BIOCHEMICAL STUDIES ON PTERIDINES IN PLANTS¹

II. BIOGENESIS OF FOLIC ACID IN GREEN LEAVES: ENZYMATIC SYNTHESIS OF DIHYDROPTEROIC ACID FROM GUANOSINE COMPOUNDS AND MECHANISM OF ITS SYNTHETIC PATHWAY²

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Reynolds and Brown (1,2) demonstrated the enzymatic net synthesis of the pteridine moiety of dihydrofolic acid from guanosine nucleotides by cell-free extracts of *Esheria coli*. Recently, a similar reaction was shown by Shiota and Palumbo (3), using the extracts of *Lactobacillus plantarum*, and, on the basis of the study of formate production in the reaction involving the removal of carbon 8 of guanine nucleotides, they suggested that GTP or GDP but not GMP is the immediate substrate. Furthermore, a recent communication with the cell-free extracts of *Salmonella typhimurium* by Dalal and Gots (4) indicated that GTP was the most efficient of the guanine nucleotides tested.

In 1961, Weygand *et al.* (5) proposed a hypothetical pathway for pteridine biosynthesis from guanosine or GMP on the basis of the observed labelled pattern in the leucopterin isolated from a butterfly *Pieris brassicae*. The over-all pathway proposed that 4-(5'-phosphoribosyl)amino-2,5-diamino-6-hydroxypyrimidine and a phosphorylated form of 2-amino-4-hydroxy-6-(D-erythro-1',2',3',-trihydroxypropyl) dihydropteridine were intermediary compounds. This pteridine was later isolated by Rembold and Buschmann (6). Jones, Reynolds and Brown (7) represented that the extracts of *E. coli* could convert this pteridine into dihydropteroic acid.

The enzymatic synthesis of dihydropteroic acid or dihydrofolic acid from 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine and *p*-aminobenzoic acid or *p*-aminobenzoylglutamic acid in the presence of ATP and Mg²⁺ was demonstrated in plants by the present authors (8,9), using the extracts of spinach leaves. Afterwards the same observations were presented by Iwai *et al.* (10) with soy bean seedlings. The authors (11) extended their studies and obtained preliminary evidence that cell-free extracts of *Brassica pekinensis* Rupr (HAKUSAI) could catalyze the conversion of guanosine compounds into dihydropteroic acid. Together

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with these findings, the study of the mechanism of the biosynthesis especially on the effectiveness of various synthetic pteridines as the donor for forming dihydropteroic acid are described in this paper.

EXPERIMENTAL

1. Materials

2-Amino-4-hydroxy-6-hydroxymethylpteridine was synthesized according to the method of Forrest and Walker (12), or Waller *et al.* (13), and monophosphate and pyrophosphate esters of this pteridine were prepared by the procedure of Shiota, Disraely and McCann (14). They were obtained as lead salts and stored in a refrigerator at 20°. When the esters were tested for the enzyme reaction, lead was removed as lead sulfide by bubbling the solution of the ester in cold 0.1 N HCl with hydrogen sulfide, followed by centrifugation (14). 2-Amino-4-hydroxy-6-(*D*-erythro-trihydroxypropyl)-pteridine was obtained from condensation of 2,4,5-triamino-6-hydroxypyrimidine sulfate (15) and *D*-ribose or *D*-arabinose according to Rembold and Buschman (6). The corresponding *L*-erythro, *D*-threo and *L*-threo isomers were synthesized by the same method by changing the sugar to *L*-arabinose, *D*-xylose and *L*-xylose, respectively. 2-Amino-4-hydroxypteridine was obtained by the procedure of Mowat *et al.* (16), pteric acid according to Waller *et al.* (17). 2-Amino-4-hydroxy-6-(*L*-arabo-1',2',3'-trihydroxy-4'-deoxybutyl)pteridine, 2-amino-4-hydroxy-6-(*D*-arabo-1',2',3',4'-tetrahydroxybutyl)pteridine and 2-amino-4-hydroxy-6-(*D*-lyxo-1',2',3',4'-tetrahydroxybutyl)pteridine were prepared by condensation of 2,4,5-triamino-6-hydroxypyrimidine (15) with *L*-rhamnose, *D*-glucose and *D*-galactose, respectively (18). The pteridines were reduced to the corresponding dihydroform by treatment with sodium hydrosulfite as suggested by Futterman (19), when the pteridines were used in the enzyme reactions.

Crystalline ATP, GTP, guanosine, guanine, DL- α -glycerophosphate and tris-(hydroxymethyl)aminomethane were purchased from Sigma Chemical Company; adenine, 3-phosphoglycerate from Nutritional Biochemicals Corporation; hypoxanthine from Zellstoffabrik Waldhof; sodium pyruvate, *L*-arabinose and *D*-arabinose from E. Merck, A. G., Darmstadt; *D*-ribose from Wako Pure Chemical Industries Ltd.; *D*-xylose and highly purified agar from Difco Laboratories; Norit A from Fischer Scientific Company; *L*-xylose and sodium glyoxylate from Tokyo Chemical Industry Co. Ltd.; Folic Acid Assay Medium "Nisan" for *Streptococcus faecalis* R (ATCC No. 8043) and yeast extract from Nissui Chemical Industries Ltd.; sodium ascorbate from Takeda Pharmaceutical Industries Ltd., and Sephadex G-50, medium, from Pharmacia, Uppsala. All other chemicals including folic acid, inosine, xanthine, *p*-aminobenzoic acid, *L*-rhamnose, *D*-glucose and *D*-galactose were of the best quality available.

2. Preparation of Cell-Free Extracts from *Brassica pekinensis* Rupr

In all the preparation and assays of the enzyme, the phosphate used was potassium salt unless otherwise stated. Two hundred grams of leaves of *Brassica pekinensis* Rupr were homogenized at 4° with 50 ml of 0.2 M phosphate buffer, pH 7.0, containing 40 mM Na₂SO₃. The homogenate was filtered through gauze and centrifuged at 10,000 rpm for one hour at 0°. To the resulting supernatant,

150 ml, *p*-aminobenzoic acid was added to give a final concentration of 2 *mM*, and then solid ammonium sulfate was added up to 0.55 saturation, stirring for 30 minutes at 4°. The precipitate was collected by centrifugation at 8,000 rpm for 20 minutes at 0°, dissolved in 0.05 *M* Tris-HCl buffer, pH 7.0, containing 0.5 *mM* *p*-aminobenzoic acid and 10 *mM* Na₂SO₃, and dialyzed against the same buffer, 2 liters for two hours under stirring at 4°. After centrifuging the dialysate at 10,000 rpm for 20 minutes at 0°, *p*-aminobenzoic acid to make a concentration of 5 *mM* and Norit A, equivalent in weight to the protein present, were added successively to the supernatant, stirring for 10 minutes at 4°. The suspension was centrifuged at 10,000 rpm for 20 minutes at 0° to remove the charcoal. The clear supernatant fluid will be referred to as the "charcoal-treated extract" in this paper (9).

A column of Sephadex G-50, medium, (2.2 × 24 cm), jacketed for cooling at 4°, was washed with 150 ml of 0.1 *M* Tris-HCl buffer, pH 7.65. Three ml of the charcoal-treated extract was applied on the top of the column, washed into the gel with 3 ml of the same buffer and developed. Light brown colored protein fractions were collected and used in the following experiments, unless otherwise mentioned.

3. Microbiological Assay

Dihydropteroic acid produced was determined by microbiological assay with *St. faecalis* R as described in the previous report (9). Although only dihydropteroic acid was produced in the enzyme reaction of *p*-aminobenzoic acid with pteridine donors (9), folic acid was used as the standard in the assay for convenience and the values indicated in this paper are expressed as folate equivalents.

4. Enzyme Assay

The enzyme reaction was carried out in the Thunberg tubes under nitrogen or hydrogen in an air incubator at 30°. The reaction was stopped by immediate cooling in an ice bath and adding 0.1 ml of mercaptoethanol. An aliquot of the mixture was used for microbiological assay as indicated before (9). Protein was determined by the method of Kalcker (20), measuring the optical densities at 260 and 280 *mμ*.

RESULTS AND DISCUSSION

1. Enzymatic Synthesis of Dihydropteroic Acid from Guanosine Compounds

Various purines were tested for enzymatic formation of dihydropteroic acid. As shown in Table 1, only guanosine and GMP were most effectively utilized in the synthesis. All the other purines were without any effect, although hypoxanthine showed a much lesser activity. The results in Table 2 represent that ATP, Mg²⁺ and phosphate were all required for the enzymatic transformation from guanosine. Higher concentration of phosphate, Mg²⁺, ATP and mercaptoethanol were found to result in remarkable inhibition of the synthesis.

2. Comparison of Effectiveness of Guanosine Compounds to Form Dihydropteroic Acid

The preparation of the enzyme for this experiment was as follows: The charcoal-treated extract was dialyzed at 4° over-night under vigorous stirring against

TABLE 1
Effectiveness of Purine Compounds as Precursor for Enzymatic Synthesis de novo of Dihydropteroic Acid

Reaction mixtures contained: *p*-aminobenzoic acid, 0.03 μ mole; $MgSO_4$, 10 μ moles; sodium phosphate, 20 μ moles, pH 8.0; mercaptoethanol, 30 μ moles; ATP, 2.5 μ moles; $CuSO_4$, 0.001 μ mole; purine derivative, 0.3 μ mole and enzyme protein, 11.7 mg of *Brassica pekinensis* Rupr in 2 ml of Tris-HCl, 0.1 *M*, pH 7.95.

Incubated for 4 hr at 30° under nitrogen. Reaction was stopped by addition of 0.1 ml of mercaptoethanol. Dihydropteroic acid was determined by microbiological assay with *S. faecalis* R.

Purine derivative	Folate equivalents formed	
	Observed	Corrected against control
	<i>mμg/4 hr in 2 ml</i>	
Control ^a	107	0
GMP	375	268
Guanosine	283	176
Guanine	61	—
Adenine	107	0
Xanthine	107	0
Hypoxanthine	156	49
Adenosine	89	—
Inosine	82	—

^a Control containing enzyme protein, $CuSO_4$, mercaptoethanol and Tris buffer.

TABLE 2
Enzymatic Synthesis de novo of Dihydropteroic Acid from Guanosine Derivatives

Reaction mixtures contained: *p*-aminobenzoic acid, 0.05 μ mole; $MgSO_4$, 10 μ moles; potassium phosphate, 20 μ moles, pH 8.0; mercaptoethanol, 30 μ moles; ATP, 2.5 μ moles; $CuSO_4$, 0.001 μ mole; guanosine derivative, 0.3 μ mole and enzyme protein, 12.4 mg of *Brassica pekinensis* Rupr in 2 ml of Tris-HCl, 0.1 *M*, pH 7.95.

Incubated for 4 hr at 30° under nitrogen.

Reaction mixture	Folate equivalents formed	
	Observed	Corrected against control
	<i>mμg/4 hr in 2 ml</i>	
Control ^a	46	0
Complete (guanosine)	174	128
Omit $MgSO_4$	66	20
Omit ATP	72	26
Omit guanosine	40	—
Omit phosphate	72	26
Omit enzyme	0	—
Guanine used	72	26
GMP used	184	138

^a Control containing enzyme protein, $CuSO_4$, mercaptoethanol and Tris buffer.

500 volumes of 0.015 *M* Tris-HCl buffer, pH 7.0. After centrifuging the dialysate at 10,000 rpm for 20 minutes at 0°, the supernatant solution was applied on a Sephadex G-50 column (2.2 $\times$ 24 cm), which had been previously equilibrated with 0.03 *M* Tris-HCl buffer, pH 7.65. The column was cooled, and developed with the same buffer. The protein emerging from the column were collected. Using the

dialyzed enzyme in the reaction, dihydropteroic acid was formed from guanosine compounds as given in Table 3. It can be seen that guanosine nucleotides were the most active, though prolonged dialysis resulted in considerable loss of the enzyme activity. Guanosine, which was active as substrate in the crude extracts, has been removed from the conversion system in the step of this purification.

TABLE 3
*Effects of Guanosine Derivatives as Precursors for Enzymatic
Synthesis de novo of Dihydropteroic Acid* (One step incubation)

Reaction mixtures contained: *p*-aminobenzoic acid, 0.04 μ mole; $MgSO_4$, 5 μ moles; potassium phosphate, 100 μ moles, pH 8.0; mercaptoethanol, 30 μ moles; ATP, 2 μ mole; guanosine derivative, 0.2 μ mole and enzyme protein, 9.3 mg of *Brassica pekinensis* Rupr in 2 ml of Tris-HCl, 0.1 M, pH 7.95.

Incubated for 4 hr at 30° under nitrogen gas.

Guanosine derivative	Folate equivalents formed
	<i>mg/4 hr in 2 ml</i>
GTP	23.3
GDP	27.9
GMP	26.4
Guanosine	0
Control ^a	0

^a Control containing enzyme protein, mercaptoethanol, potassium phosphate and Tris buffer.

In order to decide which guanosine nucleotides might be included as the direct precursor in the biosynthesis of dihydropteroic acid, the enzyme system was tried to be splitted into two stages of the reaction. In the first, the reaction system containing guanosine compound and the dialyzed enzyme was incubated at 30° for 3 hours under nitrogen. At the end of the incubation, the medium was heated at 100° for 2 minutes, and then centrifuged at 3,000 rpm for 10 minutes to remove the resulting precipitate. After 0.5 ml of the supernatant was added to the second reaction mixture, containing *p*-aminobenzoic acid, Mg^{2+} , ATP and the charcoal-treated extract obtained after gel-filtration with Sephadex G-50, the second reaction was allowed to proceed at 30° for 3 hours under nitrogen. As indicated in Table 4, GDP was utilized most efficiently as the pteridine donor for the enzymatic production of dihydropteroic acid; about three times more active than GMP and about 1.7 times as effective as GTP. When GTP was used, the conversion into dihydropteroic acid was absolutely dependent on ATP and Mg^{2+} under the conditions given in Table 4 (see Table 5). These results provided the possibility that the participating guanine congener is GTP or GDP rather than GMP or guanosine; GDP or GTP might be transformed without ATP and Mg^{2+} during the first reaction period into a pteridine which can couple with *p*-aminobenzoic acid in the presence of ATP and Mg^{2+} to form dihydropteroic acid.

These findings in the higher plant on the enzymatic conversion of guanosine compounds to dihydropteroic acid were compatible with those obtained by Reynolds and Brown (1,2) with *E. coli* system, and of Shiota and Palumbo (3) in cell-free extracts of *Lactobacillus plantarum*. Very recently, Dalal and Gots demonstrated the same observations using *Salmonella typhimurium* (4).

TABLE 4

Effects of Guanosine Derivatives as Precursors for Enzymatic Synthesis de novo of Dihydropterotic Acid (Two step incubation)

1st incubation: reaction mixtures contained: potassium phosphate, 10 μ moles; CuSO_4 , 1.5×10^{-3} μ mole; mercaptoethanol, 2 μ moles; guanosine derivative, 0.2 μ mole and enzyme protein, 9.0 mg of *Brassica pekinensis* Rupr in 1 ml of Tris-HCl, 0.02 M, pH 8.1.

Incubated for 3 hr at 30° under nitrogen. Reaction was stopped by heating at 100° for 2 min and reaction mixture was centrifuged at 3,000 rpm for 10 min. The supernatant, 0.5 ml was used in the 2nd reaction.

2nd incubation: reaction mixtures contained: *p*-aminobenzoic acid, 0.03 μ mole; potassium phosphate, 100 μ moles; MgSO_4 , 10 μ moles; ATP, 5 μ moles; mercaptoethanol, 100 μ moles; the super of 1st reaction mixture, 0.5 ml and the enzyme protein, 18.2 mg in 2 ml of Tris-HCl, 0.09 M, pH 8.2.

Incubated for 3 hr at 30° under nitrogen. Reaction was stopped by addition of 0.1 ml mercaptoethanol.

Guanosine derivative	Folate equivalents formed in 2ml of the 2nd reaction
	<i>mg/3 hr</i>
GTP	15.5
GDP	26.3
GMP	9.3
Guanosine	3.1
Control ^a	0

^a Control containing enzyme protein, CuSO_4 , mercaptoethanol, potassium phosphate and Tris buffer.

TABLE 5

Cofactor Requirements for Enzymatic Synthesis de novo of Dihydropterotic Acid from GTP

The condition used were similar to those described in Table 4.

Reaction mixture	Folate equivalents formed in 2ml of the 2nd reaction
	<i>mg/3 hr</i>
Control	0
Complete (GTP)	15.5
Omit MgSO_4	9.3
Omit ATP	6.2
Omit GTP	0
Omit enzyme	0

^a Control containing enzyme protein, CuSO_4 , mercaptoethanol, potassium phosphate and Tris buffer.

3. *Enzymatic Synthesis of Dihydropterotic Acid from 2-Amino-4-hydroxy-(D-erythro-1',2',3'-trihydroxypropyl)dihydropteridine and Its Related Compound*

The hypothetical pathway for leucopterin and xanthopterins biosynthesis from guanosine or GMP, proposed by Weygand *et al.* (5) consists of the following steps: (a) an opening of the imidazole ring of the guanine moiety and elimination of carbon 8 to give 4-(5'-phosphoribosyl)amino-2,5-diamino-6-hydroxypyrimidine, (b) Amadori rearrangement of the ribose residue to the corresponding 1'-(substituted-amino)-1'-deoxypentulose and (c) cyclization of this ketose to a phosphorylated form of 2-amino-4-hydroxy-6-(D-erythro-1',2',3'-trihydroxypropyl)dihydropteridine

which serves as the immediate precursor of xanthopterin. Reynolds and Brown (1,2) found by using C^{14} -labelled guanosine and ribose that guanosine was incorporated except for the carbon 8 into dihydrofolic acid by the cell-free extracts of *E. coli* and the ribose moiety provides carbon atoms for the formation of the pyrazine ring. Recently, Jones, Reynolds and Brown (7) also, prepared and tested the *L-threo*, *D-threo*, and *L-erythro* isomers of 2-amino-4-hydroxy-6-(*D-erythro*-trihydroxypropyl)dihydropteridine. The *D-erythro* compound was the most active among the isomers tested to yield dihydropteroic acid. Shiota and Palumbo (3) also observed using *Lact. plantarum* the dihydrofolic acid synthesis from the *D-erythro*, but not the *L-erythro* form, which had rather an inhibitory action on the production.

The present authors examined the ability of 2-amino-4-hydroxy-6-(*D-erythro*-trihydroxypropyl)dihydropteridine together with its isomers to be transformed into dihydropteroic acid in the plant system. The results are represented in Table 6. One can see that the *D-erythro* and *D-threo* compounds were the most active, and the *L-erythro* and *D-threo* revealed half the activity of the formers. In other experiments, a comparison of the relative activities of these isomers, as given in Table 7, showed that the *D-erythro* was utilized much more effectively than the other isomers. The *L-threo*, *D-threo* and *L-erythro* compounds gave 78, 45.4 and 31.8% of the activity of the *D-erythro* compound. When the *D-erythro* compound was used as a pteridine donor, the enzymatic formation of dihydropteroic acid was dependent on ATP, Mg^{2+} and *p*-aminobenzoic acid (see Table 8).

TABLE 6
Enzymatic Formation of Dihydropteroic Acid from 2-Amino-4-hydroxy-6-trihydroxypropyldihydropteridine Derivatives

Reaction mixtures contained: *p*-aminobenzoic acid, 0.03 μ mole; $MgSO_4$ 10 μ moles; mercaptoethanol, 100 μ moles; potassium phosphate (pH 8.0), 100 μ moles; dihydropteridine, 0.15 μ mole; ATP, 5 μ moles and enzyme protein, 10.1 mg of *Brassica pekinensis* Rupr in 2 ml of Tris-HCl, 0.1 M, pH 7.95. Incubated for 3 hr at 30° under nitrogen.

Trihydroxypropyl- dihydropteridine	Folate equivalents formed
	<i>mg/3 hr in 2 ml</i>
2-Amino-4-hydroxy-6-(<i>D-erythro</i> -trihydroxypropyl)- dihydropteridine	219
2-Amino-4-hydroxy-6-(<i>L-threo</i> -trihydroxypropyl)- dihydropteridine	216
2-Amino-4-hydroxy-6-(<i>L-erythro</i> -trihydroxypropyl)- dihydropteridine	125
2-Amino-4-hydroxy-6-(<i>D-threo</i> -trihydroxypropyl)- dihydropteridine	121
None	0
2-Amino-4-hydroxy-6- hydroxymethyldihydropteridine ^a	221

^a 0.05 μ mole.

TABLE 7

Relative Activities of 2-Amino-4-hydroxy-6-trihydroxypropyldihydropteridines and 2-Amino-4-hydroxy-6-tetrahydroxybutyldihydropteridines for the Enzymatic Formation of Dihydropteroic Acid

The conditons used were similar to those described in Table 6.

Polyhydroxyalkyl-dihydropteridine	Relative activity of formation of dihydropteroic acid
	<i>per cent</i>
2-Amino-4-hydroxy-6-(D-erythro-trihydroxypropyl)dihydropteridine	100
2-Amino-4-hydroxy-6-L-threo-trihydroxypropyl)dihydropteridine	78.0
2-Amino-4-hydroxy-6-(L-erythro-trihydroxypropyl)dihydropteridine	31.8
2-Amino-4-hydroxy-6-(D-threo-trihydroxypropyl)dihydropteridine	45.4
2-Amino-4-hydroxy-6-L-arabo-4'-deoxy-1',2',3'-trihydroxybutyl)dihydropteridine	117.5
2-Amino-4-hydroxy-6-(D-arabo-1',2',3',4'-tetrahydroxybutyl)dihydropteridine	69.7
2-Amino-4-hydroxy-6-(D-lyxo-1',2',3',4'-tetrahydroxybutyl)dihydropteridine	30.6

TABLE 8

Enzymatic Formation of Dihydropteroic Acid from 2-Amino-4-hydroxy-6-(D-erythro-trihydroxypropyl)dihydropteridine

Reaction mixtures contained: *p*-aminobenzoic acid, 0.03 μ mole; MgSO₄, 10 μ moles; potassium phosphate (pH 8.0), 100 μ moles; mercaptoethanol, 100 μ moles; dihydropteridine, 0.15 μ mole; ATP, 5 μ moles and enzyme protein, 10.2 mg of *Brassica pekinensis* Rupr in 2ml of Tris-HCl buffer, 0.1 M, pH 7.95.

Incubated for 3 hr at 30° under nitrogen.

Reaction mixture	Folate equivalents formed
	<i>mg/3 hr in 2 ml</i>
Complete	208
Omit MgSO ₄	0
Omit pteridine ^a	0
Omit <i>p</i> -aminobenzoic acid	95
Omit enzyme	0
Omit ATP	0
2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine used (0.05 μ mole)	188

^a 2-Amino-4-hydroxy-6-(D-erthro-trihydroxypropyl)dihydropteridine.

These findings allowed the present authors to attempt to prepare 6-butyl-dihydropteridine derivatives and to test the possibility of these substances to be utilized as the substrates in the enzymatic reaction to form dihydropteroic acid. As shown in Table 7, 2-amino-4-hydroxy-6-(L-arabo-1',2',3'-trihydroxy-4'-deoxybutyl)-dihydropteridine was equally as active as the D-erythro-trihydroxypropyldihydropteridine. 2-Amino-4-hydroxy-6-(D-arabo-1',2',3',4'-tetrahydroxybutyl)dihydropteridine and 2-amino-4-hydroxy-6-(D-lyxo-1',2',3',4'-tetrahydroxybutyl)dihydropteridine

gave the activities of 69.7 and 30.6 %, respectively, against the *D-erythro* compound. In the present investigation we can not give any answer for what means the enzyme reacts with polyhydroxyalkylpteridine in the metabolism of pteridine compounds in plants. However, it should be noted that the analogue of these substances does indeed exist in nature as active compounds. Occurrence of 2-amino-4-hydroxy-6-trihydroxypropylpteridine as a natural product was demonstrated firstly by Goto and Forrest (21), who isolated the phosphate ester of this pteridine from *E. coli*. 2-Amino-4-hydroxy-6-(*D-erythro*-trihydroxypropyl)pteridine was isolated from the pupae of the bee and hoey, and characterized by Rembold and Buschman (6). The other isomers of this pteridine have not been found. Recently, Sakurai and Goto (22) isolated 2-amino-4-hydroxy-6-tetrahydroxybutylpteridine from human urine, and, also, Goto *et al.* (23) obtained 2'- β -glucuronide of 2-amino-4-hydroxy-6-1',2',3',4',5'-pentahydroxypentylpteridine from *Mycobacterium smegmatis*, which had the activity for *Crithidia fasciculata* as a growth factor.

4. No Requirements of Mg^{2+} and ATP for Enzymatic Conversion of 2-Amino-4-hydroxy-6-(*D-erythro*-1',2',3'-trihydroxypropyl)dihydropteridine to 2-Amino-4-hydroxy-6-hydroxymethyl-dihydropteridine

Recently, using C^{14} -labelled compound, the reduced form of 2-amino-4-hydroxy-6-trihydroxypropylpteridine was proved in *Drosophila melanogaster* to be incorporated into 2-amino-4-hydroxy-6-methylpteridine, which was supposed to be derived from 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine (26).

As demonstrated in previous communications (8, 9), ATP and Mg^{2+} were required for coupling of 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine with *p*-aminobenzoic acid to produce dihydropteroic acid. In order to confirm whether ATP and Mg^{2+} are involved or not in the enzymatic conversion of 2-amino-4-hydroxy-6-(*D-erythro*-trihydroxypropyl)dihydropteridine to 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine, experiments were carried out as follows. In preincubation at 30° for one hour under nitrogen two different reaction mixtures, the trihydroxypropyldihydropteridine with the enzyme, and the same pteridine and Mg^{2+} with the enzyme, were incubated. At the end of the period, to the former reaction system was added *p*-aminobenzoic acid, ATP and Mg^{2+} , and to the latter, ATP and *p*-aminobenzoic acid. The two reaction media were reincubated at 30° under nitrogen in parallel with the control not preincubated system, and the formation of dihydropteroic acid was followed in the course of time. From the data shown in Fig. 1, one can observe that, in the reaction mixture containing trihydroxypropyldihydropteridine with the enzyme preincubated before addition of ATP, *p*-aminobenzoic acid and Mg^{2+} , dihydropteroic acid was produced with a yield higher than in the medium without preincubation. These observations suggested the possibility that the conversion of trihydroxypropyldihydropteridine to hydroxymethyl-dihydropteridine might proceed without ATP and Mg^{2+} during the preincubation period.

5. No Effectiveness of 2-Amino-4-hydroxydihydropteridine as a Donor for Enzymatic Synthesis of Dihydropteroic Acid

Forrest and Nawa (25) proposed that a three-carbon unit of the side chain of reduced 2-amino-4-hydroxy-6-(*D-erythro*-trihydroxypropyl)pteridine is cleaved to

FIG. 1 Effect of Preincubation of Enzyme and 2-Amino-4-hydroxy-6-(D-erythro-trihydroxypropyl)dihydropteridine in the Presence and the Absence of Mg^{++} on the Formation of Dihydropteroate

Reaction mixtures contained: *p*-aminobenzoic acid, 0.03 μ mole; $MgSO_4$, 10 μ moles; mercaptoethanol, 100 μ moles; potassium phosphate, (pH 8.0), 100 μ moles; 2-amino-4-hydroxy-6-(D-erythro-trihydroxypropyl)dihydropteridine, 0.15 μ mole; ATP, 5 μ moles; and enzyme protein, 11.3 mg of *Brassica pekinensis* Rupr for one hour at 30° under nitrogen. Reaction proceeded for 3 hr at 30° under nitrogen.

I, no preincubation; II, preincubation in the presence of magnesium ion and the absence of ATP; III, preincubation in the absence of both ATP and magnesium ion.

form reduced 2-amino-4-hydroxypteridine, which is then converted to reduced 2-amino-4-hydroxy-6-hydroxymethylpteridine by addition of a one-carbon unit and sepiapterin by addition of a three-carbon unit. Okada and Goto (24) found that the reduced form of 2-amino-4-hydroxypteridine-10- C^{14} was incorporated into isoxanthopterin and sepiapterin in *Drosophila melanogaster*.

The present authors examined the possibility that 2-amino-4-hydroxydihydropteridine functions as an intermediate in the enzymatic formation of dihydropteroic acid. As presented in Table 9, this pteridine failed to reveal any effects in the plant system.

Baugh and Shaw (27) have reported that the intact cells of *Corynebacterium* species can incorporate radioactivity from 2,4,5-triamino-6-hydroxypyrimidine-5- C^{14} into pteridine compounds. This possibility of the formation of dihydropteroic acid in the plant system could be excluded from the finding that under the conditions employed in Table 1 in which this pyrimidine and ribose instead of guanosine compounds were incubated with the enzyme, any compound active for *St. faecalis* R was failed to be detected.

TABLE 9

No Effectiveness of 2-Amino-4-hydroxydihydropteridine as Precursor for Enzymatic Synthesis of Dihydropterotic Acid

Reaction mixtures contained: *p*-aminobenzoic acid, 0.03 μ mole; $MgSO_4$, 10 μ moles; mercaptoethanol, 100 μ moles; potassium phosphate (pH 8.0), 100 μ moles; dihydropteridine, 0.15 μ mole; carbon donor, 0.15 μ mole; ATP, 5 μ moles and enzyme protein, 8.4 mg of *Brassica pekinensis* Rupr in 2 ml of Tris-HCl, 0.1 M, pH 7.95.

Incubated for 3 hr at 30° under nitrogen.

Reaction mixtures	Folate equivalents formed in 2 ml
	<i>mg/3 hr</i>
2-Amino-4-hydroxydihydropteridine only	0
Plus DL-glyceraldehyde	0
Plus 3-phospho-D-glycerate	0
Plus DL- α -glycerolphosphate	0
Plus sodium pyruvate	0
Plus glyoxylic acid	0
Plus acetic acid	0
Plus ethanol	0
Plus formic acid	0
Plus formaldehyde	0
Plus D-ribose	0
Plus D-arabinose	0
Plus L-arabinose	0
Plus D-xylose	0
Plus L-xylose	0
2-Amino-4-hydroxy-6-(D-erythro-trihydroxypropyl)dihydropteridine	150
2-Amino-4-hydroxy-6-hydroxymethyl-dihydropteridine ^a	150

^a 0.05 μ mole

TABLE 10

Enzymatic Formation of Dihydropterotic Acid from 2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine Pyrophosphate and p-Aminobenzoic Acid

Reaction mixtures contained: *p*-aminobenzoic acid, 0.03 μ mole; $MgSO_4$ 10 μ moles; potassium phosphate (pH 8.0), 100 μ moles; mercaptoethanol, 100 μ mole; dihydropteridine, 0.05 μ mole and enzyme protein, 12.5 mg, of *Brassica pekinensis* Rupr in 2 ml Tris-HCl buffer, 0.1 M, pH 7.95.

Incubated for 3 hr at 30° under nitrogen. Reaction was stopped by addition of 0.1 ml of mercaptoethanol. Dihydropterotic acid was determined by microbiological assay with *S. faecalis* R.

Reaction mixture	Folate equivalents formed in 2 ml
	<i>mg/3 hr</i>
Complete	217
Omit $MgSO_4$	0
Omit pteridine ^a -pyrophosphate	0
Omit <i>p</i> -aminobenzoic acid	59
Omit enzyme	0
Pteridine ^a -monophosphate used	0
Pteridine ^a used	0
Pteridine ^a plus ATP (5 μ moles)	158

^a 2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine.

6. *Enzymatic Synthesis of Dihydropteroic Acid from 2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine Pyrophosphate*

The enzymatic synthesis of dihydropteroic acid from 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine and *p*-aminobenzoic acid by the cell-free extracts of spinach leaves had been shown to require ATP and Mg^{2+} (8,9), postulating that a pyrophosphate ester of this pteridine might be involved in the formation of dihydropteroic acid. Recently, Iwai *et al.* (10) prepared the pyrophosphate ester according to the method of Shiota *et al.* (14) and found that the ester could combine with *p*-aminobenzoic acid to yield dihydropteroic acid by the extracts of soy bean seedlings in the absence of ATP. The same evidence was presented by the present authors with the enzyme preparation of *Brassica pekinensis* Rupr as given in Table 10. The pyrophosphate ester could be converted into dihydropteroic acid only in the presence of Mg^{2+} , but the monophosphate ester was not utilized.

These observations in higher plants are quite in agreement with those of Shiota *et al.* (14, 28) with *Lact. plantarum*, who demonstrated firstly these evidences, and also with that made by Weiman and Brown in *E. coli* system (29).

SUMMARY

1. Cell-free extracts of *Brassica pekinensis* Rupr (Hakusai) catalyzed the formation of dihydropteroic acid from guanosine compounds and *p*-aminobenzoic acid. This reaction required ATP, Mg^{2+} and phosphate.

2. GTP and GDP were more active in dihydropteroic acid synthesis than GMP and guanosine. When GTP was used as substrate, the synthesis was absolutely dependent on ATP and Mg^{2+} .

3. 2-Amino-4-hydroxy-6-(*D*-erythro-1',2',3'-trihydroxypropyl)dihydropteridine was the most active as a pteridine donor for the formation of dihydropteroic acid. This enzyme reaction needed ATP and Mg^{2+} . The *L*-erythro, *D*-threo and *L*-threo isomers of 2-amino-4-hydroxy-6-(*D*-erythro-trihydroxypropyl)dihydropteridine, 2-amino-4-hydroxy-6-(*L*-arabo-1',2',3'-trihydroxy-4'-deoxybutyl)dihydropteridine, 2-amino-4-hydroxy-6-(*D*-arabo-1',2',3',4'-tetrahydroxybutyl)dihydropteridine and 2-amino-4-hydroxy-6-(*D*-lyxo-1',2',3',4'-tetrahydroxybutyl)dihydropteridine were tested in the ability of enzymatic transformation to dihydropteroic acid.

4. 2-Amino-4-hydroxy-6-(*D*-erythro-1',2',3'-trihydroxypropyl)dihydropteridine seemed to convert to 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine without ATP and Mg^{2+} .

5. 2-Amino-4-hydroxydihydropteridine and 2,4,5-triamino-6-hydroxypyrimidine were not involved in the synthesis of dihydropteroic acid.

6. 2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphate could be converted into dihydropteroic acid only in the presence of Mg^{2+} . The monophosphate ester was not utilized in the conversion.

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