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The Biosynthesis of Folic Acid Compounds in Plants

III. Distribution of the Dihydropteroate-synthesizing Enzyme in Plants[1]

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Dihydropteroate synthetase was widely distributed in the higher plants tested, and a high enzyme activity was found in green leaves. In the early germination stage in pea seedlings, most of the enzyme activity existed in the cotyledon. With growth, a remarkable increase in enzyme activity in the root plus shoot was observed. The amount of *Streptococcus faecalis* R-active substances in root plus shoot rapidly increased during the germination of pea seedlings.

A large percentage of the enzyme activity was located in the mitochondrial fractions of both pea and soybean seedlings. Most of the S. *faecalis* R-active substances in pea seedlings existed in the soluble fraction from the cytoplasm.

We reported earlier (1) that *Pediococcus cerevisiae*-active substances (the citrovotum factor) in pea seeds increased during germination, and suggested that plant folate compounds were formed as a labile form of folate coenzymes, *i.e.* N^{10}-formyltetrahydrofolate and N^5, N^{10}-methenyltetrahydrofolate. Several recent works (2-4) using cell-free enzyme systems have demonstrated that the probable intermidiate in the biosynthesis *de novo* of dihydrofolic acid is dihydropteroic acid rather than *p*-aminobenzoylglutamic acid. In the biosynthesis of folate compounds, dihydropteroic acid is the first compound having a folic acid activity for microorganisms. A previous paper (5) reported a simple and rapid radioassay procedure for the activity of dihydropteroate synthetase, which catalyzed the coupling reaction of 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine (or its pyrophosphate ester) and *p*-aminobenzoic acid to yield dihydropteroic acid, using ^{14}C-labeled *p*-aminobenzoic acid as substrate.

In the present investigation, by applying the radioassay technique, the distribution and intracellular localization of dihydropteroate synthetase in plants were examined.

MATERIALS AND METHODS

1. *Materials*

2-Amino-4-hydroxy-6-pyrophosphorylmethylpteridine was prepared by the method of Shiota *et al.* (6) from 2-amino-4-hydroxy-6-hydroxymethylpteridine (7). For use as a substrate in the enzymatic reaction, 2-amino-4-hydroxy-6-pyrophosphorylmethylpteridine was reduced, by treatment with sodium borohydride, to the dihydro-derivative (8). *p*-Aminobenzoic acid labeled with ^{14}C at the carboxyl group was purchased

[1] See the previous paper, Part II (13). An outline of this report was presented at the 20th annual meeting of the Japan Vitamin Society at Kochi on April 23, 1968. This work was supported in part by a grant (for K. I.) from the Vitamin B Research Committee. Dihydropteroate-synthesizing enzyme is abbreviated to dihydropteroate synthetase in this paper.

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from Calbiochem, Los Angeles, California, through its Japanese distributor, Daiichi Pure Chemicals Co. Ltd. The specific activity of the labeled *p*-aminobenzoic acid was 10 mCi/mmole.

2. Germination of Pea and Soybean Seeds

Air-dried, healthy looking pea seeds (*Pisum sativum L.* var. Alaska) and soybean seeds (Tsurunoko) were soaked in deionized water at 25° for 18 hours, then allowed to germinate at 25° in moist vermiculite.

3. Preparation of Cell-free Extracts of Various Plants and Tissues

Each fresh weight 10 g sample was homogenized with 10 ml of 0.05 M Tris-HCl buffer at pH 7.5 containing 5 mM 2-mercaptoethanol. The homogenate was dialyzed for 5 hours against 5 liters of the homogenating solution containing 0.01 M ammonium sulfate. All operations were performed in a cold room at 5°. Dialyzed extracts prepared from several plants were used for the assay of dihydropteroate synthetase activity.

4. Measurement of Dihydropteroate Synthetase Activity

A radioassay (5) for the enzyme using ^{14}C-labeled *p*-aminobenzoic acid as substrate was made. The reaction mixture contained 10 mμmoles of 2-amino-4-hydroxy-6-pyrophosphorylmethyldihydropteridine, 2 mμmoles of labeled *p*-aminobenzoic acid, 100 mμmoles of magnesium chloride and 0.02 ml of the enzyme solution in a total volume of 0.1 ml of 0.05 M Tris-HCl buffer at pH 8.8. The reaction was caried out at 37° for 10 minutes, and was stopped by the addition of 0.1 ml of 95 % ethanol. The labeled dihydropteroic acid produced was separated from labeled *p*-aminobenzoic acid by ascending paper chromatography with 0.1 M potassium phosphate buffer at pH 7.0 as developer. Radioactivity of the paper section containing the labeled product was counted in a Tri-Carb liquid scintillation counting system (Packard Instrument Co.). The amount of dihydropteroic acid formed was determined by this method as having a specific activity of 11,980 cpm per mμmole. One enzyme unit was defined as the amount of the enzyme required to synthesize one mμmole of dihydropteroic acid under the above conditions.

5. Determination of Protein Concentration

The amount of protein in the enzyme solution was determined by the method of Lowry *et al.* (9) using bovine serum albumin as the standard.

6. Microbiological Assay

Folate compounds in pea seedlings were determined using an aseptic microbiological assay procedure with *Streptococcus faecalis* R (10) with folic acid as the standard. A fresh sample was homogenized with 2 volumes of ice-cold 0.1 M Tris-HCl buffer at pH 7.5 containing 6 mg of sodium ascorbate per ml. The homogenate was squeezed through gauze and brought to 25 ml with the same buffer. After addition of 500 mg of sodium ascorbate to 10 ml of the extracted juice, the whole was boiled in a water bath for 5 minutes and chilled. Then, extracts were centrifuged to remove the precipitate, and the supernatant was used as the sample for microbiological assay of folate compounds.

7. Isolation of Cell Particles

The medium used for the isolation of cell particles contained 0.5 M sucrose, 0.025 M Tris-HCl buffer at pH 7.5 and 5 mM 2-mercaptoethanol. Each fraction was prepared in the cold with slight modification of the method of Wilson and Shannon (11). Two hundred and fifty grains of one day-old pea or soybean seedlings were homogenized with 200 ml of the isolation medium in a mortar. The homogenates were squeezed through double layers of gauze, and differentially centrifuged at $800 \times g$ for 10 minutes (= debris and nuclei), at $40{,}000 \times g$ for 30 minutes (= mitochondria), and $114{,}000 \times g$ for 90 minutes (= microsomes). The final supernatant was used as the soluble fraction. Each precipitate was washed with the isolation medium, and suspended in 0.01 M Tris-HCl buffer at pH 7.5 containing 5 mM 2-mercaptoethanol. The enzyme activities in these suspensions and in the soluble fraction were assayed as described above.

Results and Discussion

1. Distribution of Dihydropteroate Synthetase in Plants

Dihydropteroate synthetase activity in extracts from various plants and tissues was determined. The results are shown in Table 1. Data in this table are expressed as values for one gram of fresh weight. The enzyme was widely distributed in higher plants. High enzyme activity was also found in green leaves.

2. Changes in Dihydropteroate Synthetase Activity in Germinating Pea Seeds

The changes in dihydropteroate synthetase activity in pea seeds during germination are shown in Fig. 1. The values at zero day indicate the enzyme activity just after soaking the seeds in water at 25° for 18 hours. Most of the enzyme activity was found in the cotyledons of pea seedlings in the early days. In the light, enzyme activity in root plus

TABLE 1
Distribution of dihydropteroate synthetase in extracts from various plants

Plant	Tissue	Protein	Enzyme activity	Specific activity
		mg[a]	*units*[a] ($\times 10^{-3}$)	*units/mg protein* ($\times 10^{-3}$)
Pak-choi	Leaf	35.0	507	14.4
Chinese cabbage	Leaf	8.2	144	21.1
Cabbage	Leaf	7.5	30	6.7
Radish	Leaf	38.5	973	25.4
Spinach	Leaf	52.5	238	4.5
	Petiole	15.7	0	0
	Root	27.3	77	2.1
Pea seedlings				
2 day-old	Cotyledon	29.0	64	2.2
	Shoot and root	10.5	81	7.7
10 day-old	Cotyledon	19.2	27	1.4
	Shoot	5.6	61	10.9
	Root	2.3	22	9.5
Soybean seedlings				
2 day-old	Cotyledon	28.4	682	24.0
	Shoot and root	16.9	175	10.3

[a] Data are expressed as values per one gram of fresh weight. One unit is defined as the amount of enzyme required to synthesize 1 mμmole of dihydropteroic acid under standard assay conditions.

shoot increased with the growth of pea seedlings. With the appearance of green leaves, 6 or 7 days after sowing, the enzyme activity in root plus shoot increased considerably with the decrease in activity in the cotyledon, whereas, in the dark, the enzyme activity in root plus shoot increased very slightly. The increased activity in the cotyledon decreased rapidly 4 or 5 days after sowing. This suggests that the enzyme was formed in the green leaves of peas and has a relationship to photosynthesis.

When pea seeds were soaked in deionized water at 0° (in an ice bath) for 20 hours, the amount of the enzyme in the pea seed extracts was approximately equal to that in the extracts from swollen pea seeds at 25°, as shown in Table 2. The results suggest that dihydropteroate synthetase occurs in pea seeds before germination. The enzyme seems to be formed when the peas run to seed.

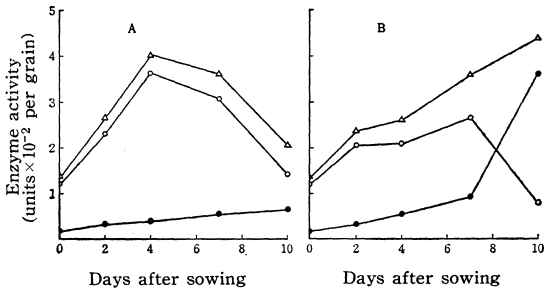

FIG. 1 *Changes in dihydropteroate synthetase activity in germinating pea seeds*
○—○, cotyledon; ●—●, root plus shoot; △—△, whole seedlings. A, in darkness; B, in light.

3. Biosynthesis of Folate Compounds in Pea Seedlings

Folate compounds contained in pea seedlings were determined by a microbiological assay with *S. faecalis* R using folic acid as the standard. It is expressed as folate equivalents. As shown in Fig. 2, in the cotyledon, the amount of folate compounds increased

TABLE 2
Comparison of enzyme activity in swollen pea seeds soaked at 25° and 0°

Soaking temp.	Protein	Enzyme activity
	mg[a]	*units*[a]
25°	1,213	10.8
0°	1,235	10.3

[a] Pea seeds were soaked in deionized water for 20 hr at 25° and 0°. Data are expressed as values per 50 grains of pea seedlings.

TABLE 3
Intracellular localization of dihydropteroate synthetase in pea and soybean seedlings

Fraction	Pea seedlings			Soybean seedlings		
	Protein	Enzyme activity	Ratio	Protein	Enzyme activity	Ratio
	mg [a]	*units* [a]	*per cent*	*mg* [a]	*units* [a]	*per cent*
Homogenate	538.0	6.02	100	1,895.0	2.03	100
Debris and nucleus	68.6	0.13	2.1	61.5	0.02	1.0
Mitochondrial	32.8	4.38	72.8	56.8	1.58	77.9
Microsomal	16.2	0	0	11.0	0	0
Soluble	420.0	1.53	25.4	1,794.0	0.29	14.3

[a] Data are expressed as values per 25 grains of one day-old seedlings. One unit is defined as the amount of enzyme required to synthesize 1 mμmole of dihydropteroic acid under standard assay conditions.

slowly, while in root plus shoot, this increase was rapid. Most of the enzyme activity existed in cotyledon in the early days of germination, as described in the preceding section, but *S. faecalis* R-active substances increased chiefly in root plus shoot. These facts suggest that folate compounds in the early germination stage of pea seeds are formed in the cotyledons and are transferred steadily into the roots and shoots.

4. Intracellular Localization of the Enzyme in Pea and Soybean Seedlings

Localization of the enzyme in various intracellular fractions from pea and soybean seedlings was investigated. In both pea and soybean seedlings, the enzyme activity was found in the mitochondrial and soluble fractions. An especcially large percentage of the activity existed in the mitochondrial fraction, as shown in Table 3. The results indicate that most of the dihydropteroate synthetase is located in the mitochondrial fractions of plant cells. Whether the enzyme activity in the soluble fraction is dependent on the isozyme is not known.

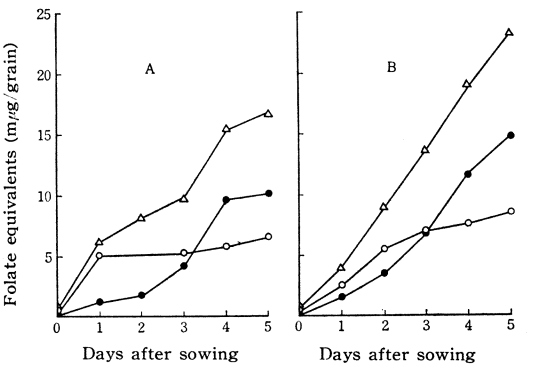

FIG. 2 *Biosynthesis of folate compounds in pea seedlings during germination*

○—○, cotyledon; ●—●, root plus shoot; △—△, whole seedlings. A, in darkness; B, in light.

Folate compounds in cell particles were determined using the microbiological assay described above. As shown in Table 4, most *S. faecalis* R-active substances were in the soluble fraction from the cytoplasm.

Iwai *et al.* (12) reported that formyltetrahydrofolate synthetase in pea seedlings, one of the enzymes which forms folate coenzymes from tetrahydrofolate, is located in the soluble fraction. These facts suggest that most folate compounds, probably up to dihydrofolic acid[4], are synthesized in mitochondria and sent to the cytoplasm, and that folate coenzymes are formed in the plant cytoplasm.

TABLE 4
Folate compounds in cell particles of pea seedlings

Fraction	Folate equivalents	Ratio
	mμg/grain	*per cent*
Homogenate	30.45	100
Debris and nucleus	0.34	1.1
Mitochondrial	6.67	21.9
Microsomal	0.03	0.1
Soluble	23.39	76.9

[4] Recent work from this laboratory indicates that the dihydrofolate synthetase in pea seedlings, which catalyzes the formation of dihydrofolic acid from dihydropteroic acid and L-glutamic acid, is also located in the mitochondrial fraction.

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