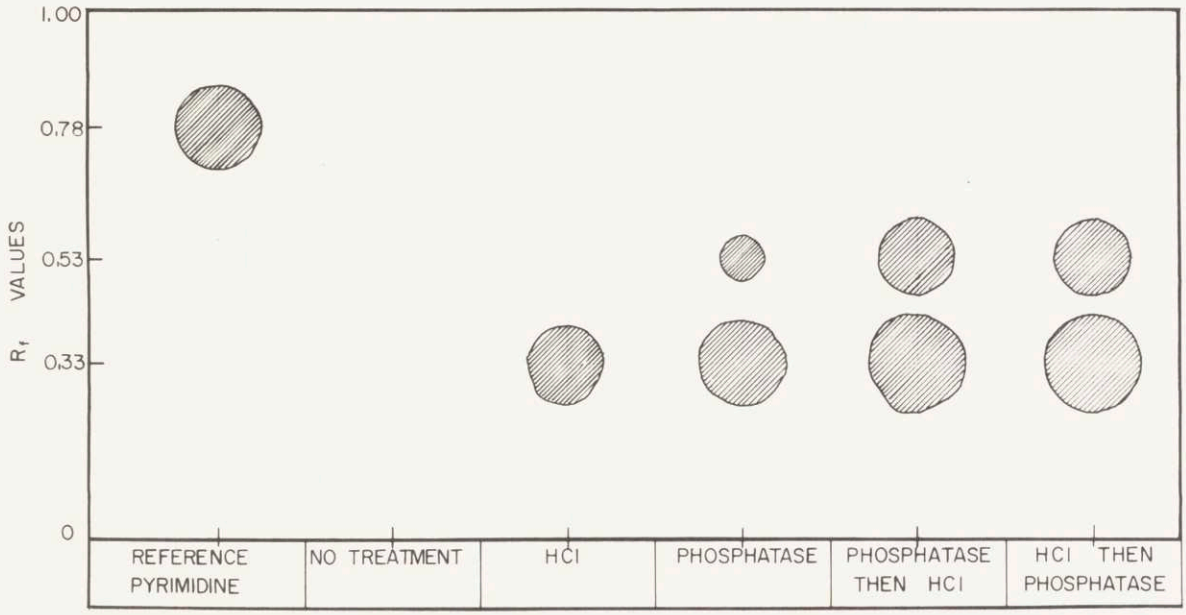

1.00
0.78
0.53
0.33
0
R_f VALUES
REFERENCE PYRIMIDINE
NO TREATMENT
HCl
PHOSPHATASE
PHOSPHATASE THEN HCl
HCl THEN PHOSPHATASE

FIGURE 9.

The question which now had to be resolved was whether these new growth zones represented two different phosphorylated pyrimidines or whether they were the result of a double-spotting of the same compound (one spot corresponding to the free phosphorylated pyrimidine and the other corresponding to a magnesium complex of the phosphorylated pyrimidine) as is encountered when nucleotides are chromatographed in the presence of Mg^{++}. To answer this question, $MgCl_2$ in a 6,000 fold molar excess as compared to the amount of intermediate, was cochromatographed with the reaction mixtures. The molar excess of this cochromatographed magnesium was over and above the 2,000 fold excess of magnesium already present in the reaction mixtures.

The developed chromatograms were again treated with either hydrochloric acid or phosphatase, and when bioautograms were prepared, it was found that the magnesium had not affected either the rate of migration of the spots as determined by their R_f values, or the relative amounts of each intermediate as determined by their spot sizes.

As a further proof that these two spots represented different compounds, the reaction mixtures were chromatographed in several different solvents and in every case, two distinct bioautographic spots were obtained. Table XIII shows the average R_f values of these two phosphorylated pyrimidine compounds in several solvent systems.

Therefore, since (a) these two phosphorylated pyrimidine compounds exhibited differential labilities to hydrochloric acid, (b) their R_f values were unaffected when they were cochromatographed with magnesium, and (c) two distinct bioautographic spots were obtained

irrespective of the solvent system used to develop the chromatograms, it was concluded that these two compounds were indeed different and that they were formed by Fraction I from pyrimidine and ATP.

In addition, it was postulated that the faster moving compound was a pyrimidine monophosphate and that the slower moving compound was a pyrophosphate derivative of pyrimidine. These postulations were based on the following considerations: (a) the R_f value of the faster moving compound suggested that this compound contained a single phosphate group; (b) the R_f value of the slower moving compound suggested that it contained either two phosphate groups or a pyrophosphate group; and (c) the acid lability of the slower moving compound is more suggestive of a pyrophosphate group (i.e., a pyrophosphoryl group is more labile to acid than an orthophosphoryl group -- Lynen, personal communication to Dr. G. M. Brown).

These two phosphorylated pyrimidine intermediates have been designated as pyrimidine I and pyrimidine II and this nomenclature will be used throughout the remaining portion of the Experimental section. Pyrimidine I refers to that phosphorylated intermediate having the higher R_f value of the two intermediates in Solvent I while pyrimidine II refers to that intermediate having the lower R_f value in Solvent I.

Enzymatic conversion of various pyrimidines to the same phosphorylated derivatives. -- At this point in the investigation, it became important to know whether the different pyrimidine substrates yielded the same or different phosphorylated pyrimidine derivatives. If different intermediates were formed from different pyrimidine substrates, this then would tend to minimize the importance of the

TABLE XIII

R_f Values of Pyrimidine and Pyrimidine Derivatives[a]

Compound	Average R_f values in the following solvent systems[b]					
	1	2	3	4	5	7
Pyrimidine	0.78	0.19	0.67	0.67	0.55	-
Bromomethylpyrimidine	0.78	0.14	0.71	0.65	0.55	-
Aminomethylpyrimidine	-	0.03	0.36	0.26	-	-
Methoxymethylpyrimidine	-	0.40	0.80	0.77	-	-
Thiamine	0.82	-	0.60	-	0.48	-
Pyrimidine sulfonate	-	0.08	0.27	0.60	-	-
Pyrimidine I[c]	0.53	-	0	-	0.20	0.52[d]
Pyrimidine II[c]	0.33	-	0	-	0.09	0.71[d]

[a]After development, the chromatograms were thoroughly air dried and the different compounds on the paper were located either by noting zones of quenching of ultraviolet light or by bioautographic techniques.

[b]See the Methods section for the numerical designation of the solvent systems.

[c]The designations pyrimidine I and pyrimidine II are explained in the text.

[d]In Solvent 7, the relative order of migration of phosphorylated compounds is reversed from most other solvent systems, i.e., the larger the degree of phosphorylation, the faster the compound moves in this solvent.

pyrimidine hydrolase in the overall biosynthesis of thiamine and would suggest that several different biosynthetic pathways exist in yeast.

In order to test this point, the different pyrimidine derivatives including thiamine were incubated with ATP, magnesium, buffer, and Fraction 1 as in Figure 5. An excess of Fraction 1 (1.0 ml.) was used to insure that all of the pyrimidine substrates had been converted to the intermediates. The chromatograms were spotted with 0.002-0.004 ml. of each reaction mixture and developed for 17 hours at room temperature in Solvents 1 and 5 (see the Methods section and Table XIII). The developed chromatograms were then treated with either 1 N HCl or phosphatase as described earlier and bioautograms were prepared. The results of this experiment showed that in both solvents, the same two phosphorylated pyrimidine intermediates were formed from every pyrimidine derivative, including thiamine.

As further proof that these intermediates were the same irrespective of the pyrimidine substrate used, 0.4 ml. aliquots of each of the above reaction mixtures were each incubated with 0.1 ml. of a phosphatase preparation for $2\frac{1}{2}$ hours at 37° and cooled in an ice bath. The phosphatase preparation was freshly prepared in 0.5 M Tris buffer, pH 8.5, at a concentration of 50 mg. of Bovine alkaline intestinal phosphatase per ml. The chromatograms were spotted with 0.002 ml. of each phosphatase-treated mixture and with reference pyrimidines, and developed for 18 hours at room temperature in Solvents 1, 2, 3 and 5 (see Methods section and Table XIII). The

subsequently prepared bioautograms showed both microbiologically and chromatographically that pyrimidine was the sole compound liberated by phosphatase action on the phosphorylated pyrimidine intermediates which had been formed from the different pyrimidine derivatives.

Therefore, on the basis of the foregoing results, it was concluded that the pyrimidine hydrolase is probably important in the conversion of the pyrimidine derivatives to free pyrimidine, and that pyrimidine, once formed is converted to the phosphorylated pyrimidine intermediates prior to the synthesis of thiamine.

Preliminary identification of pyrimidine I as pyrimidine-P. -- Chromatograms were spotted with approximately 0.004 ml. of a reaction mixture which contained both pyrimidine I and pyrimidine II. The spotted chromatograms were then developed at room temperature in Solvent 1, sprayed with acid prostate phosphomonoesterase solutions at concentrations ranging from less than 1 unit per ml. to 40 units per ml., and incubated for 1 hour at 38°. One unit of this phosphatase will liberate 0.1 mg. of phosphorus from an appropriate substrate in 15 minutes at pH 5.6 and at a temperature of 37°. Bioautograms were then prepared.

At a concentration of 10 units per ml. the bioautograms indicated that pyrimidine I had been completely hydrolyzed to pyrimidine as shown by the spot size, and that pyrimidine II had not been noticeably affected. At concentrations of 1 unit per ml. or less, neither pyrimidine was bioautographically detectable, while at 40 units per ml., both pyrimidine compounds were hydrolyzed.

Since this phosphatase is characterized as being a phosphomono-

esterase having a small amount of pyrophosphatase activity, it was reasoned that at 10 units per ml., the hydrolysis of pyrimidine I was a reflection of the primary phosphomonoesterase activity. This concentration of enzyme might not be expected to have much effect on a pyrophosphate ester. At higher phosphatase concentrations, the increasing hydrolysis of pyrimidine II was probably the result of increased pyrophosphatase activity. It was therefore concluded that pyrimidine I was probably pyrimidine-P and that pyrimidine II was very possibly pyrimidine-PP. These conclusions are consistent with other data which have been presented earlier.

Isolation of pyrimidine I and pyrimidine II by paper chromatography. -- One hundred and fifteen mμmoles of pyrimidine were incubated with 2.0 ml. of Fraction 1, 20 μmoles of ATP, 200 μmoles of $MgCl_2$, and 0.2 ml. of 1.0 M glycylglycine buffer, pH 7.4 for 3 to $3\frac{1}{2}$ hours at 37°. The volume of the reaction mixture was then reduced to approximately 0.4 ml. and the cystine sediment was centrifuged off. The clear supernatant solution was then applied to Whatman 3 mm. paper in a band $\frac{1}{4}$ of an inch wide by 18 inches in length at a distance of $1\frac{1}{2}$ inches from the bottom of the paper, and the chromatogram was developed for 15 to 16 hours at room temperature in Solvent 1. After the chromatogram was dry, four strips $\frac{1}{4}$ of an inch wide were cut out of the paper along the direction in which the solvent had moved up the paper, the strips were treated with Bovine alkaline phosphatase and bioautograms were prepared as described earlier.

The bioautograms of the four phosphatase treated strips showed 3 zones of growth. The top two had R_f values corresponding to

pyrimidine I and pyrimidine II. The small bottom, or third, spot was later found to be pyrimidine II which had moved up the paper more slowly than usual, probably as the result of the large amounts of ATP, buffer, and Mg^{++} which were present in the reaction mixture.

The three zones of pyrimidine material were then cut from the chromatogram and were separately eluted by washing the papers twice with 25-30 ml. portions of distilled water. These extracts were then taken to dryness in vacuo at room temperature over potassium hydroxide, and each residue was resuspended in 1 ml. of distilled water. These separated pyrimidine fractions were then tested in several ways discussed below.

Bioautograms of the three pyrimidine fractions and a known mixture of pyrimidines I and II showed (a) that the two bottom pyrimidine fractions were pyrimidine II with only a small amount of contaminating pyrimidine I and (b) that the top pyrimidine fraction was chromatographically pure pyrimidine I.

When the three pyrimidine fractions were heated in a boiling water bath for 30 minutes and then chromatographed with appropriate controls in Solvent I, treated with phosphatase, and bioautographed, it was found that pyrimidine II was the heat labile pyrimidine compound detected earlier, the products being pyrimidine I and pyrimidine. Pyrimidine I treated in the same manner appeared to be heat stable.

Incubation of pyrimidine I in 0.1 M glycylglycine buffer, pH 7.4, with 0.2 ml. of Fraction 1, 100 µmoles of $MgCl_2$ and $\pm$ 10 µmoles of ATP in a final volume of 0.9 ml., for three hours at 37°, followed

by chromatography of the reaction mixtures, phosphatase treatment, and the preparation of bioautograms, showed that pyrimidine I was converted to pyrimidine II in the presence of ATP, Mg^{++}, and Fraction 1. No detectable change was observed when pyrimidine II was treated in the same manner.

Incubation of pyrimidine II in 0.1 M glycylglycine buffer, pH 7.4, with 0.2 ml. of Fraction 2, 100 μmoles of $MgCl_2$, 38 μmoles of thiazole and ± 10 μmoles of ATP, followed by chromatography, phosphatase treatment, and the preparation of bioautograms in the usual manner, showed that pyrimidine II coupled with thiazole (or a thiazole intermediate) in the presence of Fraction 2 to form thiamine as measured microbiologically with L. viridescens. It was not possible to determine whether ATP was required for this coupling reaction since the isolated pyrimidine II fraction contained ATP as a contaminant from the original chromatographic separation. Under the same experimental conditions, pyrimidine I would not couple with thiazole. In order for this latter reaction to occur, Fraction 1 as well as Fraction 2 was required. These results indicated that pyrimidine II was the most direct pyrimidine precursor of thiamine.

Effect of inorganic phosphate and pyrophosphate on the formation of pyrimidine I, pyrimidine II and a thiamine compound. -- Incubation of pyrimidine, ATP, Mg^{++}, and buffer as described in Figure 5 using 0.1 ml. of the yeast autolysate prepared with toluene, followed by chromatography, phosphatase treatment, and the preparation of bioautograms, showed that all of the pyrimidine substrate had been used to form mainly pyrimidine I and only a small amount of pyrimidine II.

When this reaction mixture was made 0.1 M with respect to inorganic pyrophosphate, the relative amounts of pyrimidines I and II were not affected. But when the reaction mixture was made 0.1 M with respect to inorganic phosphate, the bioautographic results were completely reversed, resembling the results which were obtained when the partially purified Fraction I was used in place of the autolysate preparation (i.e., pyrimidine II is the main product with only a small amount of pyrimidine I being formed (see Figure 9)). Since inorganic phosphate is known to be a powerful inhibitor of many phosphatases, it might be concluded that the phosphate effect observed in this experiment had probably been the result of such an inhibition of a phosphatase. In addition, this inorganic phosphate effect suggested that in subsequent purification procedures designed to isolate these pyrimidine intermediates, it might be wise to add phosphate to the initial reaction mixture so as to favor the formation of pyrimidine II.

Incubation of the reaction mixture described above, in the presence of 120 mμmoles of thiazole and in the absence of inorganic phosphate or pyrophosphate, resulted in a good synthesis of thiamine as measured microbiologically with L. viridescens. When this last reaction mixture was prepared in 0.1 M phosphate, the thiamine synthesis was increased approximately 70%. With 0.1 M inorganic pyrophosphate, thiamine synthesis was totally eliminated. In the light of the bioautographic results presented above, the thiamine-synthesis results were interpreted on the basis that (a) any procedure which favored the formation of pyrimidine II, as inorganic phosphate was

shown to do, would be expected to favor the subsequent synthesis of thiamine, and that (b) an end product of a reaction, such as pyrophosphate liberated during thiamine synthesis, would be expected to more or less inhibit the reaction which formed that end product. However, it should be stressed that these interpretations were made on the basis of preliminary experiments.

A summary of the results obtained thus far concerning the pyrimidine-thiazole coupling enzyme system is briefly outlined below:

1. Pyrimidine derivatives $\xrightarrow[\text{pyrimidine hydrolase}]{\text{Fraction 1}}$ pyrimidine

2. Pyrimidine + ATP $\xrightarrow{\text{Fraction 1, } Mg^{++}}$ pyrimidine I + pyrimidine II

3. Pyrimidine I is probably pyrimidine-P

4. Pyrimidine II is very possibly pyrimidine-PP

5. a) Pyrimidine I + ATP $\xrightarrow{\text{Fraction 1, } Mg^{++}}$ pyrimidine II

   or

   b) Pyrimidine I $\xrightarrow{\text{Reactions (1) and (2)}}$ pyrimidine I + pyrimidine II

6. Pyrimidine II $\xrightarrow{\text{phosphatase or heat}}$ pyrimidine I + pyrimidine

7. Pyrimidine II + thiazole + (ATP?) $\xrightarrow[Mg^{++}]{\text{Fraction 2}}$ thiamine compound

8. Reaction (7) + pyrophosphate $\longrightarrow$ no thiamine synthesis

Attempts to measure adenosine nucleotides as products of the reactions by which pyrimidine phosphates are formed. -- The foregoing experiments have established that an enzyme or enzymes in Fraction I

catalyzed the formation of two phosphorylated pyrimidines (probably pyrimidine-P and pyrimidine-PP) from ATP and pyrimidine, and that pyrimidine I could be enzymatically converted to pyrimidine II in the presence of ATP. However, no information was available concerning the mechanism of formation of these two compounds. If it is assumed that pyrimidines I and II are pyrimidine-P and -PP respectively, then the question arises as to whether pyrimidine-P is a necessary intermediate in the formation of pyrimidine-PP, or whether pyrimidine-P was formed merely as a degradation product of pyrimidine-PP. If pyrimidine-P is an intermediate, then the adenosine nucleotide formed as a product in the biosynthesis of pyrimidine-PP should only be ADP according to the following reactions:

$$\text{pyrimidine} + \text{ATP} \longrightarrow \text{pyrimidine-P} + \text{ADP}$$

$$\text{pyrimidine-P} + \text{ATP} \longrightarrow \text{pyrimidine-PP} + \text{ADP}$$

On the other hand, if pyrimidine-P is formed as a degradation product of pyrimidine-PP, then the only adenosine nucleotide which should be produced would be AMP as follows:

$$\text{pyrimidine} + \text{ATP} \longrightarrow \text{pyrimidine-PP} + \text{AMP}$$

$$\text{pyrimidine-PP} \longrightarrow \text{pyrimidine-P} + \text{phosphate}$$

Finally, the remaining possibility is that pyrimidine-P and pyrimidine-PP are produced by independent mechanisms. In such a case, both ADP and AMP would be expected to be produced.

In an attempt to obtain answers to the questions, an experiment was designed to show which adenosine nucleotide or nucleotides

were products in the formation of the phosphorylated pyrimidines. For this purpose, ATP labeled with C^{14} in the C-8 position of adenine was incubated with pyrimidine, Mg^{++}, and Fraction 1 for 4 hours at 37°. Carrier amounts of non-radioactive adenosine, AMP, ADP, and ATP were then added and the reaction mixtures were chromatographed on Dowex-1-formate columns under conditions such that each of the adenosine compounds was eluted from the columns in separate fractions (see Figure 10 for details). The optical density of each eluate fraction was measured at 260 and 275 mμ in order to locate the adenosine compounds, and an aliquot of each fraction was also plated and counted for radioactivity. The elution patterns of the two pyrimidine phosphates were also determined by testing an aliquot of each fraction for pyrimidine. For this latter purpose, each aliquot was first treated with alkaline phosphatase as described earlier and then assayed for pyrimidine with S. typhimurium mutant 4-1.

Unfortunately, the results of this experiment did not yield any useful information concerning the nature of the adenosine nucleotide product of the reaction. It had been expected that the adenosine compound labeled with C^{14} would correspond to the pyrimidine product of the reaction, as discussed above. However, all of the adenosine-containing compounds were heavily labeled with C^{14} even in the control incubation mixture which was incubated in the absence of pyrimidine. In addition, the amount of C^{14} present in these adenosine compounds was far in excess of the amounts of pyrimidine phosphates formed. This result suggested that the enzyme preparation (Fraction 1) was heavily contaminated with phosphatases and perhaps myokinase.

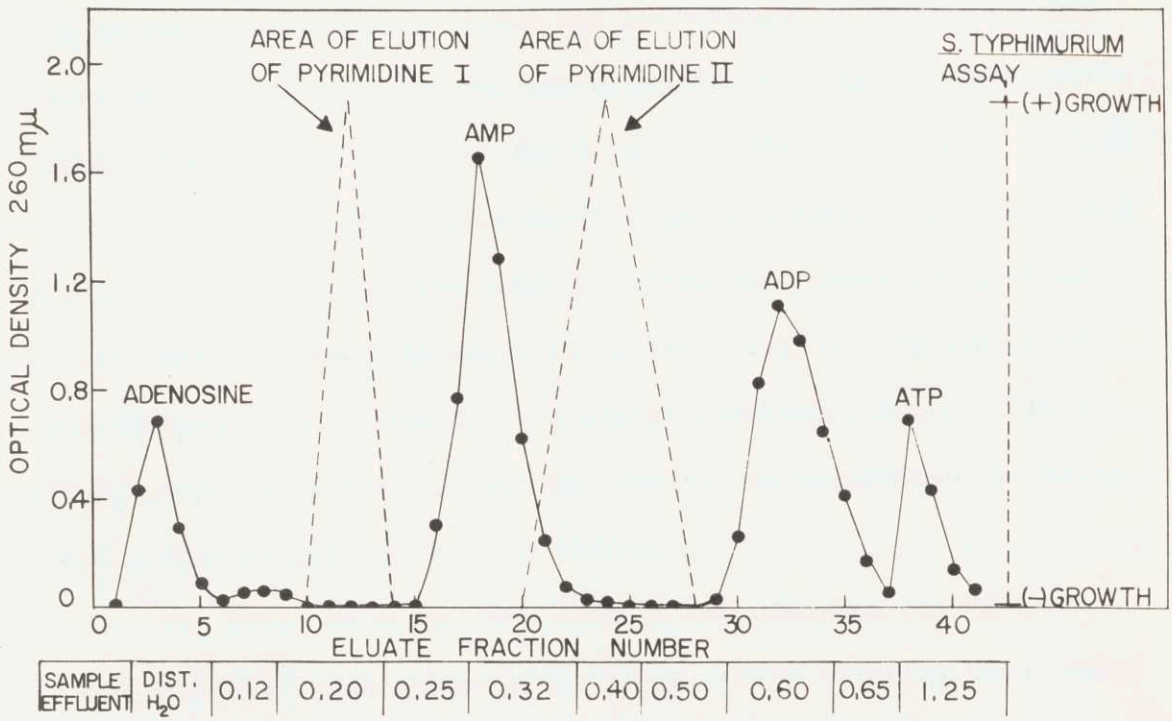
The experiment was not a total loss, however, since, in addition to separating the adenosine compounds, the procedure used was also found to be useful in separating pyrimidine I from pyrimidine II (see Figure 10) and was finally incorporated into a later procedure which was developed for the large-scale separation and isolation of these compounds. Bioautograms showed that the pyrimidine compound which had eluted first from the column corresponded to pyrimidine I while the pyrimidine compound which moved through the column more slowly corresponded to pyrimidine II. Under the experimental conditions employed, pyrimidine I resembled CMP in its elution characteristics and pyrimidine II resembled CDP.

Large scale isolation and purification of pyrimidine I and pyrimidine II. -- Preliminary experiments using dialyzed yeast autolysate prepared with toluene showed (a) that at pH 8.5 or higher, pyrimidine substrate was most efficiently utilized for the formation of pyrimidine I and pyrimidine II, (b) that the addition of potassium (as opposed to sodium) phosphate to the reaction mixture at a concentration of approximately 0.1 M favored the formation of pyrimidine II over pyrimidine I, and (c) that the autolysate preparation could handle up to 500 mμmoles of pyrimidine substrate per ml. of reaction mixture in 3 hours at 37° without any detectable substrate inhibition of the enzymes. In addition, a small scale charcoal column run was performed in order to determine whether a charcoal column procedure could be used, and if so, under what experimental conditions it should be operated. The results obtained from this charcoal column indicated (a) that all of the pyrimidine intermediates

Figure 10. A typical step-wise elution of adenosine nucleotides and pyrimidine phosphates from a Dowex-1-formate column. Five to six ml. of Dowex-1-formate resin were used to prepare a column 0.9 cm. in diameter and 8-9 cm. in length as described in detail in a later portion of this paper. The column was operated at room temperature and at a flow rate of approximately 1 ml. per minute.

A pyrimidine reaction mixture containing carrier amounts of adenosine and adenosine nucleotides (as described in the text) was adjusted to approximately pH 8 and was immediately passed through the column. The resin was washed with an aliquot of distilled water and the column was then eluted with increasing concentrations of ammonium formate in a step-wise manner as shown in the figure. The ammonium formate solutions were prepared by diluting a 5 M ammonium formate solution, at approximately pH 6.5, to the required concentrations with distilled water.

The adenosine compounds were located by 260 and 275 mμ optical density measurements of each eluate fraction, and the pyrimidine phosphates were located microbiologically as described in the text. Growth in the microbiological assay was recorded only as either plus or minus. The amount of pyrimidine present in the eluate fractions were not quantitatively determined.



SAMPLE EFFLUENT	DIST. H_2O	0.12	0.20	0.25	0.32	0.40	0.50	0.60	0.65	1.25

MOLARITY OF AMMONIUM FORMATE SOLUTIONS, pH 6.5, USED IN THE STEP-WISE ELUTION OF THE DOWEX-1-FORMATE COLUMNS

FRACTION 10.

were adsorbed on the charcoal when as little as 5 mg. of charcoal were used per ml. of reaction mixture, (b) that a scaled up version of this small column should have a satisfactory flow rate, and (c) that a recovery of the pyrimidine intermediates as high as 50% could be expected.

Preparation of the reaction mixture. -- One liter of yeast autolysate prepared with toluene was centrifuged to remove a small amount of cystine sediment and was dialyzed in versene-treated cellophane dialysis tubing at 4° for 6 hours against 10 volumes of 0.13 M (K^+) phosphate-0.02 M cysteine-0.005 M versene buffer, pH 7.0. This dialysis procedure was then repeated three more times using fresh buffer each time, and the dialysis tubing was rinsed in distilled water between each dialysis. The protein concentration of this dialyzed enzyme solution was 22.5 mg. per ml. as determined by optical density measurements at 260 and 280 mμ.

One liter of a reaction mixture was prepared containing 15,000 μmoles of neutralized ATP, 20,000 μmoles of $MgCl_2$, 500 μmoles of pyrimidine, 900 ml. of dialyzed autolysate (above) and (K^+) phosphate, versene, and cysteine which were added with the autolysate preparation. The mixture was then adjusted to pH 8.4-8.5 and was incubated for $3\frac{1}{2}$ to 4 hours at 38°. At 30 to 45 minute intervals for the first 3 hours of incubation, the reaction mixture was readjusted to pH 8.5.

After incubation, the reaction mixture was chilled in an ice bath and adjusted to pH 6.5. The protein was then denatured by swirling 50 to 60 ml. aliquots of the cold reaction mixture in a

1 liter Erlenmeyer flask immersed in a boiling water bath for 1 minute followed by a rapid cooling in an ice bath. After all the reaction mixture had been heated in this manner and cooled, the coagulated protein was centrifuged out of the reaction mixture and was washed with a small portion of distilled water. The protein wash water was then combined with the clarified reaction mixture, and the reaction mixture was stored at 4°.

A bioautogram, prepared as described earlier, of this clarified reaction mixture showed that all of the pyrimidine substrate had been used and that pyrimidine I and pyrimidine II had been formed in approximately equimolar amounts as judged by their relative spot sizes. As a result, isolation and purification procedures were begun.

Just before use, the cold reaction mixture was adjusted to pH 5.4 with concentrated hydrochloric acid and centrifuged to remove a small amount of sediment which came out of solution during adjustment of the pH. The cold supernatant solution was degassed by placing the solution under a water pump vacuum for 30 minutes and was then immediately put through the charcoal column discussed below.

Charcoal column procedure. -- A large charcoal column was prepared in a 95 mm. (inside) diameter glass tube 110 cm. in length to the bottom of which a 90 mm. polyethylene Büchner funnel had been firmly attached. First, layers of glass wool and 4 mm. glass beads were placed in the bottom of the tube to prevent the Büchner funnel openings from becoming plugged and to serve as a foundation for the remainder of the column. Next, layers of Carborundum (Grit #12), reagent-grade sand, and Johns-Manville Celite 545 were successively added

to the column, and the Celite was firmly tamped into place with a large rubber stopper just slightly smaller than the inside diameter of the glass tube. On top of the Celite, approximately 100 gm. portions of a mixture of charcoal and Celite were tamped into place. A total of 1200 to 1300 gm. of this charcoal mixture was used. The charcoal mixture consisted of a 50/50 mixture (weight/weight) of 325 mesh Norite activated charcoal and Celite 545. On top of the charcoal layer, a layer of Celite was tamped into place and layers of sand, glass wool and Carborundum were successively added to the column to keep the charcoal from floating and to prevent the column from becoming disarranged when liquid was added to the top of the column. The volume of this column, when prepared in the above manner, was approximately $2\frac{1}{2}$ to 3 liters.

Just before use, the charcoal column was degassed by passing 15 to 16 liters of cold, degassed, deionized water through the column at a flow rate of 10 to 20 ml. per minute. The degassed water was prepared by boiling deionized water for 5 to 10 minutes and cooling it to 4°. The remainder of the charcoal column procedure was performed continuously and at no time during the run was the column shut down. The procedure which was followed is a modification of the method of Pontis, Cabib and Leloir (105) which was worked out in the preliminary small scale charcoal column.

To the degassed column, the cold degassed reaction mixtures at pH 5.4 was added and passed through at a flow rate of approximately 10 ml. per minute. The walls of the glass tube were rinsed with, and the column was washed with 2 liters of cold, degassed, deionized water

at a flow rate of 10 ml. per minute. The column was then washed with 8 liters of a cold, degassed, 0.1 M versene solution, pH 7.0 at 15 to 20 ml. per minute. The versene wash was followed by a two liter water wash as above. The column eluates to this point were collected in 2 to 4 liter fractions and stored at 4°.

The column was then washed with 8 to 10 column volumes (26 liters) of an aqueous ethanol solution prepared by diluting 50 volumes of 95% ethanol to 100 volumes with deionized water. The flow rate varied from 5 to 15 ml. per minute. The first 8 liters of ethanol eluate were collected in 500 ml. fractions and the remaining 18 liters were collected in 1 to 4 liter fractions. These eluate fractions were adjusted to pH 7.0 and stored at 4°.

After all the eluate fractions had been collected, 0.1 ml. of each fraction was heated in a boiling water bath to boil off the ethanol, and was then incubated at 38° for $2\frac{1}{2}$ hours with 0.5 ml. of *E. coli* alkaline phosphatase prepared in 0.1 M Tris buffer, pH 8.5, at a concentration of 2 to 10 μgm. per ml. The samples were then diluted and a *S. typhimurium* mutant 4-1 microbiological assay was run. The results of this assay showed (a) that all of the eluate fractions up through and including the first 500 ml. of aqueous ethanol did not contain any detectable amount of the pyrimidine compounds, (b) that all of the ethanol eluate fractions, except the first 500 ml., contained the pyrimidine compounds in decreasing concentrations, and (c) that approximately a 25 to 40% recovery of the intermediates had been effected. The reason that the ethanol elution was stopped after 26 liters had been used was that the law of

diminishing returns made it impractical to continue the extraction. Although the recovery was somewhat low, the charcoal step was considered to be very worthwhile as it was possible in this single step to eliminate the large amounts of inorganic salts which were present in the reaction mixture.

The ethanol eluate fractions containing the pyrimidine intermediates were combined and concentrated in vacuo in a Precision Scientific Company Still (Catalogue #65486-Serial # F-4) at an operating temperature of 25 to 30°. The still was then rinsed twice with deionized water, and these washes were combined with the ethanol concentrates. The concentrated solution was further concentrated in vacuo in a flash evaporator at an operating temperature of 0 to 10° to a final volume of approximately 500 to 600 ml. This ethanol-free solution of pyrimidine intermediates was stored at 4° until it was used in the first Dowex column. The pH of the solutions remained fairly constant around pH 7 during the concentration procedures and eliminated the necessity for frequent pH adjustments.

Just before use, the concentrate was passed through filter paper to remove the solid impurities which had appeared during the concentration procedures and the volume was adjusted to approximately 1 liter with distilled water. The solution was then chilled in an ice bath, adjusted to pH 7.9 with ammonium hydroxide and immediately put through a Dowex-1-formate column at 4°.

First Dowex-1-formate column procedure. -- A Dowex-1-formate column 3.6 cm. in diameter and 30 cm. in length was prepared in the customary manner using 300 ml. of resin. The column was then washed

with 5 ml. of concentrated, 88% formic acid per ml. of resin at a gravity flow rate of approximately 8 ml. per minute. The column was finally washed with 15 to 25 ml. of cold, distilled water per ml. of resin also at a gravity flow rate. The effluent at the end of this water wash was at approximately pH 5, the pH of the distilled water as it is obtained from the water tap. The column was subsequently run at 4° and at a flow rate of 10 to 12 ml. per minute. The column effluent was collected in 100 to 110 ml. fractions.

To this prepared Dowex column, the ethanol-free, charcoal column concentrate, pH 7.9, (prepared above), was passed through the column and the column was washed with 1 liter of cold distilled water. A linear gradient elution of the column was then begun. The concentration gradient was scaled to increase at a rate of 0.1 M per 2 liters of eluting solution from a concentration of 0. to 0.6 M with respect to ammonium formate, pH 5.5. The optical density of each effluent fraction was measured at 260 and 275 mμ and the fractions were stored at 4°.

After all the eluate fractions had been collected, 0.1 ml. of every second fraction was incubated at 38° for 4 hours with 0.5 ml. of E. coli. alkaline phosphatase prepared in 0.1 M Tris buffer, pH 8.5, at a concentration of 2 μgm/ml. The samples were then diluted and a S. typhimurium microbiological assay was run in order to determine which fractions contained the pyrimidine compounds. The results of this assay clearly showed that the two pyrimidine intermediates had been separated from ATP and the bulk of the ADP as determined by the elution sequence (see Figure 10) as well as being partially separated

from one another. The most concentrated pyrimidine I fractions were eluted in this column procedure by ammonium formate concentrations of 0.13 to 0.17 M and the most concentrated pyrimidine II fractions were eluted by 0.21 to 0.26 M ammonium formate solutions. These results are shown in Figure 11. Subsequent recovery calculations indicated that approximately 180 μmoles of pyrimidine I and 30 μmoles of pyrimidine II were recovered in the eluate fractions.

The peak eluate fractions of each pyrimidine intermediate (#37 through #43 and #52 through #59 -- see Figure 11), which accounted for approximately 80 to 90% of each pyrimidine, were combined, adjusted to pH 6.9 to 7.0 with ammonium hydroxide, and concentrated *in vacuo* in a flash evaporator at an operating temperature of 0 to 5° to a final volume of approximately 50 to 100 ml. This concentrate was then lyophilized overnight at room temperature at less than 0.1 μ of mercury pressure using a short lead to a dry ice trap. The next morning, the sediment was resuspended in approximately 15 ml. of distilled water and was relyophilized for 2 days at room temperature in the same manner as above. The small amount of remaining residue was then dissolved in a few drops of water and again lyophilized overnight. By the use of this lyophilizing sequence, it was possible to remove all of the ammonium formate from the eluate fractions.

The lyophilization flask was washed with four, 30 to 35 ml. portions of distilled water, and the washes were combined and chilled in an ice bath. Just before use, this solution was adjusted to pH 7.9 with ammonium hydroxide and was immediately put through the second

Figure 11. The elution of pyrimidines I and II from the first large Dowex-1-formate column. The column was prepared and operated as discussed in the text. The areas of elution of pyrimidines I and II are indicated in the figures by the diagonal hatching.

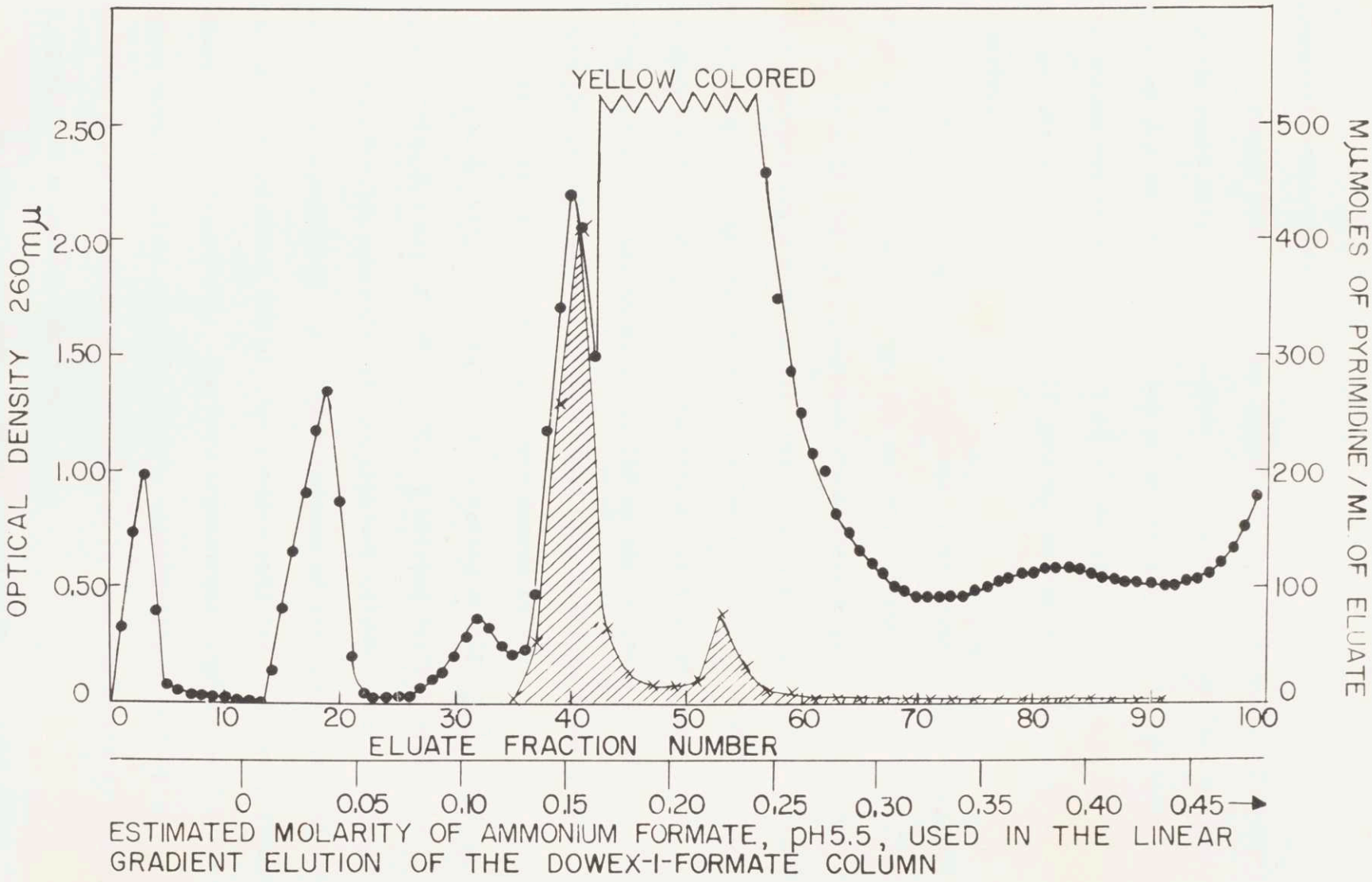

YELLOW COLORED
OPTICAL DENSITY 260mμ
2.50
2.00
1.50
1.00
0.50
0
500
400
300
200
100
0
MμMOLES OF PYRIMIDINE / ML OF ELUATE
0 10 20 30 40 50 60 70 80 90 100
ELUATE FRACTION NUMBER
0 0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 →
ESTIMATED MOLARITY OF AMMONIUM FORMATE, pH5.5, USED IN THE LINEAR GRADIENT ELUTION OF THE DOWEX-1-FORMATE COLUMN

FIGURE 11.

Dowex-1-formate column at 4°.

Second Dowex-1-Formate Column. -- One hundred ml. of Dowex-1-formate resin were used to prepare, in the manner described earlier, a column 2.2 cm. in diameter and 30 cm. in length. The operation of the column was at a temperature of 4° and at a flow rate of 10 to 12 ml. per minute. The column effluent was collected in 17 to 20 ml. fractions.

After the concentrate from the first Dowex column (adjusted to pH 7.9) had passed through the column, a linear gradient elution of the material in the column was immediately begun. The concentration gradient was scaled to increase at a rate of 0.1 M per 4 liters of eluting solution from a concentration of 0 to 0.4 M with respect to ammonium formate, pH 5.5. The optical density of every third effluent fraction was measured at 260 mμ and in those fractions having an optical density greater than 0.1, a 275 mμ reading also was taken. All of the fractions were stored at 4°.

Every third to sixth fraction having an $O.D._{260\ m\mu}$ reading greater than 0.1 was microbiologically assayed for pyrimidine as described for the operation of the previous column. The results of this assay showed that the elution pattern of the two pyrimidine intermediates resembled the elution pattern obtained from the previous Dowex column (Figure 11). The most concentrated pyrimidine I fractions were eluted by ammonium formate concentrations of 0.05 to 0.07 M; the most concentrated pyrimidine II fractions were eluted by 0.09 to 0.11 M ammonium formate.

The ultraviolet spectra of representative fractions shown to

contain pyrimidine were then determined using a Carey recording spectrophotometer. The results of these determinations indicated: (a) that fractions containing pyrimidine I were spectrophotometrically pure and were not contaminated with any of the adenine nucleotides; (b) that the U.V. spectrum of pyrimidine I was apparently identical to that of pyrimidine itself; (c) that the initial fractions containing pyrimidine II were appreciably contaminated with AMP (but not with either ADP or ATP as determined by the elution sequence -- see Figure 10); and (d) that the purest pyrimidine II (based on spectrophotometric evidence) was that which was found in the trailing edge of the portion of collective fractions which contained pyrimidine II. Recovery calculations showed that no appreciable loss of either pyrimidine I or pyrimidine II had occurred. Phosphate determinations indicated that the pyrimidine I fractions contained 3 moles of phosphate per mole of pyrimidine. The purest pyrimidine II fractions contained 4 to 5 moles of phosphate per mole of pyrimidine.

The steps described below were worked out for the further purification of pyrimidine I. However, owing to the lability of pyrimidine II on charcoal columns it was not possible to purify this compound any further, and in the futile attempts to do so, the bulk of this compound was lost.

The purification of pyrimidine I on a small charcoal column.-- A scaled-down version of the large charcoal column described earlier was prepared using 6 gms. of the charcoal mixture in a 25 mm. diameter tube. The column was then degassed as described earlier and washed successively with 100 ml. of a versene solution, 300 ml. of an ethanol solution and 1 to 2 liters of deionized water to remove any elutable

U.V. absorbing materials. The versene and ethanol solutions were prepared as indicated in the large charcoal column procedure. Approximately 11 μmoles of pyrimidine I contained in 90 ml. of the peak fractions from the second Dowex column (i.e., those fractions which contained the highest concentrations of pyrimidine I) were passed through this column at a flow rate of 1.4 ml. per minute. The charcoal column was then successively washed with 150 ml. of the versene solution and 150 ml. of deionized water at flow rates of 1.5 to 2 ml. per minute and finally, 300 ml. of the ethanol solution were passed through the column. The ethanol eluate was collected in a flask stored in an ice bath. The adsorption, versene wash, and water wash steps were performed at 4° using chilled solutions; the elution with ethanol was conducted at room temperature.

The ethanol eluate was concentrated in vacuo in a flash evaporator at an operating temperature of 0 to 5°, to a final volume of 40 to 50 ml.

The final purification of pyrimidine I on a small Dowex-1-formate column. -- Five to six ml. of Dowex-1-formate resin were used to prepare, in the manner described earlier, a column 0.9 cm. in diameter and 8 to 9 cm. in length.

The operation of the column was at room temperature and at a flow rate of 1 to 1.5 ml. per minute.

Approximately 20 ml. of the charcoal concentrate, adjusted to pH 7.9 to 8.0 with ammonium hydroxide, were passed through the column and the resin was washed with 40 to 50 ml. of deionized water. The column was then successively eluted with 40 ml. of 0.005 M, 60 ml. of 0.010 M, and 50 ml. of 0.025 M ammonium formate. The ammonium

formate solutions were prepared by diluting a 1 M ammonium formate solution, pH 4.0 to the required concentration with deionized water. The 0.010 M ammonium formate eluate was collected in 3.5 to 4 ml. fractions and the other eluates were collected in 10 ml. fractions. $O.D._{260,275m\mu}$ measurement showed that pyrimidine I had been eluted by the 0.01 M solution.

The pyrimidine I solutions obtained from this final Dowex column were used in the studies described below.

Properties of purified pyrimidine I. -- Spectrophotometric studies comparing pyrimidine and pyrimidine I under both acidic and alkaline conditions (Figure 12) showed that the ultraviolet spectra of these compounds were practically identical. The small spectral differences were in the slopes of their curves and in their relative extinction coefficients at any particular wave length. The extinction coefficients of pyrimidine were calculated directly from the spectral data, while the extinction coefficients of pyrimidine I were calculated in a less direct manner using the data obtained from a microbiological assay of phosphatase treated pyrimidine I. The same pyrimidine standard was used in both the microbiological and spectral analyses. These results indicated that pyrimidine I was as pure spectrophotometrically as the pyrimidine standard.

Measurement of the phosphate content of pyrimidine I followed by the subsequent calculation of phosphate to pyrimidine ratios revealed that pyrimidine I contained a single phosphate group. These results have been summarized in Table XIV.

Chromatography and cochromatography of isolated pyrimidine I

Figure 12. Ultraviolet absorption spectra of pyrimidine and pyrimidine I. Measurements were made in a Carey recording spectrophotometer. The pyrimidine solution was prepared in 0.010 M ammonium formate so that it would correspond to the pyrimidine I eluate obtained from the Dowex column. The blank used in the instrument was also 0.010 ammonium formate.

Figure 12 A. Ultraviolet spectra of pyrimidine and pyrimidine I in acid. The pyrimidine solution contained 115 mμmoles of pyrimidine per ml. and the pyrimidine I solution contained 46 mμmoles of pyrimidine I per ml. (the latter determined microbiologically).

Figure 12 B. Ultraviolet spectra of pyrimidine and pyrimidine I in base. The pyrimidine solution contained 96 mμmoles of pyrimidine per ml., and the pyrimidine I solution contained 46 mμmoles of pyrimidine I per ml.

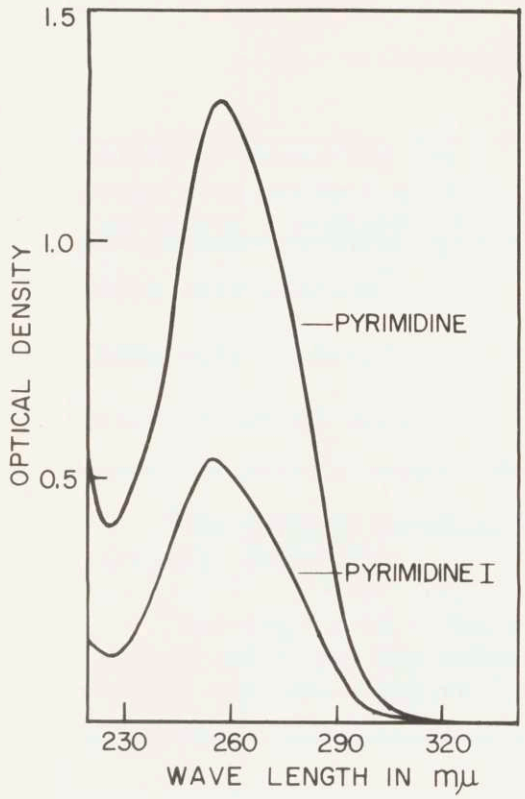


FIGURE 12 A.

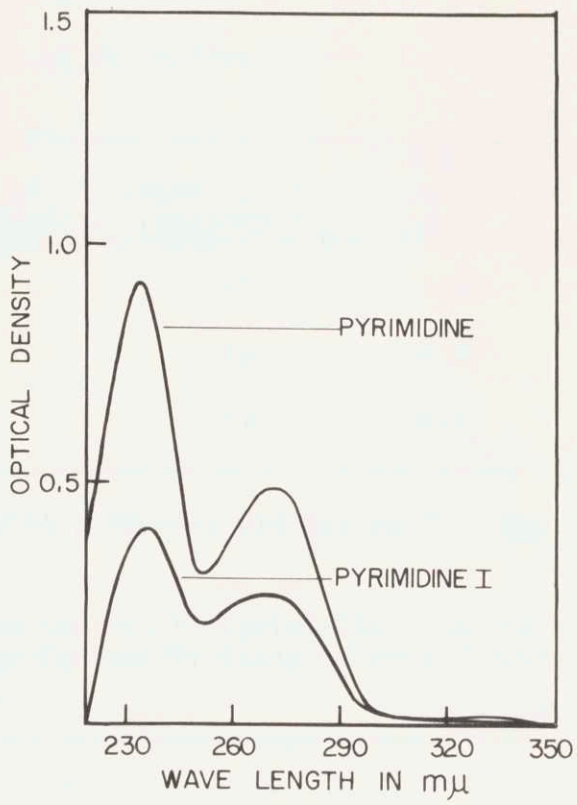


FIGURE 12 B.

FIGURE 12.

TABLE XIV

Phosphate Content of Pyrimidine I

Method of measuring the pyrimidine content of the pyrimidine I preparation	mμmoles of pyrimidine/ml.	mμmoles of phosphate/ml.[a]	phosphate to pyrimidine ratio
245mμ acid maximum[b]	48	42	0.88
260mμ acid measurement[b]	43	42	0.98
microbiological assay	46	42	0.91

[a]See Methods section. Pyrimidine I samples did not contain any inorganic phosphate.

[b]See Figure 12. The pyrimidine content of pyrimidine I was calculated using the approximate extinction coefficients of free pyrimidine at the wave lengths indicated.

with a known mixture of pyrimidines I and II showed that isolated pyrimidine I was (a) bioautographically pure and (b) identical to the pyrimidine I which had been used at the beginning of the purification scheme.

Other studies demonstrated that in order for pyrimidine I to be converted to pyrimidine II, Fraction I, ATP and Mg^{++} were required. Thiamine synthesis from pyrimidine I required these components plus Fraction II and thiazole.

The data which have been presented support the conclusion that pyrimidine I is a pyrimidine monophosphate. The evidence for such a structure includes: (a) the mobility of pyrimidine I on paper chromatograms; (b) an elution pattern from Dowex-1-formate which resembles that of CMP; (c) the enzymatic formation of pyrimidine I from pyrimidine and ATP; (d) the hydrolysis of pyrimidine I to pyrimidine by a phosphomonoesterase under conditions which do not affect what is thought to be a pyrophosphate derivative (pyrimidine II); (e) the formation of pyrimidine I from pyrimidine II by the action of either phosphatase or heat and the inhibition of phosphatase action by inorganic phosphate; (f) the enzymatic formation of a probable pyrophosphate derivative (pyrimidine II) from pyrimidine I and ATP; (g) the similarity of the ultraviolet spectra and extinction coefficients of pyrimidine and pyrimidine I under both acidic and alkaline conditions and (h) a phosphate to pyrimidine ratio of approximately one.

Examination of the structure of pyrimidine shows that a phosphate group could be easily attached at only two points in order to meet the condition that free pyrimidine be liberated by a simple phosphatase hydrolysis of the pyrimidine intermediate. These two

positions are the 4-amino and the 5-hydroxymethyl groups. The formation of a phosphoamide at the 4-amino group appears to be excluded by the stability of pyrimidine I towards 1 N HCl or temperatures of 100° for 30 minutes, and by virtue of the fact that the phosphorylation of pyrimidine is an intermediate step leading to the eventual attachment of the thiazole nitrogen to the 5-methylene carbon atom of pyrimidine. Therefore, it was concluded that pyrimidine I is a monophosphate ester of pyrimidine with the point of esterification being at the 5-methylene carbon atom.

Properties of pyrimidine II. -- Spectrophotometric studies of the partially purified, isolated pyrimidine II obtained from the second Dowex column indicated that its ultraviolet spectrum empirically resembled that of a mixture of pyrimidine and AMP. It should be pointed out that such a mixture might be expected since the elution of pyrimidine from Dowex-1-formate columns at pH 5.5 partially overlaps that of AMP.

The amount of pyrimidine present in pyrimidine II fractions, calculated by using the approximate extinction coefficients of free pyrimidine, when compared with the amount of pyrimidine determined microbiologically, indicated that pyrimidine II present in some of the fractions was at least 50% pure. This calculation was dependent upon the assumption that a pyrophosphate group attached to the 5-methylene carbon atom would not appreciably affect the pyrimidine ring resonance. If this assumption is correct, then structural considerations indicate that pyrimidine II does not contain an adenine molecule since the extinction coefficients of adenine are substantially greater than

those of this pyrimidine, and it would therefore be impossible for such a compound to give the O.D. readings which were found experimentally.

Chromatography and cochromatography of the partially purified pyrimidine II with a known mixture of pyrimidines I and II bioautographically showed that the isolated material was mainly pyrimidine II with a small amount of contamination with pyrimidine I.

Incubation of varying amounts (1 to 3 mμmoles) of the isolated pyrimidine II with Fraction 2, buffer, thiazole, ± ATP and ± Mg^{++} (concentrations and incubation conditions as in Figure 5), showed that both ATP and Mg^{++} were absolutely required, as well as Fraction 2 and thiazole, for the formation of thiamine as measured with L. viridescens. This ATP requirement suggested that either a third pyrimidine intermediate or some other intermediate (thiazole-P ?) was involved in the overall formation of thiamine. This will be discussed in more detail in a later section.

Incubation of pyrimidine II with only Fraction I and buffer, followed by the preparation of bioautograms, showed that this treatment had converted some pyrimidine II to pyrimidine I and indicated that a phosphatase was present in Fraction I. Additional experiments designed to check the reversibility of pyrimidine II formation from pyrimidine or pyrimidine I using Fraction I, buffer, Mg^{++}, ± ADP, and ± AMP yielded no information which would indicate whether or not pyrimidine I was a true intermediate in the formation of thiamine.

Indirect evidence which indicates that pyrimidine II is a pyrimidine pyrophosphate includes (a) the mobility of pyrimidine II on paper chromatograms; (b) an elution pattern from Dowex-1-formate which

resembles that of CDP; (c) the enzymatic formation of pyrimidine II from either pyrimidine and ATP or from pyrimidine-P (pyrimidine I) and ATP; (d) the formation of pyrimidine and pyrimidine-P from pyrimidine II by the action of either a phosphatase or heat and the inhibition of the phosphatase action by inorganic phosphate; (e) the lability of pyrimidine II to acid hydrolysis which liberates pyrimidine in a manner analogous to the acid hydrolysis of similar pyrophosphates; and (f) the inhibition of the enzymatic formation of thiamine activity by inorganic pyrophosphate (which presumably is a product of the reaction). In addition, since pyrimidine-P (pyrimidine I) can serve as a substrate for the enzymatic formation of pyrimidine II, and since the nitrogen of thiazole is eventually attached to the 5-methylene carbon atom of pyrimidine in the formation of thiamine, it was concluded that pyrimidine II is probably a pyrophosphate ester of pyrimidine with the point of esterification being at the 5-methylene carbon atom.

It is not considered likely that an adenine nucleotide is attached to pyrimidine II. The evidence against such a structure includes (a) the elution of pyrimidine II from Dowex-1-formate in the same manner as CDP, (b) the phosphate inhibition of the phosphatase(s) which degrade pyrimidine II to pyrimidine I, and (c) the preliminary spectral data which indicated that both pyrimidine and adenine could not be present in an adenine to pyrimidine ratio of one. This latter spectral data however, is predicated on the supposition that the extinction coefficients of pyrimidine II are reasonably close to those of pyrimidine.

Since it has not been possible to ascertain whether pyrimidine-P is an obligate intermediate in the formation of thiamine or whether it is merely a decomposition product resulting from the destruction (enzymatic or otherwise) of pyrimidine-PP, it has not been possible to suggest an enzymatic pathway for the formation of these pyrimidine intermediates from free pyrimidine. As a consequence, the enzyme or enzymes responsible for the catalytic formation of pyrimidine-PP from pyrimidine have been grouped, in a preliminary manner, under a single name, pyrimidine kinase, in order to facilitate discussion of this reaction.

Identification of the pyrimidine-thiazole coupling product as thiamine-P. -- Incubation of Fraction 1, Fraction 2, buffer, Mg^{++}, ATP, and thiazole with either pyrimidine, any of the pyrimidine derivatives (including thiamine), pyrimidine-P, or pyrimidine-PP, followed by chromatography with authentic thiamine-P in Solvents 1, 5, 6 and 7, bioautographically showed that thiamine-P was the sole product of the coupling reaction irrespective of the pyrimidine substrate used. Incubation of thiamine with Fraction 2, buffer, ATP and Mg^{++}, bioautographically showed that thiamine was not phosphorylated under these conditions. Thus, this latter experiment ruled out the possibility that thiamine was formed enzymatically and was then phosphorylated to yield thiamine-P. L. viridescens was the assay organism used in these studies. It should be noted that the nutritional requirement for thiamine of this organism can only be satisfied by thiamine phosphate esters. See Table XVI for the average R_f values of thiamine-P in several solvent systems.

The identification of thiamine-P strongly suggested that a

thiazole intermediate was involved in the coupling reaction, and further indicated that such an intermediate was very likely to be thiazole monophosphate. These conclusions are in agreement with the conclusions reached from the preincubation and rate studies described in an earlier section and from the experiment which demonstrated that ATP was still required for the formation of thiamine from thiazole and pyrimidine-PP.

Attempts to reverse the enzymatic formation of thiamine-P using buffer, ± Mg^{++} (concentrations given in Figure 5), ± Fraction 2, ± Fraction 1, ± 10 or 40 μmoles of AMP per ml. of reaction mixture, and ± 1 or 100 μmoles of inorganic pyrophosphate per ml. of reaction mixture, were completely unsuccessful. Neither a disappearance of thiamine activity as measured by L. viridescens nor an appearance of the pyrimidine intermediates on S. typhimurium bioautograms was observed. It seems likely that this apparent irreversibility is the result of an equilibrium constant which strongly favors the formation of thiamine-P.

Detection of a thiazole intermediate on paper chromatograms. -- In order to test the likely possibility that a thiazole intermediate was involved in the enzymatic formation of thiamine-P, thiazole was incubated for 3 hours at 37° with buffer, Mg^{++}, ATP (concentrations given in Figure 5), and either 0.4 ml. of Fraction 1, or 0.4 ml. of Fraction 2 (or 0.4 ml. of Fraction 2 which had been heated to 55° for 5 minutes to inactivate the Fraction 1 contaminant). Aliquots of the reaction mixtures were then spotted on two chromatograms along with thiazole controls, and the chromatograms were developed in Solvent 1

at room temperature overnight. After the chromatograms were thoroughly dry, one was treated with E. coli alkaline phosphatase as described earlier and the other was left untreated. Bioautograms prepared with the thiazole-requiring mutant of E. coli showed that a new thiazole compound had been formed only in those reaction mixtures which contained Fraction 2, ATP and Mg^{++}. In reaction mixtures containing no Fraction 2, no synthesis of this new thiazole compound was observed. In addition, it was found that the E. coli mutant responded to this thiazole compound without the phosphatase treatment of the developed chromatograms.

The R_f value of this thiazole compound in Solvent I suggested that this compound contained a single phosphate group, and since this observation was in accord with the earlier conclusion based on the finding that thiamine-P was formed by the coupling reaction, work was immediately begun to prepare chemically some thiazole-P for subsequent experiments. This preparation is described below. See Table XVI for the average R_f values of this thiazole compound and thiazole in several solvent systems.

Preparation of thiazole-P. -- Thiazole-P was prepared by a procedure similar to that used by Williams, et. al. (7) to prepare thiazole, except that thiamine-P was substituted for thiamine, and that the relative amounts of the reactants were considerably modified. Experimentally, 1 mmole of chromatographically pure thiamine-P (Nutritional Biochemical Corporation) and 2 mmoles of $NaHSO_3$ were dissolved in distilled water and the volume was brought up to 30 ml. The solution was adjusted to pH 5.2 and transferred to a 25 mm. x 200 mm.

Pyrex culture tube. After sealing off the tube, it was immersed in a boiling water bath for 50 to 60 minutes, then thoroughly chilled in an ice bath and opened. The solution was adjusted to pH 7.4 and immediately passed through a Dowex-1-formate column.

The purification of thiazole-P on a Dowex-1-formate column.-- The general method described earlier for the preparation of a Dowex column was used. Specifically, five to six ml. of Dowex-1-formate resin were used to prepare a column 0.9 cm. in diameter and 8 to 9 cm. in length. The column was operated at room temperature at a flow rate of 1 to 1.5 ml. per minute. The column eluate was collected in 10 ml. fractions.

Approximately 2 ml. of the sulfite-treated thiamine-P solution (or approximately 60 μmoles of liberated thiazole-P) were passed through the column followed by 40 ml. of distilled water. The column was then successively eluted with increasing concentrations of ammonium formate prepared by diluting a 1 M ammonium formate solution, pH 4.0, to the proper concentrations with distilled water. $O.D._{245}$, 260 and 275 mμ measurements showed that pyrimidine sulfonate was eluted by 0.015 M ammonium formate and that thiazole-P was eluted by a 0.075 M solution. The thiazole-P fractions obtained from this column were used in the following studies.

The properties of thiazole-P. -- Spectrophotometric studies comparing thiazole and thiazole-P under both acidic and alkaline conditions showed that the ultraviolet spectra of these compounds were practically identical, having both the same maxima and the same extinction coefficients at these maxima. The extinction coefficients

of thiazole were calculated directly from the spectral data, while the extinction coefficients of thiazole-P were calculated in an indirect fashion using the data obtained from a microbiological assay of phosphatase treated thiazole-P. The same thiazole standard was used both for the microbiological and spectral analyses. The results indicated that thiazole-P was as pure, spectrophotometrically, as the thiazole standard. The spectra are shown in Figure 13.

Measurement of the phosphate content of thiazole-P preparations, followed by the subsequent calculation of phosphate to thiazole ratios revealed that thiazole-P contained a single phosphate group. These results have been summarized in Table XV.

Chromatography of the isolated thiazole-P with the thiazole incubation product (prepared as described earlier) in Solvents 1, 5, 6 and 7, showed that isolated thiazole-P was (a) bioautographically pure and (b) chromatographically identical to the thiazole compound which was formed by the incubation of thiazole with Fraction 2, buffer, ATP and Mg^{++}. As further proof that these two thiazole compounds were the same, 0.1 ml. aliquots of the isolated thiazole-P and the thiazole incubation product were each incubated with 0.5 ml. of E. coli alkaline phosphatase (at a concentration of 10 μgm. per ml.) for 2 to 3 hours at 38° and cooled in an ice bath. Chromatography of these phosphatase-treated compounds along with an authentic sample of thiazole bioautographically showed that thiazole was the sole product obtained from the phosphatase treatment of the thiazole compounds. See Table XVI for the average R_f values of thiazole and thiazole-P in several solvent systems.

Figure 13. Ultraviolet absorption spectra of thiazole and thiazole-P. Measurements were made in a Carey recording spectrophotometer. The thiazole solution was prepared in 0.075 M ammonium formate so that it would correspond to the thiazole-P eluate obtained from the Dowex column. The blank used in the instrument was also 0.075 M ammonium formate.

Figure 13 A. Ultraviolet spectra of thiazole and thiazole-P in acid. The thiazole solution contained 190 mμmoles of thiazole per ml., and the thiazole-P solution contained 415 mμmoles of thiazole-P per ml. (the latter determined microbiologically).

Figure 13 B. Ultraviolet spectra of thiazole and thiazole-P in base. The thiazole and thiazole-P solutions were at the same concentrations listed for Figure 13 A.

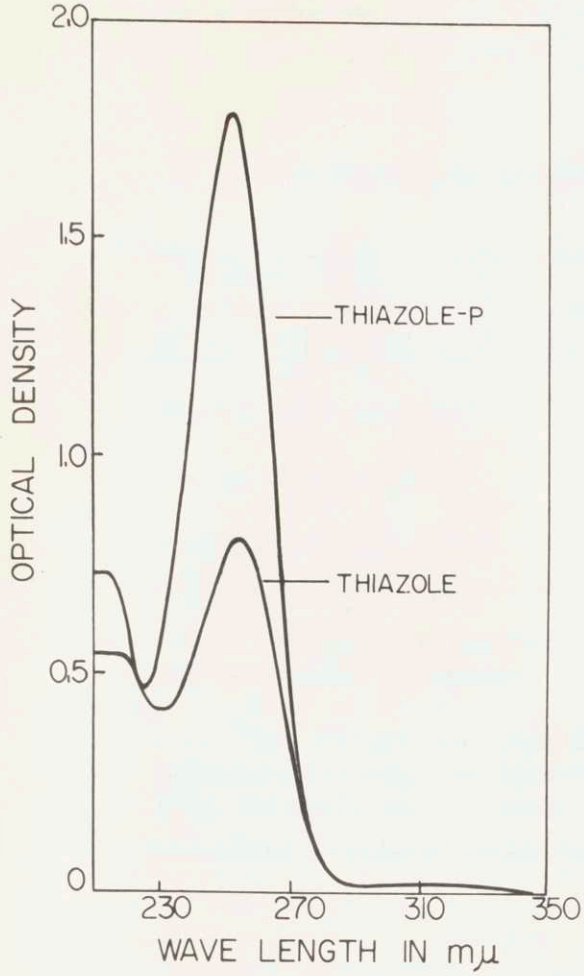


FIGURE 13 A.

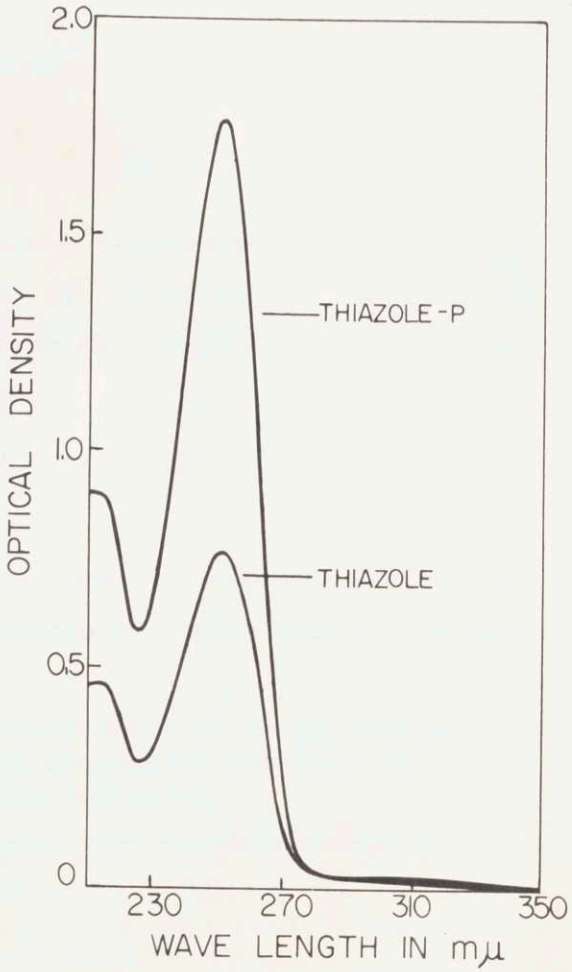


FIGURE 13 B.

FIGURE 13.

TABLE XV

The Phosphate Content of Phosphorylated Thiazole

Method of measuring thiazole content of thiazole-P preparations	mμmoles of thiazole/ml.	mμmoles of phosphate/ml.[a]	phosphate to thiazole ratios
254 mμ acid maximum[b]	417	447	1.07
251 mμ base maximum[b]	436	447	1.02
microbiological assay	415	447	1.08

[a]See Methods section. Thiazole-P samples did not contain any inorganic phosphate.

[b]See Figure 13. The thiazole content of thiazole-P was calculated using the approximate extinction coefficients of free thiazole at the wave lengths indicated.

When the synthetic thiazole-P was used as substrate along with pyrimidine-PP in the presence of Fraction 2, it was found that ATP was no longer necessary for the synthesis of thiamine-P. However, Mg^{++} was still absolutely required for this coupling reaction. The elimination of the ATP requirement by the use of thiazole-P and pyrimidine-PP suggests that these two intermediates are probably the most direct precursors of thiamine-P, and that the chemically prepared thiazole-P was able to substitute enzymatically for a required thiazole intermediate.

It would thus appear that thiazole-P and the thiazole incubation product were identical. Therefore, it was concluded that thiazole-P is an obligate intermediate in the enzymatic formation of thiamine-P. This conclusion is further substantiated by the observation that the phosphate group on thiamine-P is located at the same position on the thiazole moiety as is the phosphate group on thiazole-P.

The enzyme which catalyzes the formation of thiazole-P from thiazole and ATP in the presence of Mg^{++} has been named thiazole kinase. The enzyme which then subsequently catalyzes the formation of thiamine-P from thiazole-P and pyrimidine-PP in the presence of Mg^{++} has been designated as thiamine-P synthase.

Formation of thiamine-PP from thiamine. -- Incubation of thiamine with Fraction 1, buffer, ATP and Mg^{++} (concentrations given in Figure 5) followed by chromatography of the reaction mixtures along with an authentic sample of thiamine-PP, bioautographically showed two zones of growth, one corresponding to the thiamine substrate and the other corresponding to thiamine-PP. Thiamine-P was not detected as a product in these studies. Incubation of either thiamine-P or thiamine-PP

with Fraction 1 in the manner described above, bioautographically showed that these compounds were inert in the system, i.e., they did not yield any other thiamine compound. Similarly, incubation of either thiamine, thiamine-P or thiamine-PP with Fraction 2, buffer, ATP, and Mg^{++} did not result in the formation of any other thiamine compound.

These results show: (a) that thiamine, but not thiamine-P can serve directly as substrate for the formation of thiamine-PP; (b) that the thiamine-PP synthesizing enzyme is present in Fraction 1, but not in Fraction 2; (c) that no enzyme is present in either Fraction 1 or Fraction 2 which will dephosphorylate thiamine-P or thiamine-PP.

When thiamine was incubated with both Fraction 1 and Fraction 2 in the presence of ATP and Mg^{++}, both thiamine-P and thiamine-PP were detected in the reaction mixtures. This result was not unexpected, however, since work presented earlier has shown that thiamine can also serve as a substrate for the formation of pyrimidine II presumably by the action of pyrimidine hydrolase and pyrimidine kinase. The thiazole, which would be formed in these reactions, could then be converted to thiazole-P which would react with the pyrimidine-PP to yield thiamine-P.

The various thiamine compounds involved as products and substrates in the above reactions were identified both by their R_f values (as compared to authentic thiamine compounds) and by their use as growth factors by L. viridescens. The average R_f values of thiamine and its phosphate esters in several solvent systems can be

TABLE XVI

R_f Values of Thiamine, Thiazole and their Phosphate Derivatives[a]

Compound	Average R_f Values in the Solvents Shown[b]				
	1	3	5	6	7
Thiamine	0.82	0.60	0.48	-	-
Thiamine-P	0.70	-	0.20	0.17	0.65
Thiamine-PP	0.48	-	0.13	-	-
Thiazole	0.86	-	0.83	0.85	0.29[c]
Thiazole-P	0.66	-	0.36	0.36	0.42[c]

[a]After development, the chromatograms were thoroughly air-dried and the different compounds on the paper were located by bioautographic techniques.

[b]See the Methods section for the numerical designation of the solvent systems.

[c]In Solvent 7, the relative order of migration of phosphorylated compounds is reversed from most other solvent systems -- i.e., the larger degree of phosphorylation, the faster the compound moves in this solvent.

found in Table XVI.

It would thus appear that thiamine-PP is formed directly from thiamine by an enzyme present only in Fraction 1. The fact that thiamine-P did not serve as a substrate for the enzymatic formation of thiamine-PP ruled out the possibility that thiamine-P was an obligate intermediate in the formation of thiamine-PP. The existence of thiamine-P in biological systems is, therefore, probably the result of the degradation of thiamine-PP and the action of thiamine-P synthase.

In order to determine whether or not the thiamine-PP synthesizing enzyme was the same as the pyrimidine kinase, experiments were undertaken to locate the position of the enzyme fractions within Fraction 1 which contained the greatest amount of thiamine-PP synthesizing enzyme. The results of these experiments clearly showed that the faster eluting fractions within Fraction 1 contained the largest amount of this enzyme as judged from the relative sizes of the bioautographic growth zones, whereas maximum pyrimidine kinase activity was observed in the slower eluting fractions. It was therefore concluded that two distinct enzymes were responsible for the two reactions. The enzyme which forms thiamine-PP directly from thiamine in the presence of ATP and Mg^{++} was named thiamine pyrophosphorylase.

Evidence for the existence of a thiamine-P phosphatase. -- The earlier observation that thiamine-P did not serve in place of thiamine as a substrate for the formation of thiamine-PP suggested that thiamine-P must first be degraded to thiamine by a phosphatase in order to serve as a precursor of thiamine-PP. The use of a crude

extract as a source of the enzyme system showed that the products formed from pyrimidine and thiazole are a mixture of thiamine and thiamine-PP with only a small amount of thiamine-P. However, the use of purified enzymes (Fractions 1 and 2) yielded only thiamine-P as the sole product. Thus, it appears that the crude extract contained a phosphatase which converts thiamine-P to thiamine, and that this phosphatase was not present in Fractions 1 and 2. It appears likely that this phosphatase was separated from the other components of the enzyme system probably during the ammonium sulfate fractionation. Since this enzyme has not been extensively purified and studied in detail, no information is yet available concerning its substrate specificity and other properties.

Leder (85) has recently reported that the thiamine-synthesizing enzyme system in extracts of baker's yeast can be separated into two fractions by treatment with ammonium sulfate. He has now decided that one of these fractions is a phosphatase which is necessary for the formation of thiamine (personal communication to Dr. G. M. Brown). These results are consistent with those reported above.

Discussion. -- A summary of the reactions and intermediates involved in the biosynthesis of thiamine and its phosphate esters from the pyrimidine and thiazole moieties of thiamine is schematically presented in Figure 14. On the basis of the evidence which is currently available it cannot yet be decided whether pyrimidine-P is an obligate intermediate in the formation of pyrimidine-PP or whether the latter compound is formed directly from pyrimidine and ATP. This uncertainty is indicated by the brackets shown in the figure. The

evidence for the identity of each of the compounds shown in Figure 14 has already been summarized in previous sections of this dissertation.

The existence of pyrimidine-PP and thiazole-P as intermediates in thiamine synthesis was independently indicated by the work of Nose, et. al. (86) who prepared these compounds synthetically and found that they could be used without the addition of ATP to form a thiamine compound as catalyzed by a crude extract of yeast. The thiamine compound which was formed was not identified, but on the basis of the present investigations, was almost surely thiamine-P.

On the basis of evidence derived from a study of the nutritional requirements of a series of Neurospora mutants, Harris (83) has postulated that two pathways exist for the formation of thiamine: (a) one which couples pyrimidine with thiazole, and (b) an alternate method by which pyrimidine is coupled with a thiazole-like compound to yield a product which could then be converted to thiamine. In the light of the results presented in this dissertation, it would be interesting to know whether Harris' thiazole-like compound might not be identical with thiazole-P. Experiments should be undertaken with the use of cell-free extracts of Neurospora to settle decisively whether or not a mechanism of thiamine biosynthesis operates within this organism which is different from that which has been shown to operate in yeast.

The results of the current investigations which show that thiamine is converted to thiamine-PP without the intermediate formation of thiamine-P are identical with the results of Mano, et. al. (91) who studied this reaction in rat liver preparations. It would thus

Figure 14. The biosynthetic pathway for the formation of cocarboxylase from the pyrimidine and thiazole moieties of thiamine. See the text for a discussion of this scheme. The compounds shown in the parentheses are assumed to be the products of the reactions shown even though they have not been well characterized as such. The names of the intermediates and products have been underlined.

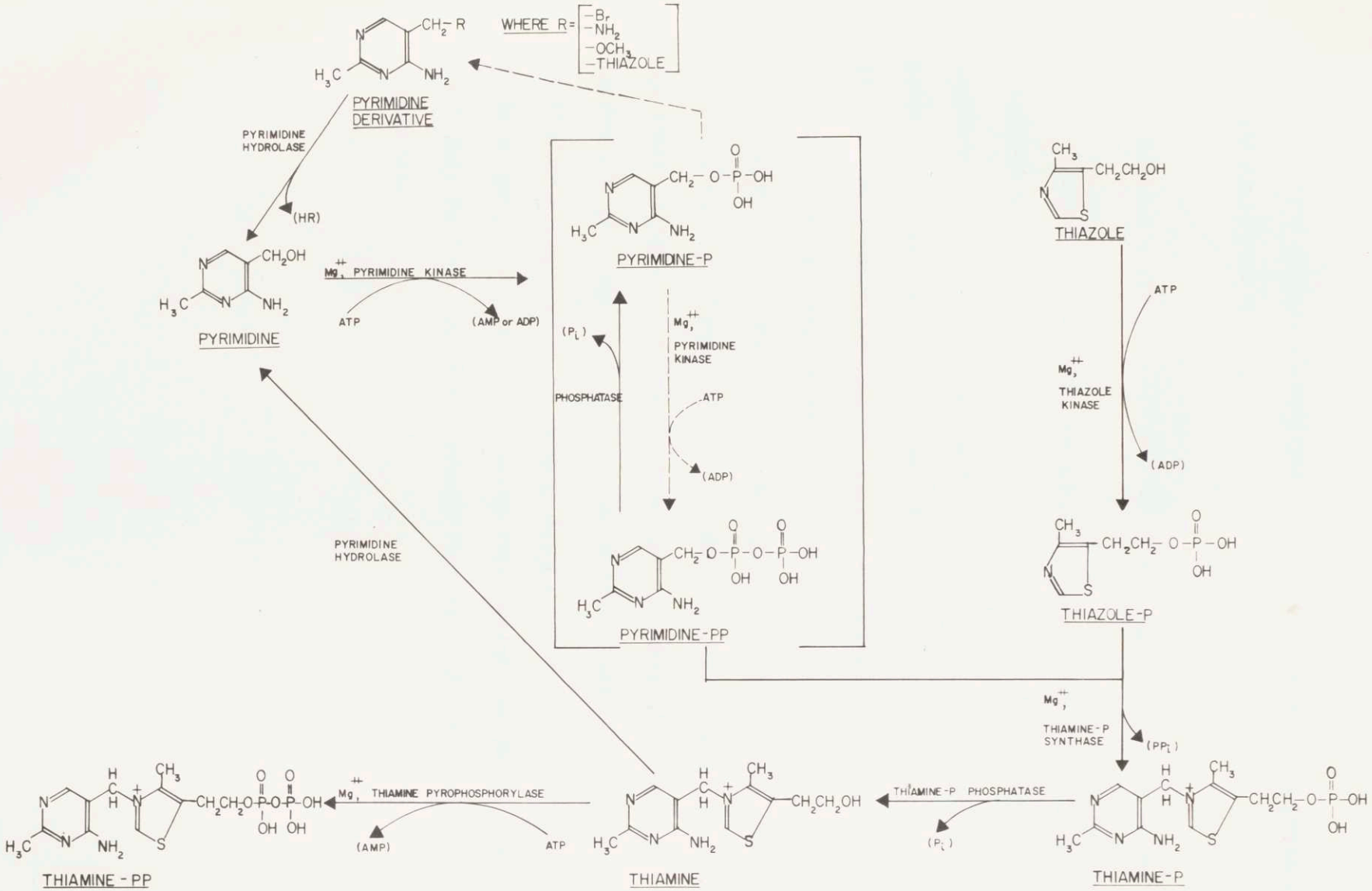

WHERE R = -Br, -NH2, -OCH3, -THIAZOLE
PYRIMIDINE DERIVATIVE
PYRIMIDINE HYDROLASE
(HR)
PYRIMIDINE
Mg++, PYRIMIDINE KINASE
ATP
(AMP or ADP)
PYRIMIDINE-P
(Pi)
PHOSPHATASE
Mg++, PYRIMIDINE KINASE
ATP
(ADP)
PYRIMIDINE-PP
PYRIMIDINE HYDROLASE
THIAZOLE
ATP
Mg++, THIAZOLE KINASE
(ADP)
THIAZOLE-P
Mg++, THIAMINE-P SYNTHASE
(PPi)
THIAMINE-P
THIAMINE-P PHOSPHATASE
(Pi)
THIAMINE
Mg++, THIAMINE PYROPHOSPHORYLASE
(AMP)
ATP
THIAMINE-PP

FIGURE 14.

appear that a general mechanism can be formulated for the synthesis of thiamine-PP as follows:

$$\text{thiamine} + \text{ATP} \xrightarrow[\text{pyrophosphorylase}]{Mg^{++},\ \text{thiamine}} \text{thiamine-PP} + \text{AMP}$$

Future work on the formation of thiamine and the subsequent formation of thiamine-PP should include attempts to separate more completely the individual enzymes involved in the system, an investigation into their enzymatic modes of action, a more complete examination of their substrate specificities, and a more complete characterization of the products of their action. Additionally, it would be very interesting to check other biological systems for the presence of one or more of these enzymes in order to gain an understanding of how other organisms synthesize thiamine. In this direction, G. L. Carlson of this laboratory has found in preliminary experiments that resting-cell suspensions of *E. coli* $K_{12\lambda}$, but not *E. coli* B, are able to form large quantities of thiamine from pyrimidine and thiazole. These observations should be pursued further by investigations at the enzyme level.

SUMMARY

Data presented in this dissertation have shown that the biosynthesis of thiamine and its pyrophosphate esters from the pyrimidine and thiazole moieties of thiamine can proceed in cell-free extracts of baker's yeast according to the series of reactions summarized below. The compounds enclosed in parentheses are assumed to be the products of the reactions shown even though they have not yet been well characterized as such. The abbreviations which are used in this summary are defined below.[1]

$$\text{pyrimidine-R} + \text{HOH} \xrightarrow{\text{pyrimidine hydrolase}} \text{pyrimidine} + (\text{HR}) \qquad (1)$$

$$\text{pyrimidine} + \text{ATP} \xrightarrow{Mg^{++},\ \text{pyrimidine kinase}} \left[\begin{array}{c}\text{pyrimidine-P} \\ \text{ATP} \downarrow Mg^{++} \\ \text{pyrimidine-PP}\end{array}\right] + (\text{ADP or AMP}) \qquad (2)$$

$$\text{thiazole} + \text{ATP} \xrightarrow{Mg^{++},\ \text{thiazole kinase}} \text{thiazole-P} + (\text{ADP}) \qquad (3)$$

$$\text{pyrimidine-PP} + \text{thiazole-P} \xrightarrow{Mg^{++},\ \text{thiamine-P synthase}} \text{thiamine-P} + (\text{PP}) \qquad (4)$$

$$\text{thiamine-P} \xrightarrow{\text{thiamine-P phosphatase}} \text{thiamine} + (\text{P}) \qquad (5)$$

$$\text{thiamine} + \text{ATP} \xrightarrow{Mg^{++},\ \text{thiamine pyrophosphorylase}} \text{thiamine-PP} + (\text{AMP}) \qquad (6)$$

---

[1]Abbreviations used are: pyrimidine, pyrimidine-P and pyrimi-

The enzyme system which carries out these reactions was partially purified and separated into two fractions by fractionation of a crude extract of baker's yeast with ammonium sulfate followed by chromatography on diethylaminoethyl cellulose columns. These two enzyme fractions were both required for the formation of thiamine activity (as measured microbiologically with Lactobacillus viridescens) from the pyrimidine and thiazole moieties of thiamine. Additional investigations have indicated that this enzyme separation was a reflection of having separated pyrimidine kinase from thiazole kinase. With the exception of thiamine-P phosphatase, which has been separated completely from the two enzyme fractions, and thiamine pyrophosphorylase which is contained in only one of the fractions, the quantitative distribution of the remaining enzymes within the two separated enzyme fractions is not precisely known.

Reaction 1 (shown above) was demonstrated by the finding that an enzyme present in yeast, presumably a pyrimidine hydrolase, could hydrolyze both aminomethyl- and methoxymethylpyrimidines to yield a compound which microbiologically and chromatographically was identical to pyrimidine. Presumably, bromomethylpyrimidine and thiamine are similarly hydrolyzed since these compounds serve as substrate for the

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dine-PP for 2-methyl-4-amino-5-hydroxymethylpyrimidine, the ortho phosphoric acid ester, and the pyrophosphoric acid ester of this compound, respectively; pyrimidine-R for pyrimidine-5-R methyl derivatives where R corresponds to a bromo, amino, methoxy, or thiazole (thiamine itself) group; thiazole and thiazole-P for 4-methyl-5-(2-hydroxyethyl)-thiazole and the ortho phosphoric acid ester of this compound, respectively; thiamine-P and thiamine-PP for thiamine mono- and di- phosphate; AMP, ADP and ATP for adenosine mono- di- and tri-phosphate; and P and PP for inorganic ortho- and pyro-phosphate.

formation of the products of reaction 2.

The two compounds shown as products of reaction 2 have both been isolated from enzymatic reaction mixtures. One compound was identified as pyrimidine-P by analyses which showed one mole of phosphorus per mole pf pyrimidine. Pyrimidine was measured spectrophotometrically as well as by microbiological assay with a mutant of Salmonella typhimurium which requires this specific pyrimidine or thiamine for growth. Since the compound which is thought to be pyrimidine-PP is quite labile, it has not been possible to isolate it in large enough quantities for accurate phosphorus analyses. Indirect evidence which indicates that it is pyrimidine-PP includes (a) an elution pattern from Dowex-1 columns which is similar to that of cytidine diphosphate, (b) its relative lability to acid hydrolysis (when compared to pyrimidine-P) to yield both the free pyrimidine and pyrimidine-P, (c) the inhibition of the formation of thiamine-P (reaction 4) by pyrophosphate (which presumably is a product of reaction 4), (d) its conversion to pyrimidine-P and free pyrimidine by phosphatases, and (e) its formation enzymatically from pyrimidine-P in the presence of ATP and magnesium ion. Whether or not pyrimidine-P is an obligate intermediate in the formation of pyrimidine-PP cannot be decided from the evidence currently available. The enzymes concerned must be further purified before this question can be answered.

Thiazole-P was prepared by cleaving thiamine-P with sulfite followed by chromatography on Dowex-1 (formate) to separate the compound from the other components of the reaction mixture. The isolated compound, which analyzed to contain the expected one mole of phos-

phorus per mole of thiazole, was shown by paper chromatography to be identical with the compound formed enzymatically from thiazole and ATP (reaction 3). The isolated thiazole-P was also able to serve as substrate along with pyrimidine-PP for the synthesis of thiamine-P.

The products of reactions 4, 5, and 6 have been identified by comparing their mobilities on paper chromatograms, in a variety of solvent systems, with the mobilities of the corresponding known compounds and by their use as growth factors by Lactobacillus viridescens. Thiamine cannot be used as a substrate in place of thiazole for reaction 3. Free thiazole or pyrimidine-P cannot be used as substrate for reaction 4 and thiamine-P cannot be used as a substrate for reaction 6. Thus, pyrimidine-PP, thiazole-P, thiamine-P and thiamine are all necessary intermediates in the biosynthesis of thiamine-PP from pyrimidine and thiazole.

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