NUTRITIONAL REQUIREMENTS FOR FOLATE COMPOUNDS AND SOME ENZYME ACTIVITIES INVOLVED IN THE FOLATE BIOSYNTHESIS

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Summary

Dihydrofolate synthetase activity is widely distributed in various microorganisms and mushrooms. Animals and microorganisms, i.e., rat and chicken (in the liver), L. casei and P. cerevisiae, which require essentially pteroylglutamic acid as a nutrient for growth showed no detectable dihydrofolate synthetase activity. S. faecalis R, which can replace pteroylglutamic acid with pteroic acid as a nutrient for growth, had little dihydropteroate synthetase activity but showed normal dihydrofolate synthetase activity. This suggests that the nutritional requirements for folate compounds shown in vivo is in good agreement with the results obtained with dihydropteroate and dihydrofolate synthetase activities in vitro, and that the pathway through dihydropteroic acid as an intermediate is the main route in folate biosynthesis in nature.

It is generally accepted that organisms that can not biosynthesize folate compounds in vivo and require them for growth lack the necessary enzyme in the folate biosynthetic pathway. Animal organisms and one group of lactic acid bacteria are known to require folate compounds for nutrition; Streptococcus faecalis R can replace pteroylglutamic acid with pteroic acid (1), Lactobacillus casei requires pteroylglutamic acid (1), and Pediococcus cerevisiae requires cofactor forms of folic acid (2). This suggests that nutritional requirements for folate compounds might result in the lack of the enzyme in the folate biosynthetic pathway. We have tried to confirm this possibility in vitro by detecting the enzymes, dihydropteroate synthetase and dihydrofolate synthetase, which are thought to be the key enzymes in the folate biosynthetic pathway.

1 Studies on the Biosynthesis of Folic Acid Compounds, XI (for Part X, see Reference (12)).
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MATERIALS AND METHODS

Materials. Hydroxymethylpterin was prepared by the method of WALLER et al. (3). For use as a substrate in the enzymatic reaction, hydroxymethylpterin was reduced to a dihydro derivative by treatment with sodium borohydride (4). p-Aminobenzoic acid labeled with 14C at the carboxyl group was purchased 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 per mmole. ATP was purchased from the Sigma Chemical Company. L-Glutamic acid, folic acid, 2-mercaptoethanol and ascorbic acid were obtained from commercial sources. Pteroic acid was provided by the Lederle Laboratories Division, American Cyanamid Company and was reduced to dihydropteroic acid by treatment with sodium dithionite as described by FUTTERMAN (5).

Measurement of dihydropteroate synthetase activity. A radioassay (6) for the enzyme using 14C-labeled p-aminobenzoic acid as the substrate was made. The reaction mixture contained 2 mµmoles of p-aminobenzoic acid-14COOH (8,000 cpm); 10 mµmoles of hydroxymethyl dihydropterin; 200 mµmoles of ATP; 100 mµmoles of magnesium chloride; 0.05 M Tris-HCl buffer (pH 8.5) containing 0.01 M 2-mercaptoethanol and a specified amount (20 µl) of cell-free extract in a total volume of 0.1 ml. The reaction was carried out at 37°C for 60 min, after which it was stopped by the addition of 0.1 ml of 99% ethyl alcohol.

The labeled dihydropteroic acid produced was separated from the labeled p-aminobenzoic acid by ascending paper chromatography with 0.1 M potassium phosphate buffer (pH 7.0) as the developer, and the 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 thus determined as having a specific activity of 11,890 cpm per mmole.

Measurement of dihydrofolate synthetase activity. Dihydrofolate synthetase activity was determined by a microbiological assay (7). The reaction mixtures contained 100 µmoles of Tris-HCl buffer (pH 9.0); 5.0 µmoles of magnesium sulfate; 5.0 µmoles of L-glutamic acid; 50 µmoles of potassium sulfate; 5.0 µmoles of ATP; 0.01 ml of cell-free extract; 0.05 µmole of dihydropteroic acid and 50 µmoles of 2-mercaptoethanol in a final volume of 1.0 ml. The reaction was carried out at 37°C for 30 min, and was then stopped by heating the entire mixture in a boiling water bath for 1 min. After diluting the reaction mixture with cold water, the amounts of dihydrofolate formed were determined by microbiological assay with L. casei in 10 ml of an assay medium for folic acid (8); these are expressed as folate equivalents.

Determination of protein. The amount of protein was determined by the method of LOWRY et al. (9), using crystalline bovine serum albumin as a standard.

Culture conditions of microorganisms. Streptococcus faecalis R, ATCC 8043, Lactobacillus casei, ATCC 7469, and Pediococcus cerevisiae, ATCC 8081 were grown at 37°C for 18 hr in submerged cultures. The growth medium for the
microorganisms contained 1% yeast extract, 0.5% peptone, 1% glucose and 0.5% Na-acetate and 0.2% monopotassium phosphate at pH 6.8.

*Saccharomyces carlsbergensis* 4228, ATCC 9080, and *Saccharomyces cerevisiae* (baker’s yeast) were grown at 30°C for 24 hr in shaking cultures. The growth medium for these microorganisms contained 3% malt extracts, adjusted to pH 5.6.

*Escherichia coli* B, *Bacillus cereus* IFO 3131, *Pseudomonas riboflavina* IFO 3140 and *Serratia indica* IFO 3759 were grown at 30°C for 18 hr in shaking cultures in modified Massen medium (3% glucose, 0.07% D,L-malic acid, 1% asparagine, 0.25% K₂HPO₄, 0.04% MgSO₄·7H₂O, 0.25% Na₂CO₃, 0.001% CaCl₂ at pH 7.0).

*Aspergillus niger* IFO 3526 and *Neurospora crassa* IFO 6068 were cultured at 25°C for 5 days in Czapek-Dox medium enriched with yeast extracts (3% sucrose, 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 0.05% yeast extract, at pH 6.0).

Mushrooms were obtained from commercial sources.

Preparation of cell-free extracts. Animal organs and mushrooms were homogenized with two volumes of isolating medium (0.05 M of Tris-HCl buffer at pH 7.5 containing 0.01 M of 2-mercaptoethanol) in a Waring blender. The homogenate was squeezed through two layers of gauze, then centrifuged at 15,000 × g for 20 min at 0-4°C. The supernatant solution was used as the cell-free extract.

Yeast cells and mold myceria collected by filtration were ground well with sand in a porcelain mortar, and were then extracted with the isolating medium. The extracts were centrifuged at 15,000 × g for 20 min in the cold.

Bacteria cells collected by centrifugation (15,000 × g for 15 min) were suspended in the isolating medium and sonicated for 10 min at 0°C with an Ultra Sonic Oscillator 4210 (Kaijo Denki). The sonicate was centrifuged at 15,000 × g for 20 min in the cold.

RESULTS AND DISCUSSION

Distribution of dihydrofolate synthetase in microorganisms and mushrooms

Dihydrofolate synthetase activities in the extracts of various microorganisms and mushrooms were measured by microbiological assays with *L. casei*. Results are shown in Tables 1 and 2. Data in Table 2 are expressed as values per one gram of fresh weight. The enzyme activity was widely distributed in microorganisms and mushrooms. High enzyme activity was found in *E. coli*, *S. indica*, *B. cereus* and *Pseudomonas riboflavin.*

Nutritional requirements for folate compounds and some enzyme activities involved in the folate biosynthetic pathway.

The relationship between nutritional requirements for folate compounds and some enzyme activities involved in the folate biosynthetic pathway tested *in vitro* using cell-free extracts of rat liver, chicken liver, *S. faecalis* R, *L. casei* and *P. cere-
visiae. Results are shown in Table 3. A very little dihydropteroate synthetase activity was found in S. faecalis R, while some extent of the enzyme activity were detected in L. casei and P. cerevisiae. On the other hand, a high dihydrofolate synthetase activity was found in S. faecalis R, whereas no enzyme activity was detected in L. casei, P. cerevisiae, rat liver and chicken liver.

Table 1. Distribution of dihydrofolate synthetase activity in microorganisms.

| Microorganism                        | Specific activity |
|--------------------------------------|-------------------|
|                                      | units/mg of protein |
| Serratia indica IFO 3759             | 3.61              |
| Escherichia coli B                   | 4.75              |
| Bacillus cereus IFO 3131             | 3.74              |
| Saccharomyces carlsbergensis         | 0.46              |
| Saccharomyces cerevisiae (Baker's yeast) | 0.42            |
| Aspergillus niger IFO 3526           | 0.14              |
| Pseudomonas riboflava IFO 3140       | 3.65              |
| Neurospora crassa IFO 6068           | 2.25              |

*a 1 unit=0.1 μmole of folate equivalent formed per 30 min under standard assay conditions.

Table 2. Distribution of dihydrofolate synthetase activity in mushrooms.

| Mushrooms                      | Protein | Enzyme activity | Specific activity |
|--------------------------------|---------|-----------------|-------------------|
| Flammulina velutipes (enokitake) | 5.11    | 3.25            | 0.64              |
| Lentinus edodes (Pileus) (shiihake) | 4.07    | 1.73            | 0.43              |
| Lentinus edodes (Stalk) (shiihake) | 4.17    | 1.27            | 0.30              |
| Pleurotus ostreatus (hiratake)    | 15.60   | 4.77            | 0.31              |
| Pholiota nameko (nameko)          | 3.06    | 0.213           | 0.07              |
| Tricholoma matsutake (matsutake)  | 5.23    | 3.50            | 0.67              |

*a 1 unit=0.1 μmole of folate equivalent formed per 30 min under standard assay conditions.

Data are expressed as values per one gram of fresh weight.

It has been reported that dihydropteroate synthetase is widely distributed in various bacteria and plants (4,10). We have also reported that dihydrofolate synthetase is widely distributed in various plants (11). As shown in Tables 1 and 2, dihydrofolate synthetase was also found in various bacteria, yeasts, molds and mushrooms. In S. faecalis R, which requires pteroic acid, folic acid or the tetrahydro form of folic acid as a nutrient for the growth, very little dihydropteroate synthetase activity was detected, while a significantly high dihydrofolate synthetase activity was observed (Table 3). In L. casei and P. cerevisiae, which require folic acid or the tetrahydro form of folic acid, respectively, as nutrient for growth, no dihydrofolate synthetase activity was detected, whereas certain dihydropteroate synthetase activity was found. Furthermore, in livers of rat and chicken, which
Table 3. Requirement for folate compounds and some enzyme activities involved in folate biosynthesis.

| Organisms                  | Dihydropteroate synthetase activity | Dihydrofolate synthetase activity | Comparative growth-promoting activity* |
|----------------------------|-------------------------------------|----------------------------------|----------------------------------------|
|                            | Specific activity (unit*/µg of protein) | Specific activity (unit*/mg of protein) | Pterioic acid | Folic acid | H₄-folic acid |
| Escherichia coli B         | 6.50                                | 4.75                             |             |           |             |
| Serratia indica            | 3.50                                | 3.61                             |             |           |             |
| Streptococcus faecalis R   | trace                               | 1.45                             | +++         | +++       | +++         |
| ATCC 8043                  |                                     |                                  |             |           |             |
| Lactobacillus casei        | 0.09                                | nd                               |             | +++       | +++         |
| ATCC 7469                  |                                     | nd                               |             |           |             |
| Pediococcus cerevisiae     | 0.25                                | nd                               |             |           | +++         |
| ATCC 8081                  |                                     | nd                               |             |           |             |
| Rat liver                  |                                     | nd                               |             |           |             |
| Chicken liver              |                                     | nd                               |             |           |             |

* 1 unit = 0.25 µmole of dihydropteroate formed per 60 min under standard assay conditions.

** 1 unit = 0.1 µmole of dihydrofolic acid formed per 30 min under standard assay conditions.

* Cited in part from the Reference (1).

nd: not detectable, H₄-folic acid: tetrahydrofolic acid.

require folic acid or the tetrahydro form of folic acid as nutrient for growth, no dihydrofolic acid synthetase activity could be detected (Table 3). These results suggest that the nutritional requirement of organisms for folic acid compounds is, at least, due to the lack of dihydrofolic acid synthetase in their cells, and support the opinion that dihydropteroate is a true intermediate in the biosynthesis of folate compounds, and that the pathway, which passes through dihydropteroic acid as the intermediate, is the main route for the biosynthesis of folate compounds in nature.

REFERENCES

1) Williams, R. J., Eakin, R. E., Beerstecher, Jr. E., and Shive, W. (1950): The folic acid group, in Biochemistry of B Vitamins, Reinhold Publishing Corp., New York, pp. 565-567.

2) Stokstad, E. L. R. (1954): Biochemical relations between citrovorum factor and PGA, in The Vitamins, ed. by Sebrell, W. H., Jr., and Harris, R. S., Academic Press, Inc., Publishers, New York, Vol. III, pp. 110-111.

3) Waller, C. W., Goldmann, A. A., Angier, R. B., Booth, J. H., Hutchings, B. L., Mowat, J. H., and Semb, J. (1950): 2-Amino-4-hydroxy-6-pteridine carboxaldehyde. J. Am. Chem. Soc., 72, 4630-4633.

4) Shiota, T., Diskraely, M. N., and McCann, M. P. (1962): Preparation of dihydropteridinediphosphate, an intermediate in dihydrofolic acid synthesis. Biochem. Biophys. Res. Commun., 7, 194-198.
5) Futtermann, S. (1957): Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. *J. Biol. Chem.*, **228**, 1031–1038.

6) Okinaka, O., and Iwai, K. (1969): A radioassay for dihydropteroate-synthesizing enzyme activity. *Anal. Biochem.*, **31**, 174–182.

7) Herbert, V. (1961): The assay and nature of folic acid activity in human serum. *J. Clin. Invest.*, **40**, 81–86.

8) Iwai, K., Okinaka, O., and Yokomizo, H. (1967): A uniform medium for microbiological determination of the B-vitamins with various lactic acid bacteria. *Vitamins (Japan)*, **35**, 387–394.

9) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

10) Okinaka, O., and Iwai, K. (1970): The biosynthesis of folic acid compounds in plants. III. Distribution of the dihydropteroate-synthesizing enzyme in plants. *J. Vitaminol.*, **16**, 196–200.

11) Ikeda, M., and Iwai, K. (1970): Biosynthesis of folic acid compounds in plants. VI. The occurrence and properties of the dihydrofolate-synthesizing enzyme in pea seedlings. *Plant & Cell Physiol.*, **11**, 639–656.

12) Ikeda, M., and Iwai, K. (1976): Some characteristics of the dihydrofolate synthetase from *Serratia indica*. *J. Nutr. Sci. Vitaminol.*, **22**, 365–373.