The Enzymatic Synthesis of Folate-like Compounds from Hydroxymethylhydropteridine Pyrophosphate

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The enzymatic synthesis of dihydropteroate or dihydrofolate from 2-amino-4-hydroxy-6-hydroxymethylhydropteridine and p-aminobenzoate or p-aminobenzoylglutamate by bacterial extracts has been shown to require adenosine triphosphate (1–3). It has been postulated that a phosphorylated pteridine intermediate is involved before synthesis of folate-like compounds. Jaenicke and Chan (4) reported that the pyrophosphate derivative of dihydropteridine was active in the enzymatic synthesis of folate-like compounds. Recently we have reported the preparation of a pteridine diphosphate that, after reduction to the dihydro level, can be coupled with p-aminobenzoate or p-aminobenzoylglutamate to yield dihydropteroate or dihydrofolate by extracts of Lactobacillus plantarum in the absence of adenosine triphosphate (5).

The purposes of this communication are to present evidence indicating that the phosphorylated pteridine active in the enzymatic synthesis of dihydropteroate or dihydrofolate is 2-amino-4-hydroxy-6-hydroxymethylpyrophosphate, and to show that cell-free extract preparations from Veillonella strain V2 carry out the enzymatic reaction as follows.

Hydroxymethylhydropteridine-PP + p-aminobenzoate → dihydropteroate + PP; (1)

Hydroxymethylhydropteridine-PP + p-aminobenzoylglutamate → dihydrofolate + PP; (2)

EXPERIMENTAL PROCEDURE

Materials—Yeast inorganic pyrophosphatase was prepared by the alternative procedure as described by Hoppel (6). Dihydrofolate and dihydropteridine were prepared by Futterman's procedure (7), and 2-amino-4-hydroxy-6-hydroxymethylpteridine by the method of Waller et al. (8).

One hundred grams of DEAE cellulose, 0.9 meq per g, were prepared for chromatographic use by alternately washing with 4 liters of 1 M NaOH, 4 liters of 1 M HCl, and 4 liters of 1 M NaOH. The DEAE-cellulose was filtered after each washing and was then resuspended in 7 liters of distilled water, adjusted to pH 7 with HCl, and washed by decantation with seven changes of 7 liters of water.

Intestinal alkaline phosphatase was obtained from Worthington Biochemical Corporation, purified calf intestinal phosphomonoesterase from Sigma Chemical Company, sodium pyrophos-
was added and the precipitates were collected by centrifugation. This procedure was repeated until the precipitates were free from chloride ions. The precipitates were dried by washing with ether. Each of the phosphorylated pteridines was further purified by repeating the chromatography on DEAE-cellulose and the subsequent steps to remove the lithium chloride. The yields of the pteridine monophosphate and pteridine diphosphate were 37 and 32 mg, respectively.

Preparation of 32P-Labeled Pteridine Diphosphate—A solution containing 1 mcg of sodium pyrophosphate-32P,-32P was lyophilized in a test tube, 20 x 150 mm. To the dried sodium pyrophosphate-32P, 50 mg of 2-amino-4-hydroxy-6-hydroxymethylpteridine and 2 g of pyrophosphoric acid were added. The tube was stoppered and the mixture was stirred for 2 hours at 60° C. The procedure for the isolation of the 32P-labeled pteridine diphosphate was similar to that described above. The yield of the pteridine diphosphate as the lithium salt was 16 mg. A freshly prepared sample gave 60,000 c.p.m. per μmole of pteridine diphosphate.

Assay of Pteridine Monophosphate and Pteridine Diphosphate—Pteridine monophosphate and pteridine diphosphate were assayed by determining their phosphorus to pteridine ratios. The pteridine concentration was obtained by measuring absorbance in 0.1 N NaOH at 253 μm with the use of a molar extinction coefficient of 23,500 \((5)\). Total phosphorus was determined by measuring the phosphate released on treatment with alkaline phosphatase. The latter assay was carried out by incubating 1 mg of alkaline phosphatase (Worthington) with the phosphorylated pteridine (total of 1 μmole of phosphate) in 3 ml of 0.1 N NaOH. The mixture was stirred for 10 minutes. The iodometric titration method (15) adapted as described in a previous communication (16) was used for the determination of labile hydrogen of dihydropteridines.

Paper Chromatography of Pteridines—Table I shows the mobilities of pteridine monophosphate, pteridine diphosphate, and dihydropteridine diphosphate in three solvents. The compounds were located under ultraviolet light.

Preparation of Dihydropteroines—The pteridines were reduced in stoppered test tubes, 20 x 200 mm, fixed for magnetic stirring. Each tube contained 1.5 μmoles of pteridine and 10 mg of NaBH₄ in a total volume of 1.5 ml. In order to initiate the reaction, 0.07 ml of 1 N HCl was added to each tube containing pteridine monophosphate, and 0.1 ml of 1 N HCl to each tube containing pteridine diphosphate. The contents of each tube were stirred at room temperature for 15 minutes. The reaction was terminated by the addition of 0.1 ml of 1 N HCl. The mixture was stirred for 10 minutes and labile hydrogen determinations were performed. The iodometric titration method (15) was used for the determination of labile hydrogen of dihydropteridines.

Table I. Paper chromatoaphy of phosphorlated pteridines

<table>
<thead>
<tr>
<th>Pteridine</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>Solvent C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pteridine monophosphate</td>
<td>0.71 (F)</td>
<td>0.76 (F)</td>
<td>0.35 (F)</td>
</tr>
<tr>
<td>Pteridine diphosphate</td>
<td>0.79 (F)</td>
<td>0.85 (F)</td>
<td>0.27 (F)</td>
</tr>
<tr>
<td>Dihydropteridine diphosphate</td>
<td>0.76 (Q)</td>
<td>0.85 (Q)</td>
<td>0.30 (F)</td>
</tr>
</tbody>
</table>

* F indicates fluorescence and Q, quenching.
and by using a molar extinction coefficient of 21,000 (7). The samples were then assayed microbiologically in 6 ml of a folic acid assay medium (Difco) supplemented with 6 mg of potassium ascorbate, pH 6.3, per ml of medium. The tubes were sterilized at 120° at 15 p.s.i. for 5 minutes and inoculated with 1 drop of an 18-hour inoculum of *S. faecalis*. Under these conditions, 18-hour assays showed dihydrofolate to be about 21.5% as active as citrovorum factor on a molar basis (Table II).

Several samples of dihydropterate preparations that had been dried by washing with ethanol and ether were weighed and then assayed microbiologically. Such preparations were about 10.4% as active as citrovorum factor (Table II).

The dihydrofolate activity obtained here differs greatly from a previously reported value (16). Perhaps more activity was observed earlier because an air-dried sample of dihydrofolate was used and the assays were performed with medium that contained only 1.33 mg of potassium ascorbate per ml. Brown, Weisman, and Mohlar (2) reported that dihydrofolate and dihydropteroate were about 50% and 40% as active, respectively, as folate for *S. faecalis*.

The absorption spectra of dihydropteroate are shown in Fig. 2. In 0.1 N NaOH, the molar extinction coefficient is 22,000 at 278 ma. At pH 7, the extinction coefficient is 20,000 at 277 ma.

**Enzymatic Synthesis of Folate-like Compounds by Several Microbial Extracts—** *L. plantarum, Lactobacillus casei, Lactobacillus bulgaricus, Leuconostoc mesenteroides P-60, Pediococcus cerevisiae, S. faecalis,* and *B. rettgeri* were grown in a medium described previously (1). *F. fusiforme* (Prevot) and *L. buccalis* were grown in a medium composed of 0.37% brain-heart infusion (Difco) and 0.2% yeast extract (Difco). Saccharomyces cerevisiae was obtained as a commercial preparation, and Veillonella strain V2 was grown in a modified Rogosa medium (18). A more detailed description of the preparation of Veillonella extracts is presented below. Cells were disrupted with a French pressure cell (Aminco); the cellular debris was removed by centrifugation; and the cell-free extracts were dialyzed overnight against 0.01 M Tris buffer, pH 7.6.

These various microbial extracts were tested for dihydropteroate or dihydrofolate synthetase from 2-amino-4-hydroxy-6-dihydropteridinylmethyl pyrophosphate and p-aminobenzoate or p-aminobenzylglutamate. Of the organisms tested, *L. plantarum*, *L. casei*, *L. mesenteroides* P-60, *P. cerevisiae*, *S. faecalis*, *L. buccalis*, and *S. cerevisiae* were inactive. The dihydropteridinylmethyl monophosphate was inactive in the synthesis of folate-like compounds by the various microbial extracts. Veillonella strain V2 showed varied activities as indicated in Table III.

### Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dihydrofolate</th>
<th>Dihydropteroate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>11.9</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>10.3</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>9.3</td>
</tr>
<tr>
<td>Average</td>
<td>21.5</td>
<td>10.4</td>
</tr>
</tbody>
</table>

| Correction factor† | 4.65 | 9.52 |

* Based on a citrovorum factor activity of 100%.
† The correction factor is the reciprocal of the relative activity.

In addition to these organisms, *Escherichia coli* (2) and a micrococcus (3) apparently possess a similar system. The extracts of *L. bulgaricus*, *L. casei*, *L. mesenteroides* P-60, *P. cerevisiae*, *S. faecalis*, *L. buccalis*, and *S. cerevisiae* were inactive. The dihydropteridinylmethyl monophosphate was inactive in the synthesis of folate-like compounds by the various microbial extracts. Veillonella strain V2 was selected for further studies because it contained relatively low pyrophosphatase activity.

**Preparation of Veillonella Extract—** Stock cultures of *Veillonella* V2 were maintained at 5° for 1 month in screw cap tubes, 16 × 130 mm, containing 13.5 ml of modified Rogosa medium as described by Mergenhagen (18). The medium is composed of 5 g of Trypticase (Baltimore Biological Laboratories), 3 g of yeast extract (Difco), 20.83 ml of 60% sodium lactate, 0.75 g of sodium thioglycolate, and 1000 ml of distilled water. All transfers were made into freshly heated and cooled medium with the use of 1 ml of *Veillonella* culture as an inoculum. For mass cultures, 5% (v/v) transfers were made, first into screw cap tubes, 16 × 130 mm, then into 500 ml of medium contained in
To the supernatant fluid, 6.2 g of ammonium sulfate were added, 27.7 g of ammonium sulfate were added. The resulting precipitate was collected by centrifugation at 38,000 x g for 20 minutes, and dissolved in 50 ml of 0.01 M Tris buffer, pH 7.4. The pH was lowered to 4.6 with 1 M acetic acid, pH 4.5. The precipitate was immediately collected by centrifugation (38,000 x g for 20 minutes) and dissolved in 50 ml of 0.02 M Tris buffer, pH 8. The pH of the above solution was lowered to 4.7, and the precipitate was collected by centrifugation and dissolved in 50 ml of 0.02 M Tris, pH 8 (D). Table IV summarizes the steps involved for the removal of pyrophosphatase activity. The table also shows that there were no differences in the activities of the various fractions for dihydrofolate synthesis and for dihydropteroate synthesis (R).

### RESULTS

#### Characterization of Pteridine Diphosphate

Pteridine monophosphate and pteridine diphosphate were analyzed by ultraviolet light absorption spectra, acid and enzymatic hydrolysis, and electrometric titration.

**Ultraviolet Absorption Spectra**—The absorption spectra of pteridine diphosphate are shown in Fig. 3. The spectra obtained at the three pH values are identical with those obtained for the nonphosphorylated pteridine (8).

The spectra of the monophosphate pteridine derivative were also measured and found to be similar to those obtained for the diphosphate derivative.

**Acid and Enzymatic Hydrolysis**—The pteridine monophosphate and diphosphate were heated at 100° in 1 N H₂SO₄. Table V summarizes the results of the acid hydrolysis experiments. The rate of release of phosphate from pteridine monophosphate was measured and found to be similar to that obtained for the diphosphate derivative.

Cells were disrupted by dispersing 4 g of dried cells in 40 ml of 0.01 M Tris buffer, pH 7.4, and passing the cell suspension through a French press. The cellular material was removed by centrifugation at 38,000 x g for 20 minutes at 5° and was processed and centrifuged three more times. The supernatant fluids were pooled and fractionated in order to remove pyrophosphatase activity. Attempts to purify the folate-synthesizing enzyme by various means were unsuccessful.

For each 100 ml of crude extract (Table IV, A), 39 g of ammonium sulfate were added. The resulting precipitate was collected by centrifugation at 38,000 x g for 20 minutes. The precipitate was dissolved in 400 ml of 0.01 M Tris buffer, pH 7.4. Protamine sulfate, 10 mg per ml, was added until the protamine sulfate-treated supernatant fluid showed a 280-mu/260-mu optical density ratio of about 0.90 (B). To each 100 ml of protamine sulfate supernatant, 27.7 g of ammonium sulfate were added. The precipitate was collected by centrifugation at 38,000 x g for 20 minutes and dissolved in 100 ml of 0.01 M Tris buffer, pH 7.4 (C), and then 17.6 g of ammonium sulfate were added. The precipitate was removed by centrifugation. To the supernatant fluid, 0.2 g of ammonium sulfate were added, and the precipitate was collected by centrifugation and dissolved in 50 ml of 0.01 M Tris buffer, pH 7.4. The pH was lowered to 4.6 with 1 M acetic acid, pH 4.5. The precipitate was immediately collected by centrifugation (38,000 x g for 20 minutes) and dissolved in 50 ml of 0.02 M Tris buffer, pH 8. The pH of the above solution was lowered to 4.7, and the precipitate was collected by centrifugation and dissolved in 50 ml of 0.02 M Tris, pH 8 (D). Table IV summarizes the steps involved for the removal of pyrophosphatase activity. The table also shows that there were no differences in the activities of the various fractions for dihydrofolate synthesis and for dihydropteroate synthesis (R).
phosphomonoesterase (Sigma) distinguished the monophosphate and diphosphate groupings of the pteridine derivatives. In other experiments, treatment with intestinal alkaline phosphatase (Worthington) released approximately 1 and 2 μmoles of phosphate per μmole of pteridine monophosphate and pteridine diphosphate, respectively. The purified calf intestinal phosphomonoesterase under these conditions released phosphorus quantitatively from adenosine monophosphate, pyridoxamine phosphate, and thiamine monophosphate. On the other hand, the phosphomonoesterase did not hydrolyze thiamine pyrophosphate and adenosine triphosphate; and 20% of the phosphate of adenosine diphosphate was released in 60 minutes.

Electrometric Titration—The titration of the pteridine derivatives and other related compounds was found to be a useful tool in ascertaining the nature of the phosphate groupings of the pteridines.

The titration of the nonphosphorylated pteridine, 2-amino-4-hydroxy-6-hydroxymethylpteridine, should show two titratable groups, one related to the amino group, the other to the hydroxyl group, with a sum of 2 equivalents of alkali consumed per mole of the pteridine. The amino group should be characterized by its low pKₐ value, and the "phenolic" hydroxyl group by its high pKₐ value. Upon titration the pteridine monophosphate should show two additional acidic groups—one in the acid pH range related to the primary phosphoryl group, and another in the intermediary pH range related to the secondary phosphoryl group. This is characteristic of the phosphoric acid residue, and the sum should be 4 equivalents of alkali consumed per mole for the pteridine monophosphate. The titration curve for the pteridine diphosphate, like that for the pyrophosphate derivative, should be similar to the pteridine monophosphate curve except for the titration of an additional primary phosphoryl group. Fig. 5 shows the titration curves of these compounds. The parent compound shows equivalence points at about pH 5 and pH 10. Although the titration of the amino group and the primary phosphoryl group for pteridine monophosphate and pteridine diphosphate cannot be clearly delineated, their titration can be seen to be completed by pH 5. Hence the titration of the nonphosphorylated, the monophosphate, and the diphosphate pteridine derivatives to pH 5 showed, as predicted, 1, 2, and 3 equivalents of acidic groups. The titrations obtained from about pH 5 through 7 for both of the phosphorylated pteridines are due to the secondary phosphoryl group. It can be seen that in this pH range the two curves are parallel and thus show the presence of only one secondary phosphoryl group. The titration of 1 equivalent of acid from about pH 7.25 to pH 10.5 for each of the compounds is due to the "phenolic" hydroxyl group.

Enzymatic Synthesis of Dihydrofolate and Dihydropterotate from Hydroxymethylpringo-PP

Some Properties of pH 4.7 Fraction of Veillonella Extracts—The rate of enzymatic synthesis of dihydrofolate or dihydropterotate by the pH 4.7 fraction of Veillonella extracts was linear up to 1300 μg per ml of protein concentration and over a period of 80 minutes. The pH optimum of the enzyme for dihydrofolate or dihydropterotate synthesis was approximately pH 8.5.

Effects of Substrate and Sulfathiazole—The rates of synthesis of dihydropterotate and dihydrofolate are shown to be dependent upon hydroxymethylhydropteridine-PP concentration (Fig. 6). The reciprocal plots of the velocity versus substrate (19) gave a

<table>
<thead>
<tr>
<th>Hydrolysis time</th>
<th>Phosphate released</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>% of total</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1.9</td>
</tr>
<tr>
<td>180</td>
<td>17.5</td>
</tr>
<tr>
<td>300</td>
<td>28.7</td>
</tr>
</tbody>
</table>

Table V: Rate of release of phosphate from pteridine monophosphate and pteridine diphosphate by acid hydrolysis

To each set of four tubes containing 0.25 ml of 2 X H₂SO₄, 0.25 ml of the appropriate phosphorylated compound (0.2 μg of phosphorus) was added. The tubes were placed in a water bath at 100°. At the time indicated, a tube from each set was removed and immediately cooled in an ice bath. The contents were subsequently analyzed for total phosphorus.
Fig. 5. Electrometric titration. Ten micromoles of each pteridine were titrated with 0.013 N KOH.

Fig. 6. Effect of concentration of hydroxymethylidihydropteridine-PP on the synthesis of dihydropterate (●) and dihydrofolate (○). Velocity is given in micrograms of product formed per mg of protein per hour, and hydroxymethylidihydropteridine-PP concentration was varied between 2 × 10⁻⁶ and 22 × 10⁻⁶ M. Experimental conditions were as described for Fig. 4. An amount of the pH 4.7 enzyme fraction equivalent to 200 μg of protein was added to each reaction mixture as a source of enzyme.

Fig. 7. Effect of p-aminobenzoylglutamate and sulfathiazole on dihydrofolate synthesis. Dihydrofolate synthesis was tested in the presence of 8 × 10⁻⁴ M sulfathiazole and 0.8 × 10⁻⁴ M p-aminobenzoate.

preparation was tested in the presence of 3.2 × 10⁻⁴ M sulfathiazole and 0.8 × 10⁻⁴ M p-aminobenzoate.

The reversibility of the inhibition caused by sulfathiazole was tested by a preliminary incubation procedure similar to that described by Brown (20). Table VI illustrates a typical experiment showing the reversal of sulfathiazole inhibition by p-aminobenzoate. It was demonstrated that when sulfathiazole and hydroxymethylidihydropteridine-PP were present in non-limiting concentrations during the first incubation mixture, the degree of reversal of inhibition by the subsequent addition of p-aminobenzoate (Table VI, Experimental Group C) was similar to that obtained when all of the components were added at the same time (Experimental Group B). Similar results were obtained for the dihydrofolate synthesis system when sulfathiazole at 4 × 10⁻⁴ M and hydroxymethylidihydropteridine-PP at 2 × 10⁻⁴ M were incubated with enzyme for 30 minutes and p-aminobenzoylglutamate at 5 × 10⁻³, 10 × 10⁻³, 30 × 10⁻³, and 70 × 10⁻³ M was added at the beginning of the second incubation period (see Table VII).

When the experiments as described in Tables VI and VII were repeated with a limiting concentration of hydroxymethylidihydropteridine-PP, 2 × 10⁻⁴ M, the inhibition that was observed was readily reversed by p-aminobenzoate or p-aminobenzoyl-
glutamate when all of the components were added together (as in Table VI, Experimental Group B). However, if the same amounts of hydroxymethylidihydropteridine-PP and sulfathiazole were added to the first incubation mixture, the subsequent addition of either p-aminobenzoate or p-aminobenzoylglutamate failed to overcome the inhibition. This observation, which was initially made by Brown (20), was called "irreversible inhibition." The experiment just described was repeated with the use of a sulfathiazole concentration that was limiting but still inhibited the dihydrofolate or dihydropterate synthesis to some extent. Under this condition, no inhibition was observed when either p-aminobenzoate or p-aminobenzoylglutamate was added at the beginning of the second incubation period.

**Stoichiometry**—The stoichiometric relation between the utilization of hydroxymethylidihydropteridine-PP and the formation of dihydrofolate and pyrophosphate was studied. The procedure was to treat a 2.2-ml sample of the incubated reaction mixture with 0.03 ml of a saturated solution of trichloroacetic acid and to remove the precipitated protein by centrifugation. Two milliliters of the supernatant fluid were then shaken for 15 minutes with 0.1 ml of an aqueous suspension of 15% Norit A (acid-washed). The charcoal was collected on a Millipore filter pad 25 mm in diameter (porosity, 0.45 μ) and washed four times with a 0.4 ml of 0.005 N HCl. The 32P-32P content of the charcoal filtrate was detected in a liquid scintillation spectrometer. The radioactivity of each eluate was determined. The results are shown in Table VIII. For each millimicromole of hydroxymethylidihydropteridine-32P that was adsorbed to the charcoal, the charcoal was eluted by treating the charcoal with four successive portions (0.5 ml each) of eluting reagent (equal volumes of 3 N NH4OH and absolute ethyl alcohol). The radioactivity of each eluate was determined. The results are shown in Table VIII. For each millimicromole of hydroxymethylidihydropteridine-PP used, 1 pmole each of inorganic pyrophosphate and dihydrofolate was formed.

**Reversibility of Reaction**—Attempts to show reversibility of the enzyme reaction as measured by the incorporation of pyrophosphate.
analogue of a folate-like compound.

According to Brown (20), who demonstrated the formation of an irreversible inhibition observed by Brown (20) was due to the depletion of the 2-amino-4-hydroxy-6-dihydropteridine-PP was also suggested by Jaenicke and Chan (4). The enzyme preparation of a Veillonella extract (free from pyrophosphatase) is described. This preparation synthesizes dihydrofolate and dihydropterote from p-aminobenzoylglutamate and p-aminobenzoate, respectively, in the presence of hydroxymethyl-dihydropteridine pyrophosphate. The pH optimum of this reaction is 8.5. The reaction rate is linear with respect to the enzyme concentration and for a period of 80 minutes. The results from stoichiometry experiments indicated that for each mole of hydroxymethyl-dihydropteridine pyrophosphate used, 1 mole each of dihydrofolate and pyrophosphate was formed.

Sulfathiazole inhibits the formation of dihydropterote and dihydrofolate by competing with p-aminobenzoate or p-aminobenzoylglutamate for the active site of the enzyme.

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