The Enzymatic Synthesis of Folate-like Compounds from Hydroxymethylhydropteridine Pyrophosphate

T. Shiota, M. N. Disraely, and M. P. McCann

From the National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014

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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-dihydropteridinylmethyl pyrophosphate, and to show that cell-free extract preparations from Veillonella strain V2 carry out the enzymatic reaction as follows.

\[
\text{Hydroxymethylhydropteridine-PP} + \text{p-aminobenzoate} \rightarrow \text{dihydropteroate} + \text{PP}_{\text{i}} \quad (1)
\]

\[
\text{Hydroxymethylhydropteridine-PP} + \text{p-aminobenzoylglutamate} \rightarrow \text{dihydrofolate} + \text{PP}_{\text{i}} \quad (2)
\]

**EXPERIMENTAL PROCEDURE**

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

One hundred grams of DEAE cellulose, 0.9 meq per gm, were prepared for chromatographic use by alternately washing with 4 liters of 1 x NaOH, 4 liters of 1 x HCl, and 4 liters of 1 x 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 mc of sodium pyrophosphate-32P-32P was lyophilized in a test tube, 20 X 150 mm. To the dried sodium pyrophosphate-32P-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°. 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 nm 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 a freshly prepared sample gave 60,000 c.p.m. per μmole of pteridine diphosphate.

**Procedure for Electrometric Titration**—The salts of the pteridines were converted to the free acid by adding each of the phosphorylated pteridines to a column of Dowex 50-H+. The column was washed with water until the emerging effluent liquid was free of fluorescence. The effluent was lyophilized. Electrometric titration of the various pteridines was done on a microscale with an automatic pH meter (Radiometer) and a titrator (Amino).

**Preparation of Dihydropteridines**—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) 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.

**Purification of Commercial Folic Acid**—Folic acid, 200 mg, was suspended in 4 ml of water, and 1 N NaOH was added dropwise until the folic acid went into solution. The folate was then added to the top of a DEAE-cellulose column, 12 X 300 mm, that was wrapped with foil to shield it from light. The column was developed by linear gradient elution; the mixing chamber contained 600 ml of 0.3 M KCl in 0.01 M Tris, pH 7.4, and the reservoir contained 600 ml of 0.6 M KCl in 0.01 M Tris, pH 7.4. Fractions were collected at a rate of 10 ml per 4 minutes. Fig. 1 shows the elution profile. Tubes 26 to 34 contained aminolevulinic acid and a strongly fluorescing compound. The folate fraction (tubes 65 to 89) was completely free of fluorescent materials. The fractions containing folate were pooled, the pH was adjusted to 3 with glacial acetic acid, and the precipitate was collected by centrifugation. The precipitate was suspended in 40 ml of distilled water and dissolved by dropwise addition of 1 N KOH. The folate was precipitated again at pH 3, washed three times with 1% acetic acid, and lyophilized (17). The yield was 190 mg.

**Folic acid was purified in a similar manner.**

**Dihydrofolate and Dihydrofolic Acid Assays**—In order to determine quantitatively dihydrofolate and dihydrofolic acid by microbiological assays using S. faecalis, it was necessary to relate the activity of these compounds to a stable reference compound, citrovorum factor, that could be used in the routine microbiological assays.

The concentrations of freshly prepared dihydrofolate samples were obtained by measuring absorbance at 283 nm in 0.1 N KOH.
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 6.5, per ml of medium. The tubes were sterilized at 120°C at 15 p.s.i. for 5 minutes and inoculated with 1 drop of an 18-hour inoculum of \textit{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 dihydropterate were about 50% and 40% as active, respectively, as folate for \textit{S. faecalis}.

The absorption spectra of dihydropterate are shown in Fig. 2. In 0.1 N NaOH, the molar extinction coefficient is 22,000 at 278 m\(\text{\mu}\). At pH 7, the extinction coefficient is 20,000 at 277 m\(\text{\mu}\).

**Enzymatic Synthesis of Folate-like Compounds by Several Microbial Extracts—**\textit{L. plantarum}, \textit{Lactobacillus casei}, \textit{Lactobacillus bulgaricus}, \textit{Lactococcus mesenteroides} P-60, \textit{Pediococcus cerevisiae}, \textit{S. faecalis}, and \textit{R. rettgeri} were grown in a medium described previously (1). \textit{F. fusiforme} (Prevot) and \textit{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 \textit{Veillonella} strain V2 was grown in a modified Rogosa medium (18). A more detailed description of the preparation of \textit{Veillonella} extracts is presented below. Cells were disrupted with a French pressure cell (Aminoex); 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 dihydropterate or dihydrofolate synthesis from 2-amino-4-hydroxy-6-dihydropyridinylmethyl pyrophosphate and \(\text{p}^{-}\)aminobenzoate or \(\text{p}^{-}\)aminobenzoylglutamate. Of the organisms tested, \textit{L. plantarum}, \textit{B. rettgeri}, \textit{Veillonella} strain V2, and \textit{F. fusiforme} (Prevot) showed varied activities as indicated in Table III.

### Table II

<table>
<thead>
<tr>
<th>Organism from which extracts were prepared</th>
<th>Citrovorum factor equivalents produced from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{p}^{-})Aminobenzoic acid</td>
</tr>
<tr>
<td>\textit{L. plantarum}</td>
<td>2.2</td>
</tr>
<tr>
<td>\textit{R. rettgeri}</td>
<td>1.2</td>
</tr>
<tr>
<td>\textit{Veillonella}</td>
<td>1.4</td>
</tr>
<tr>
<td>\textit{F. fusiforme}</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* The values are expressed as millimicrograms of citrovorum factor equivalents per mg of protein per ml of reaction mixture.

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

**Preparation of Veillonella Extract—**Stock cultures of \textit{Veillonella} V2 were maintained at 5°C for 1 month in screw cap tubes, 16 \(\times\) 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 \textit{Veillonella} culture as an inoculum. For mass cultures, 5% (v/v) transfers were made, first into screw cap tubes, 16 \(\times\) 130 mm, then into 500 ml of medium contained in
TABLE IV

**Fractionation of Veillonella extract**

The activities of the various fractions containing 0.5 mg to 1 mg of protein were determined by incubating them with 5 μmoles of Tris buffer, pH 8.5; 0.5 mg of potassium ascorbate; 0.05 μ mole of p-aminobenzoylglutamate or p-aminobenzoate; and 0.0125 μ mole of hydroxymethylidihydropterine-PP in a total volume of 0.25 ml. The reaction mixtures were incubated for 60 minutes under an atmosphere of argon. Pyrophosphatase activity was measured by incubating the various fractions (ranging from 0.1 mg to 1 mg of protein) with 25 μmoles of Tris buffer, pH 8.5, and 1 μmole of sodium pyrophosphate in a total volume of 1.5 ml. Tubes were incubated at 37°C, and samples for phosphorus analysis were removed at 0, 1, and 3 hours.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Dihydrofolate activity</th>
<th>Pyrophosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activitya</td>
<td>Total units x 10⁻⁴</td>
</tr>
<tr>
<td>A. Crude extract</td>
<td>5.3</td>
<td>11.2</td>
</tr>
<tr>
<td>B. Supernatant fluid after treatment with protamine</td>
<td>5.5</td>
<td>7.9</td>
</tr>
<tr>
<td>C. Ammonium sulfate fraction</td>
<td>6.9</td>
<td>5.8</td>
</tr>
<tr>
<td>D. pH 4.7 precipitate</td>
<td>10.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

a Specific activity is the units of enzyme per μg of protein per hour.

A unit is defined as the amount of enzyme that synthesizes 1 mg of either dihydrofolate or dihydropterate under the conditions described.

R is defined as the ratio of the specific activity of dihydropterate synthesis to the specific activity of dihydrofolate synthesis.

Pyrophosphatase activity is defined as the millimicromoles of phosphate released per μg of protein per hour.

550 ml screw cap bottles, and finally into 20 liters of medium. Incubation was for 20 hours at 37°C. The cells were harvested in a continuous flow centrifuge (Sorvall), washed twice with water, and dried by lyophilization.

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 pressure cell. The cellular material was removed by centrifugation at 38,000 × g for 20 minutes at 5°C 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 × 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 μm-260 μm 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 × 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 N acetic acid, pH 4.5. The precipitate was immediately collected by centrifugation (38,000 × 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 dihydropterate 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 of 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 Hydrolyses**—The pteridine monophosphate and diphosphate were heated at 100°C in 1 N H₂SO₄. Table V summarizes the results of the acid hydrolysis experiments. The rate of release of phosphate from pteridine monophosphate indicated that this compound contained a stable phosphate, i.e., an ester phosphate. On the other hand, the pteridine diphosphate yielded half of its phosphate by 7 minutes—a fact which suggests that the compound contains a labile phosphate, i.e., pyrophosphate. Under these same conditions approximately half of the phosphate of adenosine diphosphate and thiamine pyrophosphate is released in 7 minutes.

The possibility that the pteridine diphosphate contained a pyrophosphate grouping was further substantiated by comparing the hydrolysis of the mono- and diphosphate derivatives of the pteridine by phosphomonoesterase. Fig. 4 shows that the rate and the amount of phosphate released by purified calf intestinal...
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 pH<sub>a</sub> value, and the "phenolic" hydroxyl group by its high pH<sub>a</sub> 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 Hydroxymethylhydropteridine-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

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**Table V**

<table>
<thead>
<tr>
<th>Hydrolysis time (min)</th>
<th>Phosphate released (%)</th>
<th>Pteridine monophosphate</th>
<th>Pteridine diphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1.3</td>
<td>50.1</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>17.5</td>
<td>72.9</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>28.7</td>
<td>77.6</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 4.** Hydrolysis of pteridine monophosphate and pteridine diphosphate by phosphomonoesterase. The reaction mixture contained 5 μg of phosphomonoesterase, 200 μmoles of MgCl₂, 200 μmoles of Tris, pH 10.4, and 1 μmole of pteridine monophosphate or pteridine diphosphate in a total volume of 5 ml. The reaction mixtures were incubated at 37°.

**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 μH₂SO₄, 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.

**K<sub>a</sub>** value for hydroxymethylhydropteridine-PP of 3.12 × 10⁻⁴ M in the presence of p-aminobenzoylglutamate and 2.38 × 10⁻⁴ M in the presence of p-aminobenzoylglutamate. The **K<sub>m</sub>** value obtained from three experiments ranged from 1.32 × 10⁻⁴ to 3.78 × 10⁻⁴ M in the presence of p-aminobenzoylglutamate and from 1.22 × 10⁻⁴ to 4.02 × 10⁻⁴ M in the presence of p-aminobenzoylglutamate.

Sulfathiazole competitively inhibited the synthesis of dihydrofolate and dihydropterotate. Fig. 7 shows the inhibition of dihydrofolate synthesis. The **K<sub>m</sub>** value of 3.46 × 10⁻³ M was obtained for p-aminobenzoylglutamate. Values from three experiments ranged from 1.48 × 10⁻⁵ to 3.46 × 10⁻⁵ M. The **K<sub>i</sub>** value obtained from Fig. 7 is 0.83 × 10⁻⁴ M, and the range is from 0.83 × 10⁻⁵ to 2.14 × 10⁻⁵ M. Similar results were obtained when the enzyme preparation was tested for dihydropterotate synthesis in the presence of sulfathiazole. The **K<sub>m</sub>** value obtained from three experiments ranged from 1.58 × 10⁻⁴ to 3.75 × 10⁻⁴ M, and the **K<sub>i</sub>** value ranged from 0.78 × 10⁻⁴ to 4.32 × 10⁻⁴ M. The experimental conditions were similar to those described in Fig. 7, with the exception that the enzyme
Fig. 5. Electrometric titration. Ten micromoles of each pteridine were titrated with 0.013 N KOH.

Fig. 6. Effect of concentration of hydroxymethyl dihydropteridine-PP on the synthesis of dihydropterate (●) and dihydrofolate (○). Velocity is given in micrograms of product formed per mg of protein per hour, and hydroxymethyl dihydropteridine-PP concentration was varied between 2 × 10⁻⁶ and 22 × 10⁻⁴ 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-aminobenzoyl glutamate and sulfathiazole on dihydrofolate synthesis. Dihydrofolate synthesis was tested in the presence of 8 × 10⁻⁵ M sulfathiazole and 0.8 × 10⁻⁴ M p-aminobenzoate.

Experimental conditions were as described for Brown (20). Table VI illustrates a typical experiment showing the reversal of sulfathiazole inhibition by p-aminobenzoate. It was demonstrated that when sulfathiazole and hydroxymethyl dihydropteridine-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 hydroxymethyl dihydropteridine-PP at 2 × 10⁻⁴ M were incubated with enzyme for 30 minutes and p-aminobenzoyl glutamate 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 hydroxymethyl dihydropteridine-PP, 2 × 10⁻⁴ M, the inhibition that was observed was readily reversed by p-aminobenzoate or p-aminobenzoyle...
Under an atmosphere of argon. benzoate as indicated were made according to the conditions of incubation. The first incubation period was 30 minutes and the second, 60 minutes at 37°C. The reaction mixtures were incubated at 37°C under an atmosphere of argon.

Stoichiometry—The stoichiometric relation between the utilization of hydroxymethylhydropteridine-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 0.4 ml of 0.005 N HCl. The 32P-32P content of the charcoal filtrates was detected in a liquid scintillation spectrometer. The radioactivity of each eluate was determined. The results are shown in Table VIII.

Stoichiometry of dihydrofolate synthesis

Each reaction mixture contained 140 μmoles of Tris buffer, pH 8.5; 14 mg of potassium ascorbate; 9 mg of protein of the pH 4.7 enzyme fraction; 0.7 μmole of hydroxymethylhydropteridine-32P-32P (66,000 c.p.m. per μmole); and 14 μmoles of p-aminobenzoylglutamate, as indicated, in a total volume of 7 ml. The reaction mixtures were incubated at 37°C under an atmosphere of argon. After each sample was 0.000 5 N HCl. The 32P-32P content of the charcoal filtrate was detected in a liquid scintillation spectrometer. The hydroxymethylhydropteridine-32P-32P that was adsorbed to 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.

Reversibility of Reaction—Attempts to show reversibility of the enzyme reaction as measured by the incorporation of pyrophosphate when all of the components were added together (as in Table VI, Experimental Group B). However, if the same amounts of hydroxymethylhydodihydropteridine-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 dihydropteroate 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.

Relative rate is the observed rate of dihydropteroate synthesis without sulfathiazole divided by the observed rate of dihydropteroate synthesis with sulfathiazole the pH 4.7 enzyme fraction). The additions of sulfathiazole, 8 × 10^-6 M; hydroxymethyldihydropteridine-PP, 2 × 10^-4 M; and p-aminobenzoate concentration X 100. The rates without sulfathiazole were designated 100%.

Additions of sulfathiazole, 8 × 10^-6 M; 0.5 mg of potassium ascorbate; and 200 μg of protein (the pH 4.7 enzyme fraction). The additions of sulfathiazole, 8 × 10^-6 M; hydroxymethyldihydropteridine-PP, 2 × 10^-4 M; and p-aminobenzoate as indicated were made according to the conditions of incubation. The first incubation period was 30 minutes and the second, 20 minutes at 37°C. The reaction mixtures were incubated under an atmosphere of argon.

Additions made after first and before second incubation period

% of each eluate was determined. The results are shown in Table VIII. For each millimicromole of hydroxymethylhydropteridine-PP used, 1 mpmole each of inorganic pyrophosphate and dihydrofolate was formed.

Reversal by p-aminobenzoylglutamate of sulfathiazole inhibition of synthesis of dihydrofolate

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Substrates present during first incubation period</th>
<th>p-Aminobenzoate</th>
<th>Other</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>5</td>
<td>Hydroxymethylhydodihydropteridine-PP</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>5</td>
<td>Sulfathiazole</td>
<td>42</td>
</tr>
<tr>
<td>C</td>
<td>Sulfathiazole and hydroxymethylhydodihydropteridine-PP</td>
<td>5</td>
<td>Hydroxymethylhydodihydropteridine-PP</td>
<td>44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Incubation time</th>
<th>Hydroxymethylhydropteridine-32P-32P used</th>
<th>Pyrophosphate formed</th>
<th>Dihydrofolate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>90</td>
<td>9.3</td>
<td>10.6</td>
<td>9.9</td>
</tr>
<tr>
<td>Without p-aminobenzoate</td>
<td>180</td>
<td>12.5</td>
<td>12.5</td>
<td>13.2</td>
</tr>
</tbody>
</table>

Additions made after first and before second incubation period

A 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

B 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

C 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

D 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

E 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

F 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

G 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

H 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

I 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

J 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

K 5 M Sulphathiazole | 0 |

Additions made after first and before second incubation period

L 5 M Sulphathiazole | 0 |
phosphate-P32P into the charcoal fraction or by the appearance of arylamine-positive material have been unsuccessful.

**Discussion**

The information gained from the electrometric titration and the acid and enzymatic hydrolysis of the phosphorylated pteridine derivatives indicates that a pyrophosphate grouping is present in the pteridine diphosphate. The electrometric titration data also show that the amino and hydroxyl groups of the pteridine moiety of the compound are still present and not substituted. These conclusions are substantiated by the fact that the absorption spectra of the pteridine diphosphate are qualitatively and quantitatively identical with the spectra of the nonphosphorylated pteridine. These various lines of evidence indicate that the phosphorylated pteridine is the 2-amino-4-hydroxy derivative that has the pyrophosphate grouping esterified with the hydroxymethyl group attached to the 6-position of the pteridine.

The enzymatic synthesis of dihydrofolate or dihydropteroreate from 2-amino-4-hydroxy-6-pteridinylmethyl pyrophosphate was also suggested by Jaenicke and Chan (4). Although direct evidence for enzymatic phosphorylation of the dihydropteriderine derivative is still lacking, pyrophosphorylation rather than successive phosphorylation of the dihydropteridine by ATP is indicated since the hydroxymethyl-dihydropteridine monophosphate was inactive even in the presence of ATP (5).

The similarity of the dihydrofolate- and the thiamine monophosphate-synthesizing systems has been pointed out by Leder (21) and by Brown et al. (2).

It is possible that dihydrofolate and dihydropteroreate are synthesized by different enzymes. So far, we have not been able to demonstrate the presence of more than one enzyme in *Veillonella* extracts. Although the apparent KI values for the sulfonamide-enzyme systems for dihydrofolate and dihydropteroreate synthesis were similar, these results do not exclude the existence of different enzyme systems. The KI value obtained in the case where dihydropteroreate is synthesized agrees with that of Brown (20), when his data are treated according to Dixon (22).

Merola and Koft (3) and Wolf and Hotchkiss (23) have also shown that sulfonamides competitively inhibit the synthesis of folate-like compounds in a cell-free system.

The "irreversible inhibition" observed by Brown (20) was shown to be due to the depletion of the 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine, which combines with the inhibitor during the preliminary incubation period. The results presented in this paper, which showed "irreversible inhibition" when a limiting amount of hydroxymethyl-dihydropteridine-PP was used, and the relatively little inhibition when a limiting concentration of sulfonamide was used, can be explained according to Brown (20), who demonstrated the formation of an analogue of a folate-like compound.

**Summary**

A chemically prepared pteridine diphosphate was characterized as 2-amino-4-hydroxy-6-pteridinylmethyl pyrophosphate. Reduction of this compound by sodium borohydride results in the formation of the corresponding dihydropteridine compound. This latter compound can be used directly, in the absence of adenosine triphosphate, as substrate in the enzymatic synthesis of dihydropteroreate or dihydrofolate by enzymes present in cell-free extracts of several microorganisms.

Dihydropteroreate and dihydrofolate were found to be utilized, respectively, about 17.8% and 22.8% as well as citrovorum factor as a growth factor for *Streptococcus faecalis* under the conditions described.

The preparation of a *Veillonella* extract (free from pyrophosphate) is described. This preparation synthesizes dihydrofolate and dihydropteroreate from 2-amino-4-hydroxy-6-pteridinylmethyl and 2-amino-4-hydroxy-6-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 dihydropteroate and dihydrofolate by competing with 2-amino-4-hydroxy-6-pteridinylmethyl pyrophosphate for the active site of the enzyme.

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The Enzymatic Synthesis of Folate-like Compounds from Hydroxymethyldihydropteridine Pyrophosphate
T. Shiota, M. N. Disraely and M. P. McCann


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