The Biosynthesis of Folic Acid Compounds in Plants

II. Some Properties of Dihydropteroate-synthesizing Enzyme in Pea Seedlings.¹

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Dihydropteroate-synthesizing enzyme was found in 25 to 45 % saturated fraction with ammonium sulfate of the extracts from pea seedlings, and some properties of the enzyme were investigated. The optimum pH for the enzyme activity was found to be 8.5 to 9.0 under the experimental conditions. In the enzyme reaction, Mg^{2+} was the most effective as cofactor, and the effect could partially be replaced by Mn^{2+} , whereas divalent heavy metal ions inhibited the enzyme activity. This enzyme was also inhibited by *p*-chloromercuribenzoate. The Michaelis constant for 2-amino-4-hydroxy-6-pyrophosphorylmethyldihydropteridine was found to be 7.1×10^{-5} M. It has been suggested that the enzyme reaction proceeds stoichiometrically as shown in Reaction (a), and the reaction is reversible.

In the preceding paper (1), it was presented that the cell-free extracts of pea seedlings contained the enzyme system which catalyzed the formation of dihydropteroic acid or dihydrofolic acid from synthetic 2-amino-4-hydroxy-6-pyrophosphorylmethyldihydropteridine and *p*-aminobenzoic acid or *p*-aminobenzoylglutamic acid. This extracts also could catalyze the formation of dihydrofolic acid from the above phosphorylated pteridine, *p*-aminobenzoic acid and L-glutamic acid. From these results, along with the nature of the substrate and the cofactor requirements in each enzyme reaction, it was proposed that the synthesis of dihydrofolic acid in plants proceeded by the following enzymatic reactions:

2-Amino-4-hydroxy-6-pyrophosphorylmethyldihydropteridine

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The abbreviations used are the same as in the previous paper (1).

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The enzyme which catalyzes *Reaction (a)* has been found in the preparations from *Veillonella* strain V2, and other several microorganisms including *Lactobacillus plantarum*, *Butyribacterium rettgeri* and *Fusobacterium fusiforme* by Shiota *et al.* (2,3), in the extracts of *Escherichia coli* by Weisman and Brown (4), in the cell-free preparation of *Pneumococcus* by Ortiz and Hotchkiss (5). In the present paper, we wish to report on the partial purification of the enzyme from pea seedlings and some properties of the enzyme including the stoichiometry and the reversibility of the enzymatic reaction.

EXPERIMENTAL

1. Materials

2-Amino-4-hydroxy-6-pyrophosphorylmethylpteridine was synthesized by the phosphorylation of 2-amino-4-hydroxy-6-hydroxymethylpteridine (3) which was prepared by the method of Waller *et al.* (6) as described in the previous paper (1). Pteric acid was kindly provided by Dr. T. H. Jukes, the Lederle Laboratories Division, American Cyanamid Company. Both compounds were reduced to the dihydro derivatives by the treatment with sodium borohydride (3) just before the use for experiments.

2. Determination of Pteridine Concentration

The concentration of pyrophosphorylmethylpteridine was estimated by measuring the absorbance in 0.1 N NaOH at 253 m μ , using a molar extinction coefficient of 23,500 (2). The concentration of pteric acid was determined by the absorbances at 256, 275 and 365 m μ at pH 11 with the use of a molar extinction coefficient of 25,900, 24,000, and 8,120, respectively (7).

3. Measurement of Enzyme Activity

The standard assay system contained 0.05 μ mole of pyrophosphorylmethyldihydropteridine, 0.05 μ mole of *p*-aminobenzoic acid, 1.0 μ mole of magnesium chloride and 0.05 ml of enzyme fraction in a total volume of 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.8). After two minutes of preincubation, the reaction was initiated

FIG. 1 *Response Curve of Streptococcus faecalis R*
—○—, dihydroptericoic acid; —●—, pteric acid; —Δ—, folic acid.

by the addition of pteridine as substrate and incubated at 30° for 30 minutes under the air³. The reaction was stopped by adding 0.05 ml of 14 M 2-mercaptoethanol and chilled immediately in an ice bath. Dihydropteroic acid formed was determined by an aseptic microbiological assay procedure with *Streptococcus faecalis* R (ATCC 8043) as described in the previous paper (1). A typical growth response curve of *S. faecalis* R to graded amounts of folic acid, pteric acid or dihydropteroic acid is shown in Fig. 1. The enzyme activity was expressed as the amount of dihydropteroic acid formed, and one unit of the enzyme activity was defined as the amount which formed 0.1 μ mole of dihydropteroic acid in 30 minutes under the experimental conditions. Specific activities are expressed as units per mg of protein under the same conditions as described above.

4. Other Analytical Methods

Inorganic pyrophosphate was determined as phosphorus by the method of Chen *et al.* (8) after the degradation to inorganic phosphate by autoclaving with 0.5 N sulfuric acid at 15 lbs. for 15 minutes. The protein in the enzyme preparation was measured by the method of Lowry *et al.* (9), with crystalline bovine serum albumin as the standard.

RESULTS AND DISCUSSION

1. Fractionation of Dihydropteroate-synthesizing Enzyme

Pea seeds (*Pisum sativum* L., cultivated variety Alaska) were germinated as described in the previous report (1). Three hundred grams of one day-old pea seedlings were homogenized with 450 ml of 0.05 M Tris-HCl buffer of pH 7.5 containing 0.01 M 2-mercaptoethanol in a Waring blender. The homogenate was squeezed through a gauze and centrifuged at 15,000 $\times g$ for 30 minutes in the cold. To 460 ml of the supernatant solution, solid ammonium sulfate was added gradually

TABLE 1
Enzyme Activity of Various Fractions from Extracts of Pea Seedlings

Fraction	Total protein	Total activity	Specific activity	Recovery
	mg	units ^a	units ^a /g protein	per cent
0-60 % saturated fraction with ammonium sulfate	4,988	2,275	0.46	100
Protamine supernatant	4,293	2,254	0.53	99
0-25 % saturated fraction with ammonium sulfate	200	0	0	—
25-45 % saturated fraction with ammonium sulfate	1,400	1,425	1.02	63
45-60 % saturated fraction with ammonium sulfate	810	0	0	—

^a 1 unit = 0.1 μ mole of Dihydropteroic acid formed/0.5 ml/30 minutes under the standard assay conditions.

³ It was confirmed in the preliminary experiments that the dihydropteroate synthesis was not changed in either aerobic or anaerobic atmosphere under the experimental conditions. For the longer incubation period, however, an anaerobic condition described in the preceding paper (1) was used.

to make 60 % saturation, the pH being maintained at 7.5 with 5 *N* ammonium hydroxide, and the mixture was stirred for one hour. The protein precipitated was collected by centrifugation and dissolved in 0.01 *M* Tris-HCl buffer of pH 7.5 containing 0.01 *M* 2-mercaptoethanol. The solution was dialyzed overnight in a Visking cellophane tubing against 20 liters of the same buffer. To 172 ml of this dialysate, 9 ml of 1 % protamine sulfate was added slowly, adjusting pH to 7.5, and the resulting precipitate was removed by centrifugation. The supernatant solution was fractionated with ammonium sulfate into three fractions, *i. e.*, "0 to 25 %", "25 to 45 %", and "45 to 60 %" saturated fractions with ammonium sulfate, and each fraction was collected, dissolved and dialyzed as described above. All operations were performed at below 5°. The enzyme activities of these fractions are summarized in Table 1. Since all of the enzyme activity was found in the "25 to 45 %" saturated fraction, this fraction was used as the enzyme solution. The enzyme solution did not contain any measurable folic acid activity for *S. faecalis R* used as a test microorganism in the experiments.

2. Time Course of the Enzyme Reaction

Under the standard assay conditions described above, the reaction proceeded linearly for at least 40 minutes and the dihydropteroate synthesis was proportional to enzyme concentration as shown in Fig. 2 and 3.

3. Effect of pH on the Enzyme Activity

The optimum pH for the enzyme activity was found to be 8.5 to 9.0 as shown in Fig. 4. The buffers used were Tris-maleate buffer for the pH 7.0 to 8.5, Tris-HCl buffer for pH 7.5 to 9.0, and glycine-NaOH buffer for the pH 9.0 to 10.5 at the final concentrations of 0.2 *M*, respectively.

4. Influence of Divalent Cations

The requirement of Mg^{2+} for the dihydropteroate synthesis was confirmed as shown in Table 2. Subsequently, the influence of various divalent cations on the enzyme activity was investigated at the final concentration of 2 *mM* in their chloride

FIG. 2 Time Course of the Reaction

FIG. 3 Relationship Between Enzyme Concentration and Dihydropteroate Synthesis

FIG. 4 Effect of pH on the Reaction
 —○—, Tris-HCl buffer; —●—, Tris-maleate buffer
 —△—, glycine-NaOH buffer.

TABLE 2
 Component Study for Dihydropteroate Synthesis

Omission	Dihydropteroic acid formed μmoles
None	110.5
Pyrophosphorylmethyldihydropteridine	0
<i>p</i> -Aminobenzoic acid	0
MgCl ₂	37.5
Enzyme	0

TABLE 3
 Influence of Divalent Cations on Dihydropteroate Synthesis

Divalent cation	Dihydropteroic acid formed μmoles
None	45.8
Mg ²⁺	109.0
Mn ²⁺	94.8
Ca ²⁺	58.3
Ba ²⁺	42.7
Co ²⁺	41.7
Ni ²⁺	38.5
Fe ²⁺	0
Cd ²⁺	0
Cu ²⁺	0
Hg ²⁺	0

The standard assay method was used, except that the divalent cations indicated were added at the final concentration of 2×10^{-3} M, respectively.

forms. The results are shown in Table 3. Mg²⁺ was the most effective to promote the enzyme activity, and the effect could partially be replaced by Mn²⁺, and by Ca²⁺ to a lesser extent. On the other hand, divalent heavy metal ions such as Hg²⁺, Cu²⁺, Cd²⁺ and Fe²⁺ inhibited the enzyme activity significantly.

5. Inhibition by *p*-Chloromercuribenzoate

The enzyme activity was found to be inhibited by the addition of *p*-chloromercuribenzoate (PCMB) as shown in Table 4. The enzyme was inhibited 86 % of the

TABLE 4
Inhibition of Dihydropteroate Synthesis by *p*-Chloromercuribenzoate

<i>p</i> -Chloromercuribenzoate added	Dihydropteroic acid formed	Inhibition
<i>M</i>	μmoles	per cent
None	119	0
1×10^{-4}	111	7
4×10^{-4}	56	53
1×10^{-3}	16	86

The standard assay method was used, except that the indicated amount of *p*-chloromercuribenzoate was added.

activity by 10^{-3} *M* PCMB, and 53 % by 4×10^{-4} *M* PCMB. These results, along with the inhibition by heavy metal ions, suggest that the sulfhydryl-group of the enzyme is closely related to the enzyme activity. Further studies on the inhibition of the enzyme by PCMB are now in progress by using a further purified enzyme preparation.

6. Effect of Concentrations of 2-Amino-4-hydroxy-6-pyrophosphorylmethyl-dihydropteridine and Mg^{2+}

The rate of the dihydropteroate synthesis as a function of concentration of the substrate, pyrophosphorylmethyldihydropteridine, was plotted according to the method of Lineweaver and Burk (10). The result is shown in Fig. 5. The Michaelis constant for pyrophosphorylmethyldihydropteridine was found to be 7.1×10^{-5} *M* from Fig. 5. Shiota *et al.* (3) have reported the *K_m* value of 3.12×10^{-4} *M* for pyrophosphorylmethyldihydropteridine in the enzymatic reaction by the cell-free preparations from *Veillonella*.

The effect of concentration of Mg^{2+} on the dihydropteroate synthesis is shown in Fig. 6. The optimal concentration of Mg^{2+} was observed at around 2 *mM* under the standard assay conditions, and the higher concentration of Mg^{2+} had shown a tendency to inhibit the enzyme activity.

FIG. 5 Effect of Substrate Concentration on Dihydropteroate Synthesis

FIG. 6 Effect of MgCl_2 Concentration on Dihydropteroate Synthesis

7. Participation of Inorganic Pyrophosphate in the Reaction

The products formed in the enzyme reaction were found to be dihydropteroate and inorganic pyrophosphate, and the equimolar amounts of both compounds were formed simultaneously. The reaction mixture containing $0.5 \mu\text{mole}$ of pyrophosphorylmethyldihydropteridine, $1.0 \mu\text{mole}$ of *p*-aminobenzoic acid, $5.0 \mu\text{moles}$ of MgCl_2 and 20 mg of enzyme in a total volume of 5.0 ml of 0.05 *M* Tris-HCl buffer at pH 8.8 was incubated at 30° for 2 hours under an atmosphere of argon. An aliquot (1 ml) of the reaction mixture was used to estimate the dihydropteroate formed. To 4 ml of the remaining reaction mixture, 50 mg of Norit A was added to adsorb phosphorylated pteridine used as substrate and other organic compounds, stirred for 15 minutes, and removed by filtration through Toyo No. 6 filter paper. A portion (2.5 ml) of the filtrate was mixed with 0.5 ml of 3 *N* sulfuric acid, autoclaved at 15 lbs. pressure for 15 minutes and used for the determination of the phosphate. The blank cuvette contained all of the components which was incubated for zero minute, and the amount of pyrophosphate formed in the enzyme reaction was obtained after subtracting the value in the blank cuvette. The results, as shown in Table 5, indicates that the reaction proceeds stoichiometrically as presented in Reaction (a).

TABLE 5
Stoichiometry of Dihydropteroate Synthesis

Product	Amount
	<i>mμmoles</i>
Pyrophosphate	2.8
Dihydropteroic acid	2.8

Reaction mixtures contained $0.5 \mu\text{mole}$ of pyrophosphorylmethyldihydropteridine, $1.0 \mu\text{mole}$ of *p*-aminobenzoic acid, $5.0 \mu\text{moles}$ of MgCl_2 , and 20 mg of enzyme in a total volume of 5.0 ml of 0.05 *M* Tris-HCl buffer (pH 8.8).

The reaction was carried out at 30° for 2 hours under an atmosphere of argon. The amount of pyrophosphate formed was obtained after subtracting the value in the blank cuvette.

TABLE 6
Influence of Phosphates on Dihydropteroate Synthesis

Addition	Concentration	Dihydropteroic acid formed
	<i>M</i>	<i>μmoles</i>
None		118 (100 %)
Pyrophosphate	1×10^{-2}	86 (72.8)
	2×10^{-2}	84 (71.2)
	1×10^{-1}	59 (50.0)
	2×10^{-1}	46 (39.0)
Orthophosphate	2×10^{-2}	117 (99.1)
	1×10^{-1}	112 (94.9)
	2×10^{-1}	110 (93.2)

The standard assay method was used, except that the phosphates were added at the indicated concentrations, respectively.

The dihydropteroate synthesis was significantly inhibited by the addition of inorganic pyrophosphate, whereas orthophosphate had no effect as shown in Table 6. From these results, it may be probable that the *Reaction (a)* is reversible and the dihydropteroate-synthesizing enzyme catalyzes the reversible reaction.

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