The Enzymatic Synthesis of Hydroxymethyldihydropteridine Pyrophosphate and Dihydrofolate*

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ABSTRACT: The enzymes involved in the reactions leading to dihydrofolate from extracts of *Lactobacillus plantarum* were resolved into two fractions by DEAE-cellulose chromatography.

Fraction I catalyzed the pyrophosphorylation of 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine (hydroxymethyldihydropteridine) to 2-amino-4-hydroxy-6-pyrophosphorylmethyl-7,8-dihydropteridine (hydroxymethyldihydropteridine pyrophosphate). Fraction II coupled the latter pteridine with *p*-aminobenzoylglutamate or *p*-aminobenzoate to give dihydrofolate or dihydropteroate. These conclusions were based on experimental findings which show that the reaction catalyzed by fraction I requires adenosine triphosphate and hydroxymethyldihydropteridine. Furthermore, the isolated product of this reaction had a spectrum similar to a dihydropteridine, contained two phosphate moieties, incorporated ^{32}P indicating the transfer of the P–^{32}P residue from adenosine triphosphate-γ-^{32}P, and when chromatographed with carrier hydroxymethyldihydropteridine pyrophosphate gave the expected specific activity in fractions collected from a DEAE-cellulose column. Fraction II utilized the product of the above enzymatic reaction or synthetic hydroxymethyldihydropteridine pyrophosphate to synthesize dihydrofolate or dihydropteroate with *p*-aminobenzoylglutamic acid or *p*-aminobenzoic acid, respectively. A sigmoidal kinetic response was observed with each cosubstrate. This fraction was completely inactive in a system containing hydroxymethyldihydropteridine, adenosine triphosphate, and *p*-aminobenzoylglutamic acid or *p*-aminobenzoic acid. We suggest that the enzymes of fractions I and II be named hydroxymethyldihydropteridine pyrophosphokinase and dihydropteroate or dihydrofolate synthetase, respectively.

The involvement of hydroxymethyldihydropteridine[1] and hydroxymethyldihydropteridine-PP in the enzymatic synthesis of dihydropteroate or dihydrofolate has been demonstrated in several bacteria (Brown *et al.*, 1961; Shiota and Disraely, 1961; Weisman and Brown, 1964; Shiota *et al.*, 1964; Ortiz and Hotchkiss, 1966) and more recently in plants (Mitsuda *et al.*, 1965; Mitsuda and Suzuki, 1968; Iwai *et al.*, 1968; Iwai and Okinaka, 1968).

The conversion of hydroxymethyldihydropteridine into either dihydropteroate or dihydrofolate proceeds by the two reactions (Brown *et al.*, 1961; Shiota and Disraely, 1961; Weisman and Brown, 1964; Shiota *et al.*, 1964; Richey and Brown, 1969) given below.

hydroxymethyldihydropteridine + ATP ⟶ hydroxymethyldihydropteridine-PP + AMP (1)

hydroxymethyldihydropteridine-PP + AB or ABG ⟶ dihydropteroate or dihydrofolate + PP (2)

While this manuscript was in preparation, Richey and Brown (Richey and Brown, 1969) reported the separation and purification of two enzyme preparations from *Escherichia coli*, one of which catalyzed the pyrophosphorylation of hydroxymethyldihydropteridine to hydroxymethyldihydropteridine-PP (reaction 1). The second enzyme fraction catalyzed the synthesis of dihydropteroate from hydroxymethyldihydropteridine-PP and AB (reaction 2). They proposed that the first enzyme be named hydroxymethyldihydropteridine pyrophosphokinase and the second dihydropteroate synthetase. To facilitate continuity we have adopted the above enzyme nomenclature.

We wish to report here the results of experiments with two enzyme fractions obtained from extracts of *Lactobacillus plantarum* which indicate that one of the fractions contains hydroxymethyldihydropteridine pyrophosphokinase and the other dihydropteroate synthetase. The results herein confirm and extend those recently reported by Richey and Brown (Richey and Brown, 1969).

Materials and Methods

Published procedures were used to prepare hydroxymethylpteridine (Waller *et al.*, 1950) and hydroxymethylpteridine-PP (Shiota *et al.*, 1964). The hydroxymethyldihydropteridine, prepared by dithionite reduction (Friedkin *et al.*, 1962), was stored frozen as a suspension in 0.005 N HCl. Hydroxymethyldihydropteridine-PP was prepared fresh by adding 0.5 or 1.0 μmole of hydroxymethylpteridine-PP, 0.2 ml of potassium ascorbate (20 mg/ml) (pH 7.0), and 0.05 ml of sodium dithionite (100 mg/ml) into a tube in a total volume of 0.5 ml. The solution was allowed to stand at room temperature for at least 15 min before use.

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[1] The abbreviations used are: hydroxymethylpteridine, hydroxymethyldihydropteridine, and hydroxymethyldihydropteridine-PP for 2-amino-4-hydroxy-6-hydroxymethylpteridine, the 7,8-dihydro form, and the 6-pyrophosphorylmethyl-7,8-dihydro forms, respectively; ABG for *p*-aminobenzoylglutamic acid; and AB for *p*-aminobenzoic acid.

The concentration of dihydropteridine compounds was determined at 330 mμ (pH 7.1), using a molar extinction coefficient of 6200 reported for dihydrobiopterin (Nagai, 1968) and for dihydroneopterin (Fukushima and Akino, 1968). Previously we had reported a value of 3700 for this compound (Shiota and Disraely, 1961). In the light of this discrepancy we have reexamined the absorbancy of freshly prepared dihydropteridine by the dithionite reduction method and have obtained values in close agreement with those reported for dihydrobiopterin and dihydroneopterin (Nagai, 1968; Fukushima and Akino, 1968).

Dihydrofolate or dihydropteroate was assayed by a microbiological assay using folic acid as the standard and *Streptococcus faecalis* as the assay organism similar to the procedure used previously (Shiota *et al.*, 1964). Potassium ascorbate was not routinely used. However, when AB and ABG were tested at equimolar levels for comparative purposes, potassium ascorbate was employed and the appropriate correction factors (Shiota *et al.*, 1964) were applied. These results are expressed as folate equivalents.

Protein was determined by the biuret method (Layne, 1957), using crystallized bovine albumin as the standard. DEAE-cellulose was prepared for chromatography as described previously (Shiota *et al.*, 1964). Phosphorus was determined by the procedure of Lowry (Lowry, 1957) after an alkaline phosphatase treatment (Shiota *et al.*, 1964).

Radioactivity was measured in a Packard scintillation spectrometer with the use of a scintillation fluid composed of 10 ml of 0.4% 2,5-diphenyloxazole in toluene and 6 ml of absolute ethanol.

Myokinase (1 mg converts 775 μmoles of ADP to AMP and ATP per minute at pH 7.6, 37°), hexokinase (1 unit will catalyze the phosphorylation of 1 μmole of glucose by ATP per minute at pH 8.5, 25°), and RNase (Type XII A) were purchased from Sigma Chemical.

ATP-γ-^{32}P was kindly provided by Dr. George Sachs.

Assay Procedures. The assays for the enzymes which catalyze reactions 1 or 2 depend upon the amount of dihydropteroate or dihydrofolate synthesized and are quantitated in folate equivalents by microbiological assay. The details of the enzyme assay procedures are described below. Assay 1 was performed by incubating enzyme fractions with 0.1 mM hydroxymethyldihydropteridine, 1.0 mM ABG, 1.0 mM ATP, 10 mM $MgCl_2$, 20 mM Tris buffer (pH 8.0), and 2.0 mM potassium ascorbate in a total volume of 0.25 ml for 1 hr under argon at 37°. Assay 2 which was performed as assay 1 is supplemented with an excess of standard fraction II (5 units). Standard fraction II was obtained by a previous DEAE-cellulose fractionation of a 45% ammonium sulfate fraction from an extract of *L. plantarum* and standardized by assay 3 which is described below. Since fraction II is active with hydroxymethyldihydropteridine-PP and ABG and is completely inactive with hydroxymethyldihydropteridine, ABG, and ATP, standard fraction II was used routinely to assay the product of fraction I. Assay 3 was carried out as described for assay 1 above except that 0.2 mM hydroxymethyldihydropteridine-PP was employed in place of hydroxymethyldihydropteridine. ATP was omitted from this assay. In each of the above assay procedures, one unit of fraction I or II is defined as that amount of enzyme which will synthesize 1 μg of folate equivalent under the described conditions.

Polyacrylamide Gel Electrophoresis of Fraction II. The preparation of the polyacrylamide gel (7%) and the procedure of electrophoresis (Polyanalyst, Buchler Instruments) were those described by Davis (Davis, 1964). The electrophoresis (4.5 mA/gel slab, pH 9.5) was terminated when the tracking dye emerged from the gel. The gel was stained with 1% Amido-Schwarz in 7% acetic acid and destained electrophoretically (12.5 mA/gel slab). For the enzyme assays, the gels were sliced (1 mm), macerated, and assayed as described above.

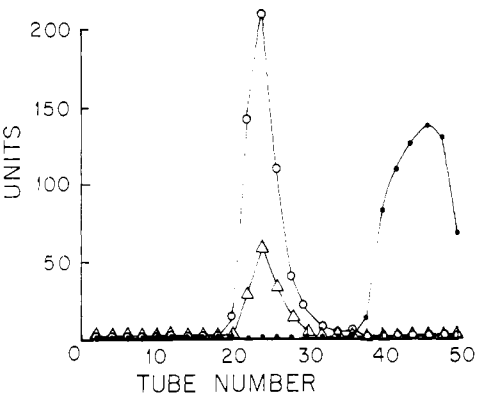

FIGURE 1: DEAE-cellulose chromatography of dialyzed 45% ammonium sulfate fraction. Dialyzed 45% ammonium sulfate fraction (0.5 g of protein) was added to a DEAE-cellulose column (2.7 × 33 cm) and the column was developed by linear gradient elution with the use of 550 ml of 0.01 M Tris buffer (pH 8.0) in the mixing flask and 550 ml of 0.2 M KCl in 0.01 M Tris buffer (pH 8.0) in the reservoir. Fractions were collected at a rate of 20 ml/tube per 8 min. The DEAE-cellulose fractions were assayed by assay 1 (△); assay 2 (○); assay 3 (●), described under Materials and Methods.

Purification of L. plantarum Extracts. Extracts of *L. plantarum* were prepared by disrupting a mixture of 25 g of dried cells (Shiota, 1959), 275 g of 0.1-mm glass beads (Dragen-Werk, George Wild, Bayreuth, Western Germany), and 60 ml of 0.01 M Tris buffer (pH 8.0) in a vibrating mill (Vibra-Cell Mill, Shuco Scientific Co.) at 5° for 30 min. The procedure which is to be described for the purification of the enzyme preparation was performed at 5°. This mixture was suspended with 62.5 ml of 0.01 M Tris buffer (pH 8.0) and centrifuged for 20 min at 30,000*g* and the supernatant fluid was set aside. The beads and cellular material were vibrated again for 30 min, suspended in 62.5 ml of buffer, and centrifuged and the supernatant fluid was accumulated as described above. This process was repeated four times. To the supernatant fluid (250 ml), 25 mg of RNase was added and the mixture stored overnight at 5°. The RNase treated extract was centrifuged to remove particulate material. To the supernatant fluid, solid ammonium sulfate was added to 45% saturation. The precipitate was collected by centrifugation, resuspended in 60 ml of 0.01 M Tris buffer (pH 8.0), and dissolved by dialysis against the same buffer for 17 hr.

DEAE-Cellulose Chromatography of Dialyzed 45% Ammonium Sulfate Fraction. The dialyzed 45% ammonium sulfate fraction of *L. plantarum* which contains fractions I and II was fractionated on DEAE-cellulose to effect the separation of the two enzymes. These results are presented in Figure 1. Assay 1 revealed enzyme activity in tubes 20–32 (fraction I); by assay 2, a greater activity is seen. On the other hand, assay 3 showed activity only in tubes 38–50 (fraction II). The tubes

TABLE I: The Sequence of the Reactions Catalyzed by Fractions I and II.[a]

Additions Made in the First Incubation Period	Additions Made in the Second Incubation Period	Folate Equivalent (mμg)
Fraction I	None	780
Fraction I	Fraction II	2690
Fractions I & II	None	2000
Fraction II	Fraction I	750
Fraction II	None	0

[a] The reaction mixture contained: hydroxymethyldihydropteridine, 0.6 mM; ABG, 1.0 mM; ATP, 1.0 mM; $MgCl_2$, 10 mM; Tris buffer, 20 mM, pH 8.0; potassium ascorbate, 2.0 mM and fraction I (125 μg of protein) and/or fraction II (248 μg of protein) as indicated. The final volume of each mixture was adjusted to 0.25 ml. At the end of 1 hr, each tube from the first incubation period was placed in a boiling water bath for 1 min, cooled and additions were made as indicated above for the second incubation period. The tubes were incubated under an atmosphere of argon at 37° for an additional hour. The reaction was terminated by placing the tubes in a boiling water bath for 2 min, and the content from each mixture was assayed for dihydrofolate.

TABLE II: The Phosphorous Content and the Specific Activities of DEAE-Cellulose Fractions.[a]

Tube Number	Phosphate (μmole/μmole)	Specific Activity (cpm/μmole)
32		306,069
33		308,545
34		308,415
35	1.9[b] 1.84[c]	296,065

[a] Phosphorous was determined as indicated under Materials and Methods. [b] Based on the specific activity of ATP-γ-^{32}P of 306,100 cpm/μmole. [c] Based on the molar extinction coefficient of 6200 (pH 7.1), at 330 mμ for hydroxymethyldihydropteridine.

containing each enzyme were pooled and concentrated by dialysis against polyethylene glycol (Carbowax 20 M, Mann) for further studies. Assays of enzymes in fractions I and II which were concentrated 20-fold indicated that these enzymes were clearly separated. Fraction I appears to have two properties according to assays 1 and 3. By assay 1, fraction I should contain some fraction II; however, no fraction II activity could be detected when assay 3 was performed on this preparation. In view of this, it is difficult to explain the low but definite ability of fraction I to synthesize dihydrofolate from hydroxymethyldihydropteridine, ATP, and ABG. The increased dihydrofolate found by assay 2 of fraction I suggested that the enzyme in this fraction synthesized an intermediate which acted as a substrate for the standard fraction II. Fraction II catalyzed the synthesis of dihydrofolate from hydroxymethyldihydropteridine-PP and ABG (assay 3) and was completely inactive when hydroxymethyldihydropteridine was used as the substrate (assays 1 and 2). These studies indicate that the DEAE-cellulose chromatography of the dialyzed 45% ammonium sulfate fraction resulted in the clear separation of two distinct enzymes.

The DEAE-cellulose step resulted in a 50-fold and a 4-fold purification for enzymes in fractions I and II, respectively. Magnesium chloride (10 mM) was required by fraction I to synthesize a product which was converted into 1040 mμg folate equivalents by standard fraction II. Omission of Mg^{2+} resulted in the failure of fraction I to synthesize this product. Fraction II synthesized 1290 mμg in the presence and 640 mμg folate equivalents in the absence of 10 mM $MgCl_2$. Both enzymes were inactivated by heating to 60° for 10 min.

The Sequence of the Reactions Catalyzed by Fractions I and II. The sequence of the reactions catalyzed by fractions I and II was determined by performing a two-stage incubation experiment. The first stage was carried out by incubating fraction I, fraction II, or fractions I and II with hydroxymethyldihydropteridine, ABG, ATP, potassium ascorbate, and $MgCl_2$ as indicated in Table I. The results presented in Table I show that fraction I alone catalyzes the synthesis of dihydrofolate (780 mμg). However, when fraction I in the first stage was followed by fraction II in the second stage the amount of dihydrofolate synthesized was three times as much as the amount produced by fraction I alone. In the case where the order of the addition of the fraction was reversed, the amount of dihydrofolate synthesized after the second-stage incubation period was no greater than that found when fraction I was incubated alone. These results indicate that fraction I has the property of converting hydroxymethyldihydropteridine in the presence of ABG and ATP to dihydrofolate and that it produces an intermediate from hydroxymethyldihydropteridine which is converted by fraction II to dihydrofolate.

The Isolation and the Identification of the Product from Fraction I. An experiment was performed in order to identify the product synthesized by fraction I by incubating fraction I with hydroxymethyldihydropteridine and ATP-γ-^{32}P as indicated in Figure 2A. This figure shows the DEAE-cellulose fractionation of the reaction mixture. There are two major radioactive peaks, tubes 18–21 and tubes 31–36. The first radioactive peak contains a 330-mμ absorbing material. The radioactive compound in this peak was identified as glucose-6-^{32}P by thin-layer chromatography using two different solvents (1-butanol–pyridine–H_2O, 6:4:3, and 1-butanol–1-propanol–H_2O, 1:1:1). The 330-mμ absorbing material was provisionally identified as hydroxymethyldihydropteridine by absorption spectra. The second radioactive peak contained a material which absorbs at 330 mμ and has cosubstrate activity. The minor peak (tubes 48–50) associated with absorption at 330 mμ and with radioactivity was not identified.

The absorption spectrum of fraction 35 presented in Figure 3 is identical with that of hydroxymethyldihydropteridine-PP. It is worthy to note that even though no precaution was taken to prevent oxidation, the pteridine was still reduced after the first DEAE-cellulose fractionation. The phosphorus analyses of fraction 35 indicated that the dihydropteridine contains two phosphates/mole (Table II). The specific activity of

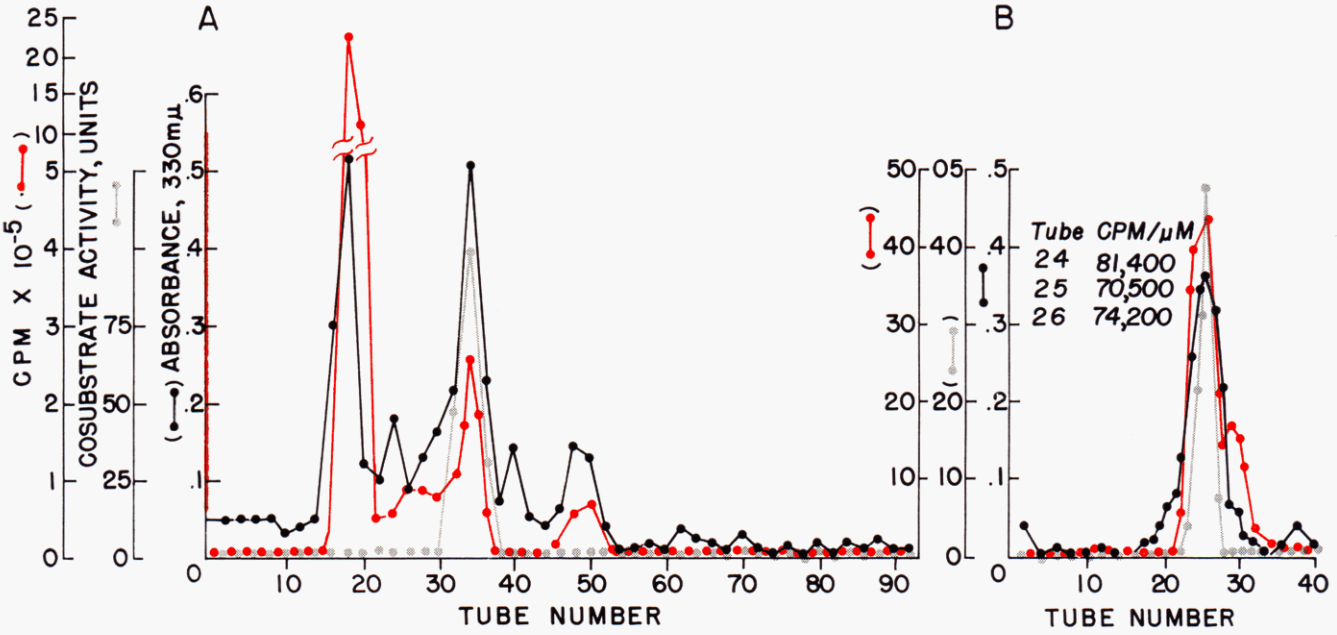

FIGURE 2: The separation and identification of the phosphorylated intermediate. (A) The purification of the phosphorylated intermediate produced by fraction I by DEAE-cellulose chromatography. The reaction mixture contained fraction I, 19 mg of protein, ATP-γ-^{32}P, 1.0 mM, 306,100 cpm/μmole; hydroxymethyldihydropteridine, 0.6 mM; $MgCl_2$, 10 mM; potassium ascorbate, 2.0 mM; and Tris buffer, 20 mM (pH 8.0) in a total volume of 25 ml. The mixture was incubated for 12 hr under an atmosphere of argon at which time 0.1 mg of myokinase, 150 units of hexokinase, and 100 μmoles of glucose were added (Weisman and Brown, 1964) and the mixture was allowed to incubate an additional 30 min. The mixture was then added to a DEAE-cellulose column (2.7 × 33 cm) and the column developed by linear gradient with the use of 500 ml of 0.02 M Tris buffer, pH 9.1, in the mixing chamber and 500 ml of 0.2 M LiCl in 0.02 M Tris buffer, pH 9.1, in the reservoir. Ten-milliliter fractions were collected and the fractions assayed for radioactivity, absorbance at 330 mμ, and cosubstrate activity with ABG in dihydrofolate synthesis by the standard fraction II. The latter assay was performed by incubating 0.15 ml of the DEAE-cellulose fractions with ABG, 1mM; $MgCl_2$, 10 mM; potassium ascorbate, 2 mM; and Tris buffer, 20 mM (pH 8.0), at 37° for 1 hr under an atmosphere of argon. The reaction mixtures were then assayed for dihydrofolate and the results expressed as units of cosubstrate. One unit of cosubstrate is that amount of cosubstrate which is converted to 1 μg of folate equivalent under the condition described above. (B) The chromatography of the mixture of radioactive cosubstrate fraction (Figure 1A) and carrier hydroxymethyldihydropteridine-PP by DEAE-cellulose fractionation. The contents from fractions 34, 35, and 36 (Figure 2A) were mixed with 3 μmoles of carrier hydroxymethyldihydropteridine-PP, (70,000 cpm/μmole) and applied to a DEAE-cellulose column (2.7 × 33 cm) and the column was developed as in Figure 2A. The assays of the fractions were as described in Figure 2A.

fractions 32 through 35 is relatively constant and compares very well with the specific activity of the ATP-γ-^{32}P (306,000 cpm/μmole). The close agreement between the specific radioactivity of the hydroxymethyldihydropteridine-PP^{32}P clearly indicates the pyrophosphorylation of the hydroxymethyldihydropteridine. To further substantiate the identity of the product, the contents from fractions 34, 35, and 36 were pooled and mixed with carrier hydroxymethyldihydropteridine-PP. The resulting specific activity was 70,000 cpm/μmole. This mixture was added to a DEAE-cellulose column and the fractionation and assays were performed as before. The results which are presented in Figure 2B indicated that there were two radioactive substances. All of the cosubstrate material was eluted with the slightly faster radioactive peak. The absorption spectra of fractions 24–27 indicated that they contained a dihydropteridine while tubes 29 and 30 contained an oxidized pteridine. It appears that oxidation of some of the dihydropteridine took place after the addition of carrier. The active cosubstrate isolated from the DEAE-cellulose column was eluted together with the carrier hydroxymethyldihydropteridine-PP in fractions 24, 25, and 26. All three had essentially the expected specific activity of 70,000 cpm/μmole. The slower moving radioactive material is the oxidized phosphorylated pteridine.

Effects of Varying the Concentration of Fractions I and II on Hydroxymethyldihydropteridine-PP and Dihydrofolate Synthesis. The effects of varying the concentration of fraction I on dihydrofolate synthesis were studied by the three routine

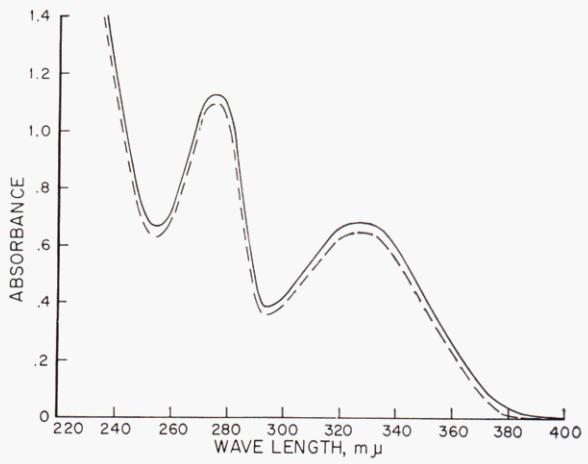

FIGURE 3: Absorption spectra of fraction 35 (—) (Figure 2A) and hydroxymethyldihydropteridine-PP (– –) at pH 7.1.

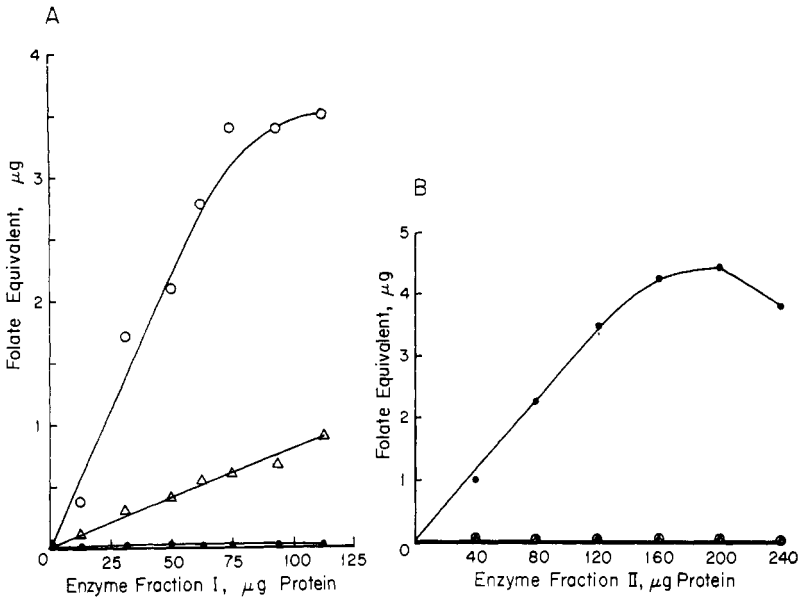

FIGURE 4: The effects of varying the concentration of fractions I and II on hydroxymethyldihydropteridine-PP and dihydrofolate synthesis. Assay 1 (△), assay 2 (○), and assay 3 (●) are described under Materials and Methods, and the amounts of fractions I and II are as indicated.

assay procedures and are shown in Figure 4A. In the presence of hydroxymethyldihydropteridine-PP and ABG (assay 3), fraction I was completely inactive with respect to dihydrofolate synthesis over the concentration range indicated. However, the rate of synthesis of dihydrofolate from hydroxymethyldihydropteridine and ABG in the presence of ATP (assay 1) by fraction I increased linearly over an approximate tenfold increase in the concentration of protein. Fraction I synthesized dihydrofolate by assay 1 but not by assay 3. This phenomenon was briefly commented upon earlier. When fraction I was assayed with hydroxymethyldihydropteridine, ABG, ATP, and an excess of standard fraction II (assay 2), the maximum rate of the pyrophosphorylation reaction could be measured indirectly. The rate of this reaction increases linearly with increasing concentration of fraction I up to approximately 70 μg of protein. We believe that based on these observations, assay 2 is a valid measure of the cosubstrate production by fraction I. Results of increasing amounts of fraction II on dihydrofolate synthesis as measured by all three assays are presented in Figure 4B. No dihydrofolate synthesis occurred when assays 1 and 2 were employed, whereas by assay 3, the rate of dihydrofolate synthesis increased linearly to about 160 μg of protein. At higher enzyme concentrations, inhibition was observed.

Effects of Varying the Concentrations of Hydroxymethyldihydropteridine and Hydroxymethyldihydropteridine-PP on Hydroxymethyldihydropteridine-PP and Dihydrofolate Synthesis. The results in Figure 5A show that hydroxymethyldihydropteridine is without activity when tested with fraction II. Fraction I exhibited some increase in activity as the concentration of hydroxymethyldihydropteridine is increased. When fraction I is incubated with standard fraction II (assay 2), the rate of hydroxymethyldihydropteridine-PP synthesis as measured indirectly by dihydrofolate synthesis is maximal at 0.8 mM. Figure 5B shows the effects of increasing concentrations of hydroxymethyldihydropteridine-PP on fractions I and II. Fraction I is completely inactive. Fraction II follows a sigmoidal kinetic response with increasing concentrations of hydroxymethyldihydropteridine-PP. The ABG concentration was constant. When a similar experiment was performed using an equivalent concentration of AB in place of ABG, again a sigmoidal kinetic response was obtained.

In Figure 6, the saturation curves from fraction II by AB or ABG are presented. Fraction II again exhibited a sigmoidal response with respect to each of these cosubstrates.

The following compounds at 1 mM concentration had no effect on fraction II: sodium pyrophosphate, AMP, sodium glutamate, pteroyltriglutamate, GTP, and sodium formate.

Polyacrylamide Gel Electrophoresis of Fraction II. Fraction II was subjected to disc gel electrophoresis (Davis, 1964) in order to obtain information regarding the ability of dihydropteroate synthetase to use either AB or ABG. Four gel slabs were prepared with fraction II (200 μg); one was stained for protein and three were sliced. One set of slices was assayed by a modified assay 1 (AB used in place of ABG), the second set of slices by a modified assay 3 (AB used in place of ABG), and the third set of slices by the usual assay 3 employing ABG. The following results were obtained: the set of slices assayed by a modified assay 1 (AB used in place of ABG) showed no activity, whereas the set assayed by the modified assay 3 (AB used in place of ABG) contained activity at 19 mm and by assay 3 (ABG) at 20 mm.

Discussion

Two enzyme fractions have been obtained from cell-free extracts of *L. plantarum*. Fraction I was shown to catalyze the pyrophosphorylation of hydroxymethyldihydropteridine by ATP to produce hydroxymethyldihydropteridine-PP. We have therefore concluded that fraction I contains hydroxymethyldihydropteridine pyrophosphokinase. These observations are in complete agreement with those reported by Richey and Brown for a highly purified enzyme preparation of *E. coli* (Richey and Brown, 1969).

The second enzyme fraction from *L. plantarum* was found to catalyze the synthesis of dihydrofolate or dihydropteroate from chemically prepared hydroxymethyldihydropteridine-PP or the product from fraction I and ABG or AB. Although we

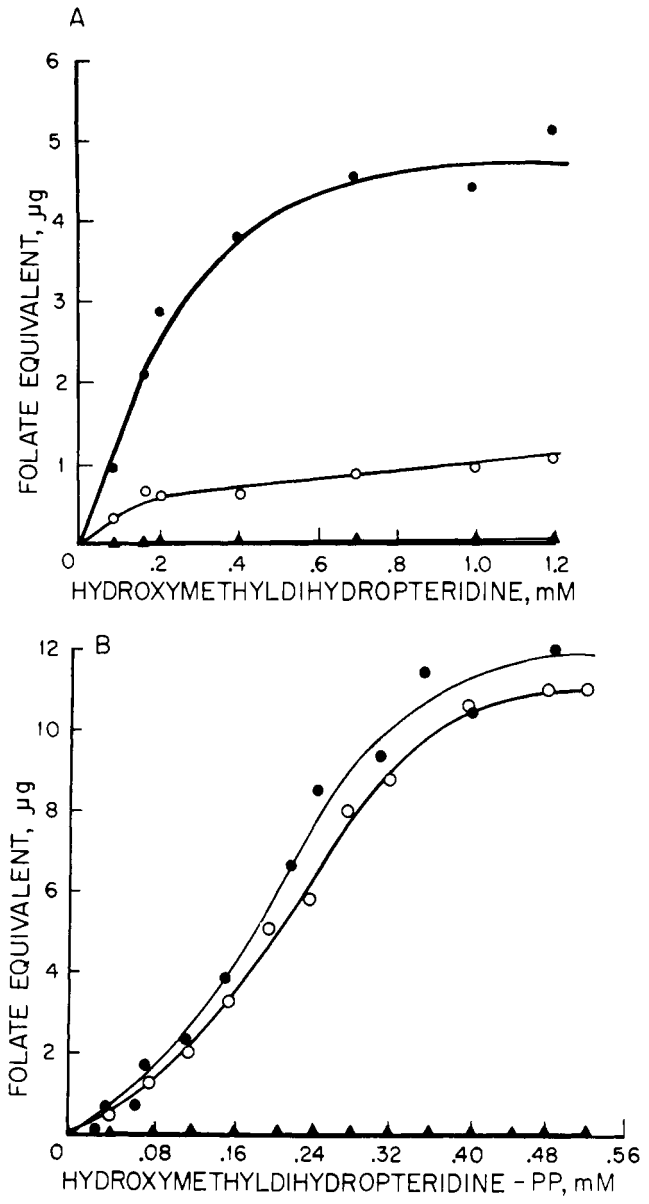

FIGURE 5: The effects of varying the concentration of hydroxymethyldihydropteridine on hydroxymethyldihydropteridine-PP synthesis (A) and hydroxymethyldihydropteridine-PP on dihydropteroate and dihydrofolate synthesis (B). The assay procedures are described under Materials and Methods and the amounts of fractions I and II were 100 and 160 μg, respectively. In A, enzyme fraction I (○), enzyme fraction I plus standard enzyme fraction II (●), and enzyme fraction II (▲) were incubated at the concentrations of hydroxymethyldihydropteridine indicated. In B, enzyme fraction II plus AB (●), enzyme fraction II plus ABG (○), and enzyme fraction I plus AB or ABG (▲) were incubated at the concentrations of hydroxymethyldihydropteridine-PP shown. The ABG in assay 3 was replaced by an equimolar concentration of AB.

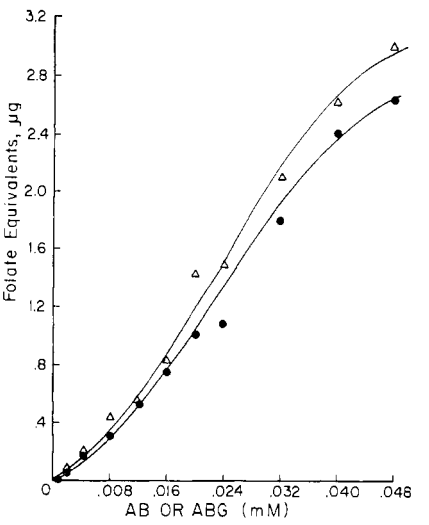

FIGURE 6: Effects of AB (●) or ABG (△) concentration on dihydropteroate or dihydrofolate synthesis. Assay 3 was employed except the concentrations of AB and ABG were varied as indicated. The protein concentration of the fraction II was 160 μg.

did not investigate the products which were formed from synthetic hydroxymethyldihydropteridine-PP, the response by the microbiological assay indicated that a "folate-like" compound was synthesized. In a previous communication (Shiota *et al.*, 1962) crude enzyme preparations from *L. plantarum* utilized AB or ABG with synthetic hydroxymethyldihydropteridine-PP to synthesize dihydropteroate or dihydrofolate as revealed by a bioautographic procedure. Hence we have assumed that fraction II similarly synthesized these products. We have no information regarding the release of inorganic pyrophosphate as the other product in the above reaction. However, in view of the specificity of fraction II, that is, (a) its complete lack of activity with hydroxymethyldihydropteridine, ATP, and ABG, and (b) its requirement for either the product of the fraction I catalyzed reaction or the chemically prepared hydroxymethyldihydropteridine-PP and AB or ABG (for dihydropteroate or dihydrofolate synthesis) these facts would suggest that this enzyme is similar to the dihydropteroate synthetase reported by Richey and Brown (Richey and Brown, 1969).

In contrast to *E. coli* (Richey and Brown, 1969), the hydroxymethyldihydropteridine pyrophosphokinase from *L. plantarum* is sensitive to heat. Magnesium was reported to be required by the *E. coli* dihydropteroate synthetase and similarly the *L. plantarum* enzyme was found to be stimulated by Mg^{2+} ions.

The synthesis of dihydrofolate from hydroxymethyldihydropteridine, ATP, and ABG by fraction I implied that it contained some fraction II. However, this explanation was discounted since fraction I was completely inactive with hydroxymethyldihydropteridine-PP and ABG. In view of the activity of hydroxymethyldihydropteridine and the inactivity of hydroxymethyldihydropteridine-PP we would like to offer a possible explanation for this phenomenon. Fraction I may contain an enzyme complex with the two activities (hydroxymethyldihydropteridine pyrophosphokinase and dihydropteroate synthetase) which catalyze the synthesis of dihydrofolate without the involvement of a free intermediate (hydroxymethyldihydropteridine-PP). A second observation was that supplementation of fraction I with an exogenous dihydropteroate synthetase increased the amount of dihydrofolate synthesized. This increase of dihydrofolate cannot be easily explained by a coupled reaction between the enzyme complex and the exogenous dihydropteroate synthetase since such a reaction would require a free intermediate. Since hydroxymethyldihydropteridine-PP was synthesized by fraction I, an

additional enzyme in fraction I is indicated. This additional enzyme is proposed to be the hydroxymethyldihydropteridine pyrophosphokinase. Perhaps the 45% ammonium sulfate fraction contains three forms of the enzyme relevant to dihydrofolate synthesis, one of which is the complex, the second the hydroxymethyldihydropteridine pyrophosphokinase, and the third the dihydropteroate synthetase. Hence, the DEAE-cellulose chromatography separates the complex and the pyrophosphokinase which we have designated fraction I from the synthetase designated fraction II.

Weisman and Brown (Weisman and Brown, 1961) previously reported that the dihydropteroate synthetase activity for ABG in *E. coli* is preferentially lost or decreased by heat or by a purification procedure. They proposed the possible existence of two enzymes, one specific for AB and the other for ABG. However, they have not excluded the possibility for a single complex which can be dissociated into two fractions. The results presented in this paper indicate that the relative activities of AB and ABG for the dihydropteroate synthetase of *L. plantarum* are equivalent. Furthermore, the results obtained from polyacrylamide gel electrophoresis of fraction II suggest that the dihydropteroate synthetase from *L. plantarum* is able to utilize either AB or ABG.

The sigmoidal kinetic response to increasing concentration of each substrate revealed by the dihydropteroate synthetase is suggestive of an allosteric enzyme (Cohen, 1965). Inhibition of the dihydropteroate synthesis by inorganic pyrophosphate (Ortiz and Hotchkiss, 1966; Iwai and Okinaka, 1968) and by dihydropteroate have been reported (Richey and Brown, 1969). The thorough study of this enzyme has been hampered by the assay procedure which depends upon the microbiological assay for dihydropteroate or dihydrofolate. However, as indicated in the text, we have not found any significant inhibition or stimulation by the compounds tested.

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