The Biosynthesis of Thiamine

I. ENZYMATIC FORMATION OF THIAMINE AND PHOSPHATE ESTERS OF THE PYRIMIDINE MOIETY OF THIAMINE*

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The biosynthesis of thiamine appears to be accomplished with the initial formation of the pyrimidine and thiazole moieties by independent biosynthetic pathways, followed by a final step in which 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-(β-hydroxyethyl) thiazole are joined together to give thiamine. This conclusion was indicated originally from the observations that certain thiamine-requiring microorganisms can utilize the combination of hydroxymethylpyrimidine1 plus thiazole in place of thiamine, a fact which suggests that these organisms cannot synthesize either hydroxymethylpyrimidine or thiazole, but are able to form thiamine from these two compounds. The nutritional and genetic studies on a series of thiamine-requiring Neurospora mutants by Tatum and Roll (1) and Harris (2) tended to support the above conclusion, although some anomalous results obtained by these workers led them to postulate the existence of Neurospora of a second pathway in which hydroxymethylpyrimidine is condensed with a thiazole precursor followed by the conversion of the unknown condensation product to thiamine (2).

The first relevant enzymatic evidence was provided by Harris and Yavit (3), who reported that extracts of bakers' yeast could catalyze the formation of thiamine from hydroxymethylpyrimidine, thiazole, and ATP in the presence of magnesium ions. They also reported that a synthetic monophosphate ester of hydroxymethylpyrimidine (pyrimidine-P) could replace the hydroxymethylpyrimidine and ATP requirements. The latter observation suggested that pyrimidine-P is an intermediate which reacts with thiazole to form thiamine and that this phosphate ester is formed enzymatically from hydroxymethylpyrimidine and ATP. However, investigations by Leder (4) indicated that ATP is the reactive intermediate, and also that this intermediate reacts with thiazole monophosphate (thiazole-P), rather than thiazole, to yield thiamine monophosphate (thiamine-P) as the product. These conclusions resulted from the observations of Nose et al. (5) and of Leder (6) that synthetic pyrimidine-PP and thiazole-P are converted enzymatically in the absence of ATP to a compound identified (6) as thiamine-P, and also from the observations of Camiener and Brown (7) who detected the absence of ATP by purified enzymes, whereas thiazole and hydroxymethylpyrimidine or pyrimidine-P were converted to thiamine-P only in the presence of ATP and the proper phosphorylating enzymes. The present paper describes the experiments which led to the detection of phosphate esters of hydroxymethylpyrimidine as intermediates and also the details of the isolation and identification of these compounds. The second paper in the series (8) describes the fractionation of the enzyme system and presents the evidence for the enzymatic conversion of pyrimidine-PP and thiazole-P to thiamine-P.

MATERIALS AND METHODS

Materials—Generous amounts of hydroxymethylpyrimidine, methoxymethylpyrimidine, bromomethylpyrimidine, and thiazole were kindly supplied by Merck and Company, Inc. Aminoethylpyrimidine was purchased from the California Corporation for Biochemical Research, crystalline ATP from the Pabst Laboratories, and Dowex 1 from the Dow Chemical Company. Alkaline phosphatase purified from Escherichia coli was a gift from Drs. F. Rothman and C. Levinthal; acid prostatic phosphatase was a gift from Dr. Gerhard Schmidt.

Phosphate Determinations—Inorganic phosphate was determined by the procedure of Lowry and Lopez (9). Total phosphate (inorganic plus organic) was determined by the method of Koerner and Sinsheimer (10). Microbiological Assays—Thiamine was determined by microbiological assay with Lactobacillus viridescens (ATCC No. 12706) according to the general directions recommended by Deibel et al. (11). The basal growth medium (11) used was modified to contain 1-asparagine (2 g per liter) and the following vitamins in amounts per liter of medium: pantothenic acid, 1.0 mg; niacinamide, 1.0 mg; riboflavin, 1.0 mg; p-aminobenzoic acid, 2.0 mg; and ATP. However, investigations by Leder (4) indicated that yeast preparations could convert pyrimidine-P and thiazole to thiamine only if ATP were also supplied. The present paper describes the enzymatic conversion of pyrimidine-PP and thiazole-P to thiamine-P.

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1 The abbreviations used are: hydroxymethylpyrimidine for 2-methyl-4-amino-5-hydroxymethylpyrimidine; bromomethylpyrimidine, methoxymethylpyrimidine, and aminoethylpyrimidine for the corresponding 2-bromomethyl, 5-methoxymethyl, and 5-aminoethyl derivatives of 2-methyl-4-aminopyrimidine; thiazole for 4-methyl-5-(β-hydroxyethyl)thiazole; and EDTA for ethylenediaminetetraacetic acid.
mg; pyridoxine, 4.0 mg; folic acid, 0.2 mg, and biotin, 0.02 mg. The thiamine-free yeast extract component of the medium was prepared according to the directions of Niven and Smiley (12). These modifications resulted in more reproducible results and also in more rapid growth of the organism so that assay tubes, when incubated at 37°, had to be read after a 5- to 8-hour incubation period. Growth was estimated turbidimetrically in a Coleman Junior spectrophotometer (660 mμ). Phosphorylated forms of thiamine are somewhat less active than thiamine in this assay, and the pyrimidine and thiazole moieties of the vitamin are completely inactive.

The microorganism used for assay of the pyrimidine moiety of thiamine was a mutant (designated as ATh4) of Salmonella typhimurium, which was obtained from Dr. H. Ozeki of the Department of Genetics, Cold Spring Harbor, New York. This mutant is characterized by having a nutritional requirement for adenine plus either thiamine or the pyrimidine moiety of thiamine. The pyrimidine moiety can be supplied as either hydroxymethylpyrimidine or bromomethylpyrimidine, but neither aminomethylpyrimidine nor methoxymethylpyrimidine can be utilized. Assay tubes (18 × 150 mm) were prepared in the customary manner (13); those used for the standard curve were prepared to contain from 0 to 10^-6 μmoles of thiamine per 10 ml of growth medium. One drop of a suspension of washed cells, adjusted so that it was only faintly turbid to the eye, was used to inoculate each assay tube. The tubes were incubated, without shaking, at 37° for 18 to 24 hours, and growth was estimated turbidimetrically. The basal medium contained, per liter: K2HPO4, 21 g; KH2PO4, 9 g; sodium citrate-2H2O, 1.0 g; MgSO4, 0.1 g; ammonium sulfate, 2.0 g; mannitol, 20 g; thiamine-free yeast extract, 10 g; and the same vitamins that were used in the growth medium for L. viridescens. Stock cultures were carried on nutrient agar and were transferred weekly in order to maintain vigorous cultures.

**Paper Chromatographic and Bioautographic Methods—**Paper chromatograms (Whatman No. 1 paper) were developed by the ascending technique. The solvents used will be indicated as the results are presented. Zones of migration of growth-promoting pyrimidine compounds were located by bioautography (14). For this purpose the developed chromatogram was placed in contact with the surface of solid medium (the S. typhimurium basal medium described above solidified with 2% agar and seeded with a culture of S. typhimurium mutant ATh4) containing a 5-mm layer in a sterile 22 × 30 cm Pyrex baking dish. After about 5 minutes, the chromatogram was removed and the plate was covered and incubated for 16 hours at 37°. The resulting growth zones corresponded to zones of migration of the pyrimidine compounds present on the original chromatogram. The inoculum used to seed the plates in these studies consisted of a suspension of washed cells taken from a 24 hour culture grown on a nutrient agar slant. One milliliter of this suspension (absorbancy reading, about 0.10) was used to inoculate 200 ml of the sterile basal growth medium before preparation of the plate.

**Preparation of Cell-free Extracts—**Extracts of bakers' yeast were prepared according to the directions given in Paper II of this series (8).

**RESULTS**

**Enzymatic Synthesis of Thiamine from Hydroxymethylpyrimidine and Thiazole—**The data of Table I show that cell-free extracts of bakers' yeast were able to convert hydroxymethylpyrimidine and thiazole to thiamine only if both ATP and Mg++ were added to the reaction mixture. Dialysis did not appreciably reduce the activity of the enzyme system, nor did the addition of possible sources of cofactors (commercial yeast extracts and "boiled juice") have any effect on thiamine production by a dialyzed enzyme preparation (Experiment 2, Table I).

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Thiamine equivalents synthesized* (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>17.1</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>0</td>
</tr>
<tr>
<td>Minus Mg++</td>
<td>0</td>
</tr>
<tr>
<td>Minus hydroxymethylpyrimidine</td>
<td>0</td>
</tr>
<tr>
<td>Minus thiazole</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>14.0</td>
</tr>
<tr>
<td>Minus Mg++</td>
<td>0</td>
</tr>
<tr>
<td>Plus yeast extract (Basamin), 1.0 mg</td>
<td>12.9</td>
</tr>
<tr>
<td>Plus yeast extract (Difco), 1.0 mg</td>
<td>10.2</td>
</tr>
<tr>
<td>Plus boiled extract, 0.1 ml</td>
<td></td>
</tr>
</tbody>
</table>

* Although it is known that under the given conditions in this experiment, and in subsequent ones to be discussed, the products were thiamine-P and thiamine-PP as well as thiamine, the results nevertheless are expressed as thiamine equivalents because the data were obtained from a microbiological assay in which the growth response to thiamine was used to construct the standard curve.

**Use of Various Pyrimidine Compounds as Substrates for Thiamine Synthesis—**Other pyrimidines which differ from hydroxymethylpyrimidine and thiazole which were supplied. Also, the enzyme system could not be saturated with ATP and Mg++ at the levels at which these two components were tested; i.e. thiamine synthesis was still increasing at the highest concentrations at which ATP and Mg++ were tested.

**Table 1**

<table>
<thead>
<tr>
<th>Substrate and cofactor requirements for thiamine synthesis by extracts of bakers' yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>The complete reaction mixture contained hydroxymethylpyrimidine, 58 μmole; thiazole, 58 μmole; ATP, 10 μmole; MgCl2, 10 μmole; and crude extract of bakers' yeast, 0.1 ml, in a total volume of 1 ml of 0.1 M phosphate buffer, pH 6.9. Incubation was for 3 hours at 37°. Reactions were stopped by heating at 100° (water bath) for about 5 minutes. In Experiment 2, a dialyzed enzyme preparation was used in place of the crude extract. Bolled extract was prepared by heating the crude extract at 100° for 5 minutes, followed by centrifugation to remove denatured protein.</td>
</tr>
</tbody>
</table>
Fig. 1. Thiamine synthesis as a function of the concentrations of hydroxymethylpyrimidine (A), thiazole (A), ATP (B), and Mg$^{++}$ (B). Reaction mixtures and incubation conditions were as described in Table I, Experiment I, except that the concentrations of the components were varied as indicated.

Table II

Ability of various pyrimidine compounds to serve as enzymatic precursors of thiamine

The reaction mixtures and incubation conditions were identical with those described in Table I except that the indicated pyrimidine was substituted for hydroxymethylpyrimidine as substrate.

<table>
<thead>
<tr>
<th>Pyrimidine used as substrate</th>
<th>Thiamine equivalents produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxymethylpyrimidine</td>
<td>21.6</td>
</tr>
<tr>
<td>Bromomethylpyrimidine</td>
<td>20.0</td>
</tr>
<tr>
<td>Aminomethylpyrimidine</td>
<td>7.6</td>
</tr>
<tr>
<td>Methoxymethylpyrimidine</td>
<td>8.4</td>
</tr>
</tbody>
</table>

enzymatically to hydroxymethylpyrimidine before their utilization for thiamine synthesis. To test this hypothesis, the aminomethyl- and methoxymethylpyrimidines were incubated, in separate experiments, with the enzyme preparation in the absence of thiazole and ATP. The reaction mixtures were then analyzed for the appearance of hydroxymethylpyrimidine by bioautography. It was evident from the bioautogram obtained (Fig. 2) that aminomethylpyrimidine and methoxymethylpyrimidine had been converted enzymatically to hydroxymethylpyrimidine. It is presumed that hydroxymethylpyrimidine is formed from bromomethylpyrimidine in a similar manner; however, this could not be proven in the manner described above because bromomethylpyrimidine (unlike the aminomethyl and methoxymethyl compounds) is active in replacing hydroxymethylpyrimidine as a growth factor for the *X. typhimurium* mutant. Also, the two compounds have identical *R*$_F$ values on paper chromatograms.

Whether or not the same enzyme is responsible for the conversion of all of these pyrimidines to hydroxymethylpyrimidine cannot be decided until enzyme fractionation and purification studies are undertaken.

**Enzymatic Formation of Phosphorylated Pyrimidine Intermediates**—Efforts were made to provide evidence for the possible formation of phosphorylated forms of hydroxymethylpyrimidine and thiazole as intermediates in thiamine synthesis. For this purpose, the enzyme preparation was incubated with ATP and either hydroxymethylpyrimidine or thiazole for 1 hour. The missing component (either hydroxymethylpyrimidine or thiazole) then was added to the reaction mixture, and the rate of formation of thiamine was compared to the rate observed in a control reaction mixture to which all components were added at the same time. The data (Fig. 3) show definitely that the preliminary incubation of hydroxymethylpyrimidine with ATP and the enzyme preparation resulted in an increased rate of thiamine synthesis. No increase was noted in the reaction mixture which contained "preincubated" thiazole. These results suggested that during the preliminary incubation there developed a phosphorylated form of hydroxymethylpyrimidine which served as a more direct precursor of thiamine. Although these experiments did not indicate the existence of a phosphorylated thiazole intermediate, they likewise did not exclude such a possibility, since in experiments of this type one cannot expect to detect an intermediate unless its formation is a rate-limiting step in the reaction sequence.

The formation of phosphorylated forms of hydroxymethylpyrimidine by the enzyme system was corroborated by paper chromatographic methods. A small aliquot of an incubated reaction mixture which initially contained hydroxymethylpyrimidine, ATP, Mg$^{++}$, and the enzyme preparation (see Table I for concentrations) was chromatographed according to the pro-
Fig. 3. Effect on the rate of thiamine synthesis of "preincubation" of hydroxymethylpyrimidine and thiazole with ATP and enzyme preparation. See the text for the procedure. The concentrations of the components of reaction mixtures and the incubation conditions were as described in Table I.

Fig. 4. A drawing of bioautograms which illustrates the migration characteristics on paper chromatograms and the stability to phosphatases and HCl of phosphorylated forms of hydroxymethylpyrimidine formed enzymatically. Small samples (0.002 to 0.005 ml) of a reaction mixture (described in the text) were spotted on paper strips, and the chromatograms were developed with isobutyric acid-NH$_2$OH-water (18:3:99, volume per volume). The chromatograms were treated with either n HCl, alkaline phosphatase, or prostatic phosphatase (as shown in the figure and according to the directions given in the text) before the preparation of the bioautogram.

phosphatase-treated reaction mixtures which, before treatment, had contained both of the pyrimidine derivatives.

**Isolation of Pyrimidine Derivatives I and II**

A 1 liter reaction mixture was prepared with 15 mmoles of ATP, 20 mmoles of MgCl$_2$, 0.5 mmoles of hydroxymethylpyrimidine, and 900 ml (20 g of protein) of an extract of bakers' yeast previously dialyzed for a total of 18 hours against 3 (6 hours each) successive 10-liter portions of 0.13 M potassium phosphate buffer (pH 7.0), which also contained, as protective agents, EDTA (0.02 M) and cysteine (0.02 M). The mixture was adjusted to pH 8.4 and incubated for 3.5 hours at 37°. After incubation, the reaction mixture (in 50- to 60-ml lots) was heated briefly at 100°, and the coagulated protein was collected by centrifugation and discarded. Bioautograms prepared from aliquots of the clarified reaction mixture showed that the hydroxymethylpyrimidine substrate had been completely converted to pyrimidine derivatives I and II and that the two compounds were present in approximately equimolar quantities. The remainder of the reaction mixture was adjusted to pH 5.4, centrifuged to remove a small amount of insoluble material, and stored at 4° until it was passed through the charcoal column described next.

**Adsorption on and Elution from Charcoal—**A glass column, 95 mm (diameter) × 110 cm (length), was packed with 1200 g of a charcoal mixture consisting of equal parts (weight per weight) of Celite 545 and 325 mesh Norit. The packed column was degassed by passing 15 to 16 liters of cold, degassed, deionized water through it (flow rate, 10 to 20 ml per minute). The cold
reaction mixture then was passed through the column (flow rate, 10 ml per minute) and the column was washed successively with 2 liters of cold, degassed water, 8 liters of 0.1 M EDTA solution, pH 7.0, and finally another 2 liters of water. A total of 20 liters (about 10 column volumes) of an aqueous ethanol solution (50 volumes of 95% ethanol plus 50 volumes of water) were then passed through the column at a flow rate which varied from 5 to 15 ml per minute; the first 8 liters of the ethanol eluate were collected in 500-ml fractions and the remaining 18 liters in 1- to 4-liter fractions and the fractions were stored at 4°C. The above procedure was suggested by the work of Pontis et al. (15), who used a similar one to purify certain nucleoside diphosphate sugars on charcoal columns.

To test each eluate fraction for the presence of phosphorylated forms of hydroxymethylpyrimidine, 0.1-ml aliquots were heated (100°C) to boil off the ethanol, then incubated at 37°C for 2 hours with about 2 μg of alkaline phosphatase in 1.0 ml of 0.1 M Tris buffer (pH 8.5), and finally assayed for hydroxymethylpyrimidine content with the *S. typhimurium* mutant. All of the fractions, except the first 500-ml fraction, contained phosphorylated hydroxymethylpyrimidine, although the fractions which were collected contained only small amounts. Although the recovery of phosphorylated hydroxymethylpyrimidine from the column was only 30 to 40%, the step was nevertheless considered to be worthwhile since it served to eliminate large amounts of inorganic salts which were present in the reaction mixture and which would otherwise have interfered with subsequent purification on ion exchange columns.

The ethanol eluates were concentrated under reduced pressure to 500 to 600 ml and stored at 4°C until passage through the first Dowex column.

**First Dowex 1 Column.—**A 300-ml portion of Dowex 1-formate ion exchange resin was used to prepare a column 3.6 cm in diameter and 30 cm long. The column was washed with 1500 ml of 85% formic acid followed by about 6 liters of distilled water until all of the formic acid had been washed from the resin. The ethanol-free effluent from the charcoal column was adjusted to pH 7.9 and passed through the column. After the column had been washed with 1 liter of distilled water, a linear concentration gradient elution was begun. The gradient was scaled to increase at a rate of 0.1 M ammonium formate per 2 liters of eluting solution from an initial concentration of 0 to a final concentration of 0.6 M ammonium formate. One hundred fractions of 100 to 110 ml each were collected (flow rate, 10 to 12 ml per minute).

The absorbancy of each fraction was measured at 260 and 275 μm, and aliquots of each fraction were treated with alkaline phosphatase and assayed for hydroxymethylpyrimidine with the *S. typhimurium* mutant. Two peaks containing phosphorylated hydroxymethylpyrimidine were obtained. It was determined by the bioautographic methods described earlier that the major peak, shown in Fig. 5, contained about 480 μmoles of pyrimidine derivative I and that the minor peak contained approximately 30 μmoles of pyrimidine derivative II. It will be noted from Fig. 5 that most of the pyrimidine derivative I fractions were free from contaminating ultraviolet-absorbing material but that the pyrimidine derivative II fractions contained large amounts of these contaminants. It was decided to put the fractions containing the phosphorylated hydroxymethylpyrimidine through a second Dowex 1 column in an effort to rid these compounds of the contaminating substances. Accordingly, the fractions which contained most of the pyrimidine derivative I (37 to 43) and pyrimidine derivative II (52 to 59) were combined, adjusted to pH 7.0 with ammonium hydroxide, and concentrated under reduced pressure to a volume of about 75 ml. This concentrate was then lyophilized, taken up in 15 ml of distilled water, and relyophilized for 2 days in order to remove the ammonium formate. The residue was dissolved in about 120 ml of water and adjusted to pH 7.9 before application to the second Dowex column.

**Second Dowex 1 Column.—**Dowex 1-formate resin, 100 ml, was used to prepare as described earlier a column 2.2 cm in diameter and 30 cm long. After the ammonium formate-free eluates from the first Dowex column had been allowed to pass through the column, a linear concentration gradient elution, scaled to increase from 0 to 0.4 M ammonium formate at a rate of 0.1 M per 4 liters of eluting solution, was applied to the column. Fractions were collected in 17 to 20 ml amounts at a flow rate of 10 to 12 ml per minute.

As before, each fraction was analyzed for phosphorylated hydroxymethylpyrimidine and for ultraviolet-absorbing material. The elution pattern from this column was similar to that shown in Fig. 5 for the first Dowex 1 column, except that the fractions containing the phosphorylated forms of hydroxymethylpyrimidine were found to be less contaminated with other ultraviolet-absorbing material by several fold. Pyrimidine derivative I was eluted by 0.05 to 0.07 M ammonium formate and pyrimidine derivative II by 0.09 to 0.11 M ammonium formate. Both compounds were obtained in quantitative yields. Examination of the fractions containing pyrimidine derivative I in a Cary recording spectrophotometer indicated that this compound was free from contaminating ultraviolet-absorbing materials as judged by a comparison of its spectrum with that of authentic hydroxymethylpyrimidine. The presence of the phosphate group apparently does not noticeably alter the spectrum of the compound. Similar spectrophotometric analyses of the pyrimidine derivative II fractions showed that these fractions were still appreciably contaminated with ultraviolet-absorbing material. Only that portion of pyrimidine derivative II which was recovered in the trailing edge of the pyrimidine derivative II peak was reasonably free from contaminating substances.

Phosphate determinations revealed that the pyrimidine deriva-
tive I fractions contained 3 moles of phosphate per mole of hydroxymethylpyrimidine and also suggested that inorganic phosphate was present as a contaminant. The pyrimidine derivative II fractions contained 4 to 5 moles of phosphate per mole of hydroxymethylpyrimidine. In order to obtain pyrimidine derivative I free of inorganic phosphate, the fractions containing this compound were passed through another charcoal column. Owing to its lability on charcoal columns, pyrimidine derivative II could not be purified further in this manner and, in unsuccessful attempts to do so, much of this compound obtained from the Dowex column was lost.

Purification of Pyrimidine Derivative I on Charcoal Column—Six grams of the charcoal mixture described earlier were packed into a column 25 mm in diameter. Before adsorption of pyrimidine derivative I, the column was degassed as described earlier and washed successively with 0.1 M EDTA (100 ml), 300 ml of 47.5%, ethanol, and 2 liters of deionized water. Eleven micro-moles of pyrimidine derivative I contained in 90 ml of the peak fractions from the second Dowex 1 column were passed through the column at a flow rate of 1.5 ml per minute. The column was then washed successively with 150 ml of 0.1 M EDTA, 150 ml of deionized water, and finally 300 ml of 47.5% ethanol. The pyrimidine derivative I-containing ethanol eluate was collected and concentrated under reduced pressure to a volume of 40 to 50 ml.

Purification of Pyrimidine Derivative I on Third Dowex 1 Column—Dowex 1-formate resin, 5 ml, was used to prepare a column 0.9 cm in diameter and 8 cm long. A 20-ml portion of the concentrated eluate from the charcoal column was adjusted to pH 8.0 with ammonium hydroxide and passed through the column. The resin was then washed with 40 to 50 ml of deionized water. The following solutions of ammonium formate were allowed to pass successively through the column: 40 ml of 0.005 M, 60 ml of 0.01 M, and 50 ml of 0.025 M. Fractions were collected in 10-ml volumes at a flow rate of the column of about 1 ml per minute. Spectrophotometric and microbiological determinations showed that pyrimidine derivative I had been eluted from the column by the 0.01 M ammonium formate.

Pyrimidine derivative I contained in these fractions was used to determine the properties of the compound.

Properties of Pyrimidine Derivative II—Experiments designed to establish elution patterns from Dowex 1 columns revealed that pyrimidine derivative II is eluted (see Fig. 5 for conditions) immediately after AMP and immediately before ADP; ATP was eluted after ADP. Thus pyrimidine derivative II obtained from the second Dowex 1 column, although not spectrophotometrically pure, nevertheless was free from ATP and pyrimidine-P (established by bioautography) and was therefore suitable for use in enzyme experiments. The original yeast enzyme system was separated into two enzyme fractions, designated as Fraction 1 and Fraction 2 (8). Fraction 2 was capable of forming thiamine-P directly from thiazole-P and pyrimidine derivative II, but not from pyrimidine-P (see Table IV).

Fig. 6. The ultraviolet absorption spectra of hydroxymethylpyrimidine and pyrimidine derivative I in 0.05 M ammonium formate at pH 4.0 (A) and pH 10.5 (B). The pyrimidine derivative I solutions contained 46 μmoles per ml. Hydroxymethylpyrimidine concentrations were 116 μmoles per ml (A) and 96 μmoles per ml (B).

<table>
<thead>
<tr>
<th>Substrates added</th>
<th>Enzyme fraction added</th>
<th>Thiamine-P equivalents produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrimidine derivative II</td>
<td>2</td>
<td>6.8</td>
</tr>
<tr>
<td>Pyrimidine derivative II</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pyrimidine derivative I</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Pyrimidine derivative I + ATP</td>
<td>2 and 1</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Pyrimidine derivative I contained in these fractions was used to determine the properties of the compound.

Properties of Pyrimidine Derivative I and Its Identification as Pyrimidine-P—Fig. 6 shows the similarity of the absorption spectra of isolated pyrimidine derivative I and hydroxymethylpyrimidine in acid and alkaline solutions. Phosphate analyses revealed that pyrimidine derivative I contained 1 mole of phosphate per mole of hydroxymethylpyrimidine (Table III). No inorganic phosphate could be detected in the pyrimidine derivative I preparation. These data indicate that pyrimidine derivative I is a monophosphate ester of hydroxymethylpyrimidine (pyrimidine-P). The stability of the compound to acid indicates that the phosphate group is not attached to the amino group of the hydroxymethylpyrimidine, but instead is esterified with the hydroxymethyl group.

Properties of Pyrimidine Derivative II—Experiments designed to establish elution patterns from Dowex 1 columns revealed that pyrimidine derivative II is eluted (see Fig. 5 for conditions) immediately after AMP and immediately before ADP; ATP was eluted after ADP. Thus pyrimidine derivative II obtained from the second Dowex 1 column, although not spectrophotometrically pure, nevertheless was free from ATP and pyrimidine-P (established by bioautography) and was therefore suitable for use in enzyme experiments. The original yeast enzyme system was separated into two enzyme fractions, designated as Fraction 1 and Fraction 2 (8). Fraction 2 was capable of forming thiamine-P directly from thiazole-P and pyrimidine derivative II, but not from pyrimidine-P (see Table IV). Pyrimidine-P could be used for thiamine-P synthesis only if Fraction 1 and ATP were also supplied (Table IV). Further investigations have shown that
Fraction 1 catalyzes the conversion of pyrimidine-P to pyrimidine derivative II (detected by bioautography) in the presence of ATP. Pyrimidine derivative II was destroyed quite readily by heating an aqueous solution at 180° for 20 to 30 minutes, or by treatment with 1 N HCl at room temperature. The products in both instances were free hydroxymethylpyrimidine and pyrimidine-P. These results suggest that pyrimidine derivative II is a pyrophosphate ester of hydroxymethylpyrimidine (pyrimidine-PP). Other observations which support this suggestion are: (a) the migration characteristics on paper chromatograms in several solvent systems which resemble those of cytidine diphosphate, and (b) the elution pattern from Dowex 1-formate columns which also resembles that of cytidine diphosphate.

The results described above also show that pyrimidine derivative II (pyrimidine-PP) is the pyrimidine compound which is the immediate precursor of thiamine.

**DISCUSSION**

The data described in this paper clearly show that both pyrimidine-P and what has been identified tentatively as pyrimidine-PP are formed by incubating an enzyme preparation from yeast with hydroxymethylpyrimidine and ATP. The experiments also show that pyrimidine-PP is the "activated" pyrimidine compound used directly for the enzymatic synthesis of thiamine. It is likewise clear that pyrimidine-PP can be degraded, enzymatically and chemically, to pyrimidine-P, and that pyrimidine-P can be converted enzymatically to pyrimidine-PP in the presence of ATP. The question arises as to whether pyrimidine-P is a necessary intermediate in the formation of pyrimidine-PP or whether pyrimidine-P is formed merely as a degradation product of pyrimidine-PP. In an effort to clarify this point, preliminary experiments were performed with ATP containing C14-labeled adenine to determine whether ADP or AMP is the product of the formation of the phosphorylated pyrimidines. In the absence of interfering side reactions, this experiment could determine whether pyrimidine-PP is formed directly from hydroxymethylpyrimidine or whether it arises via the intermediate formation of pyrimidine-P. However, the enzyme which was used (purified Fraction I) was found to be so contaminated with phosphatase, and perhaps myokinase, that all of the adenosine-containing compounds (including adenosine itself) analyzed were equally radioactive. The elucidation of the exact mechanism of formation of pyrimidine-PP therefore must be postponed until the enzymes have been further purified.

**SUMMARY**

Cell-free extracts of bakers' yeast catalyzed the conversion of 2-methyl-4-amino-5-hydroxymethylpyrimidine (hydroxymethylpyrimidine) and 4-methyl-5-(β-hydroxyethyl)thiazole to thiamine compounds (either thiamine, thiamine monophosphate, or thiamine pyrophosphate) which are active in supporting the growth of *Lactobacillus viridescens*. Adenosine triphosphate and magnesium ions were also required in the system.

Other pyrimidine compounds which contained bromomethyl, aminomethyl, or methoxymethyl groups in place of the hydroxymethyl group could also be used by the enzyme system for the synthesis of thiamine. It was shown that these pyrimidines were converted enzymatically to hydroxymethylpyrimidine before being used for thiamine synthesis.

Evidence was presented for the enzymatic formation of a phosphorylated derivative of hydroxymethylpyrimidine (formed in the presence of adenosine triphosphate) which serves as an intermediate in thiamine formation. Two phosphorylated derivatives of hydroxymethylpyrimidine were detected in enzymatic reaction mixtures. Procedures for the isolation of these compounds were described. One derivative was identified by its stability characteristics, phosphate content, and ultraviolet absorption spectrum as the monophosphate ester of hydroxymethylpyrimidine. The other derivative was identified tentatively as the pyrophosphate ester of hydroxymethylpyrimidine. The pyrophosphate ester was shown to be the intermediate active in thiamine formation. Whether the monophosphate ester is an intermediate in the formation of the pyrophosphate ester or whether it is merely a degradation product of the pyrophosphate compound cannot be decided from the available information.

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