STUDIES ON DIHYDROFOLATE SYNTHETASE

1975

MASAMICHI IKEDA

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CONTENTS

ABBREVIATIONS

PABA, *p*-aminobenzoic acid

PABG, *p*-aminobenzoylglutamic acid

AMP, adenosine triphosphate

ADP, adenosine diphosphate

ATP, adenosine monophosphate

CTP, cytidine triphosphate

GTP, guanosine triphosphate

ITP, inosine triphosphate

UTP, uridine triphosphate

ATP-γ-^{32}P, ^{32}P-labeled ATP at the γ-position

ATP-U-^{14}C, uniform ^{14}C-labeled ATP

Pi, inorganic orthophosphate

PPi, inorganic pyrophosphate

Km, Michaelis constant

Ki, inhibitor constant

PCMB, *p*-chloromercuribenzoate

Glu, glutamic acid

FA, folic acid

FAH_2, 7,8-dihydrofolic acid

H_2-pteroate or H_2pte, 7,8-dihydropteroate

min, minute

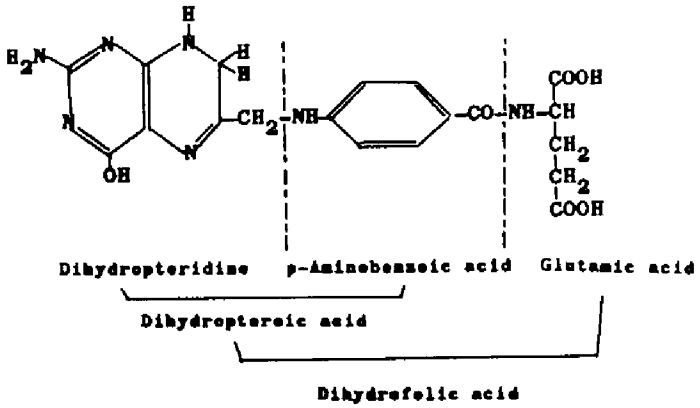
CHAPTER I

INTRODUCTION

Folic acid and its related compounds were found in liver extracts as an essential factor which some lactic acid bacteria require on growth. Using *Streptococcus lactis* as a test organism, it was shown that in addition to liver, kidney, mushroom, yeast, and particularly green leaves and grass contain the factor. The name folic acid (*folium* = leaf) was given to the substance in 1941 by Mitchell *et al.*[1)]

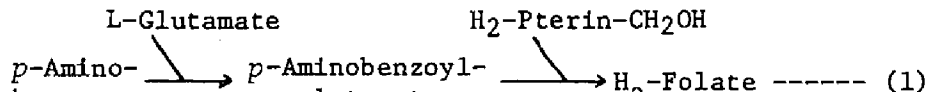
Many of the reduced derivatives of folic acid prepared as results of chemical studies of growth factors have subsequently been found to serve directly as coenzymes in the transfer of one-carbon units. These are generically called as folic acid coenzymes. The participation of the folic acid coenzymes in reactions leading to synthesis of purines[2,3)] and to thymine[4,5)], the methylated pyrimidine of DNA, emphasizes the fundamental role of folic acid in growth and reproduction of cells. Because the blood cell are subject to a relatively rapid rate of synthesis and destruction, it is not surprising that interference with red blood cell formation would be an early sign of a deficiency of folic acid, or that the folic acid antagonists would readily inhibit the formation of leukocytes.

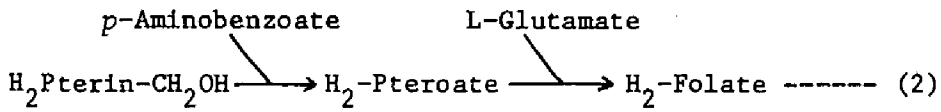
The formula for dihydrofolic acid is presented below.



This substance contains three components : dihydropteridine residue, *p*-aminobenzoic, and glutamic acids. As shown above, other related compounds are dihydropteroic and *p*-aminobenzoyl-glutamic acids.

Since the enzyme systems in several bacteria[6-10] and in plants[11-14] utilize either PABA or PABG for the formation of either dihydropteroic acid or dihydrofolic acid, respectively, the two routs for biosynthesis of dihydrofolic acid are postulated as following equation (1) and (2).





Thus the question that arises is whether PABG or PABA is the normal substrate for the biosynthesis of dihydrofolic acid.

Katsunuma *et al.*[15,16] firstly propsed that folic acid was formed *via* PABG as an intermediate using extracts of *Mycobacterium avium*. However, the postulated intermediates have not been isolated nor shown to be capable of participation in the biosynthesis of PABG under the experimental conditions employed[6,11,17] ; nor has further work with a purified enzyme system been reported.

On the other hand, it has been postulated by Brown *et al.*[6,8] and Iwai *et al.*[11] that *in vivo* biosynthesis of folic acid proceeds exclusively by way of dihydropteroate, which is formed by condensation of PABA with H_2-pterin-CH_2OH and which then reacts with L-glutamate to form H_2-folate as equation (2). This has been verified after partial purification of the dihydrofolate-synthesizing enzyme from *Escherichia coli* by Griffin and Brown[18], but the thorough study of this enzyme has been hampered by its unstability and by the assay procedure which depends upon the microbiological assay for H_2-folate.

Streptococcus faecalis R essentially requires pteroic acid, or the tetrahydro-form of folic acid as a nutrient on growth, *Lactobacillus casei* requires folic acid or the tetrahydro-form of

folic acid, and *Pediococcus cerevisiae* requres the tetrahydro-form of folic acid.[19,20] Moreover, animals such as rat and chicken[21] require folic acid or the tetrahydro-form of folic acid for growth. These are generally accepted to be dependent on the differences of the enzyme systems of folate biosynthesis.

In the present studies, from the point of view observed in the previous studies as described above, it was attempted to clarify the biosynthetic pathway of folate compounds and to estimate the reaction mechanism of dihydrofolate synthetase (EC 6.3.2.12). It was also attempted to investigate the relationship between the nutritional requirements for folate compounds *in vivo* and the enzyme systems which biosynthesize folate compounds *in vitro*. The details of these experimental results are described in the following chapters.

CHAPTER II

DISTRIBUTION AND INTRACELLULAR LOCALIZATION OF DIHYDROFOLATE SYNTHETASE IN PLANTS

1. Introduction

To study the biosyntheses and functions of living materials, it is important to investigate the localization of enzymes on biosynthetic pathways. There is little information about the intracellular distribution of folate-linked enzyme.

Bocchieri and Koft[22] reported that the coupling activity which catalyzes the condensation of the pteridine precursor and *p*-aminobenzoic acid is bound to the cell membrane fraction of *Staphylococcus epidermidis*. Okinaka and Iwai[23] demonstrated that the dihydropteroate-synthesizing enzyme is localized in the mitochondrial fraction of pea seedlings and spinach leaves. Furthermore, folate reductase and dihydrofolate reductase have been found in mitochondria from rat livers by Noronha and Sreenivasan.[24] It was demonstrated that serine hydroxymethyl transferase and 10-formyl tetrahydrofolate synthetase are localized in mitochondria by Clandinin and Cossins.[25] These results suggest the possibility that the enzymes of folate compound biosynthesis are contained in cell particles or in the cell membrane in plants and animals.

However, there is no information about the intra-

cellular localization of the dihydrofolate synthetase which catalyzes the condensation of dihydropteroic and L-glutamic acids.

Therefore, the distribution and the intracellular localization of the dihydrofolate synthetase in higher plants were examined. The results will be described in this chapter.

2. Materials and Methods

Chemicals.

ATP was purchased from the Sigma Chemical Company. Pteroic acid was a gift from Dr. G. Toennies and Dr. John A. King. Dihydropteroic acid was prepared by the methods of Futterman.[26] Other chemicals, of the highest quality commercially available, were, purchased from Nakarai Chemicals Ltd., Kyoto.

Plant materials.

Spinach was purchased commercially. Air-dried, healthy looking pea seeds (*Pisum sativum* L. var. Alaska) were soaked in deionized water at 25°C for 18 hours, then were allowed to germinate at 25°C in moist vermiculite. The values at zero day indicate enzyme activity just after soaking the seeds in water at 25°C for 18 hours.

Preparation of the particulate fraction.

All operations were carried out at 2-4°C. The medium used to isolate the cell particles contained; 0.5 M sucrose, 0.05 M Tris-HCl buffer at pH 7.5 and 50 mM 2-mercaptoethanol. Each

fraction was prepared in an ice bath using the modified method of Fujiwara *et al.*[27] Fifty grams of pea seedlings (6 days old) or spinach leaves were homogenized with 100 ml of isolation medium in a mortar. The homogenates were squeezed through four layers of gauze, then centrifuged succesively : at 100 x g for 5 min (debris and nuclei) ; at 1000 x g for 12 min (chloroplasts) ; at 20,000 x g for 30 min (broken chloroplasts and mitochondria) ; and at 105,000 x g for 90 min (microsomes). The final supernatant was used as the soluble fraction. Each precipitate was washed with the isolation medium, then suspended in 0.01 M Tris-HCl buffer at pH 7.5 containing 50 mM 2-mercaptoethanol. The enzyme activity in these suspensions and in the soluble fraction was assayed by a microbiological procedure.

Standard assay conditions.

The reaction mixtures contained Tris-HCl buffer (100 μmoles, pH 8.8) ; magnesium sulfate (5.0 μmoles) ; potassium sulfate (50 μmoles) ; L-glutamic acid (5.0 μmoles) ; 2-mercaptoethanol (50 μmoles) ; ATP (5.0 μmoles ; enzyme (0.05 ml) and dihydropteroic acid (0.05 μmole) in a final volume of 1.0 ml.

The reaction was carried out at 37°C for 30 min, then it was stopped by heating the whole in a boiling water bath for one min. After diluting the reaction mixture with cold water, the amounts of dihydrofolate formed were determined by a microbiological assay with *Lactobacillus casei* ATCC 7469 in 10 ml of an assay medium for folic acid[28]; these were expressed as folate

equivalents. One unit of dihydrofolate synthetase catalyzes the formation of 0.1 nmole of folate equivalent per 30 min under standard assay conditions. The specific activity of dihydrofolate synthetase is expressed in terms of units per mg of protein.

Determination of protein.

The amount of protein was determined by the method of Lowry *et al.*[29] using crystalline bovine serum albumin as the standard.

3. Results

Distribution of the dihydrofolate synthetase in plants.

The dihydrofolate synthetase activity in extracts from various plants and tissues was measured by a microbiological assay method. The results are shown in Table 1. Data in this table are expressed as values for one gram of fresh wight. The enzyme was widely distributed in higher plants. High enzyme activity was also found in green leaves.

Biosynthesis of folate compounds in pea seedlings.

The concentrations of folate compounds in pea seedlings during germination were determined by a microbiological assay by using *L. casei*, as shown in Figure 1. The amount of folate compounds rapidly increased during germination. An especially high increase was observed 2 days after sowing during germination. The amount of folate compounds were, thus, found to increase with the growth of the pea seedling, and for 2 days after sowing the

formation was markedly accelerated.

Table 1

Distribution of the Dihydrofolate Synthetase in Plants

Plant	Tissue	Protein (mg)	Enzyme activity (units/g of fresh weight)	Specific activity (units/mg of protein)
Lettuce	leaves	7.85	1.14	0.145
New York lettuce	leaves	2.80	0.35	0.125
Chinese cabbage	leaves	2.10	0.66	0.314
Chinese cabbage sp.	leaves	4.90	1.07	0.218
Cabbage	leaves	3.50	0.70	0.200
Swiss chard	leaves	9.28	1.71	0.184
Pea seedlings (1 day-old)	seedlings	52.47	2.89	0.055
Pea seedlings (11 day-old)	cotyledon	34.78	6.60	0.190
	shoot	4.25	1.83	0.430
	root	3.91	0.17	0.043
Etiolate seedlings of Black Mappe beans	seedlings	2.89	1.06	0.367
Spinach	leaves	7.73	3.80	0.491

1 unit = 0.1 nmole of folate equivalent formed per 30 min under the standard assay conditions.

Changes in the dihydrofolate synthetase activity of germinating seeds.

The large increase observed for the folate compound contents of pea seedlings suggests that the net biosynthesis of these compounds occurs during germination. Consequently an increase in the dihydrofolate synthetase on the biosynthetic pathway of folate compounds is to be expected ; so, changes in enzyme activity were determined in pea seeds during germination, as shown in Figure 2. The values at zero day indicate the enzyme activity just after soaking the seed in water for 18 hour at 25°C.

Clearly the increase in folate compound contents was in proportion to the increase in enzyme activity. In the light, enzyme activity increased with the growth of pea seedlings. The maximum activity was obtained 8 days after sowing. In the dark, the enzyme activity was at its maximum at 6 days. The increased activity decreased rapidly from 8 days after sowing.

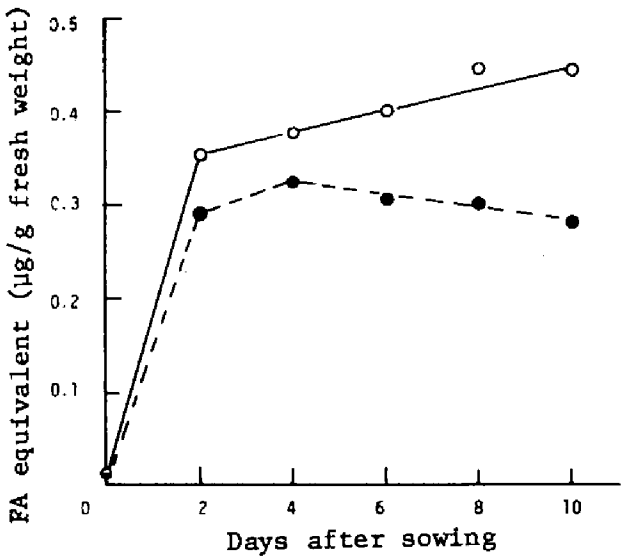


Fig. 1. Biosynthesis of Folate Compounds in Pea Seedlings during Germination

Intracellular localization of dihydrofolate synthetase.

Localization of the enzyme in various intracellular fractions from pea seedlings and spinach leaves was investigated. Results are shown in Tables 2 and 3. In both pea seedlings and spinach leaves, a high specific activity was obtained in the mitochondrial

fraction. Therefore, it seems that the enzyme is localized in mitochondria. The enzyme also existed partly in the choroplast and soluble fractions. These localizations were obscure because of contamination from the other fractions.

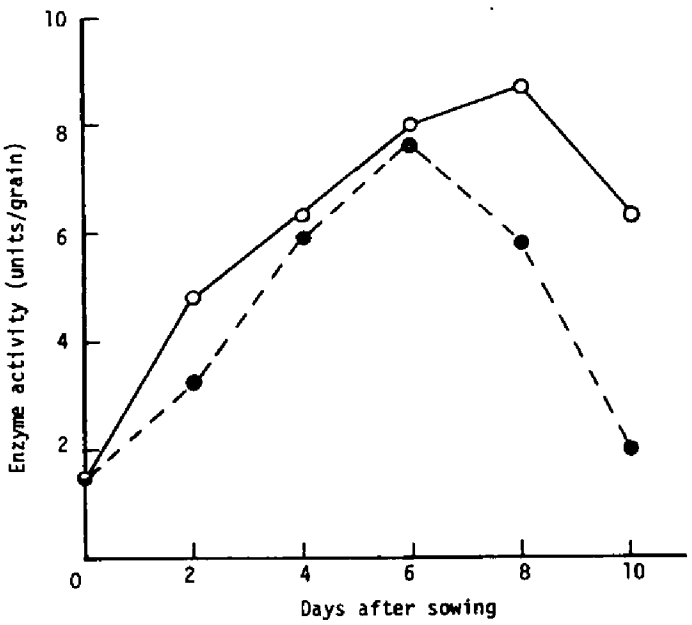


Fig. 2. Changes in Dihydrofolate Synthetase Activity in Germinating Pea Seeds

Stability of the dihydrofolate synthetase in various storage states.

A previous report[60] showed that whereas the dihydrofolate synthetase activity in cell free extracts from pea seedlings was very unstable, even when the extracts were stored at 0°C, the

Table 2

Intracellular Localization of the Dihydrofolate Synthetase in Pea Seedlings

Fraction	Protein	Enz. act.	Specific act.	Ratio
	mg	units*	units*/mg protein	%
Homogenate	2679	253	0.094	100
Debris & Nuclear	827	31	0.037	12.3
Chloroplast	369	39	0.106	15.4
Mitochondrial	171	102	0.597	40.3
Microsomal	38	0	0	0
Soluble	1190	57	0.048	22.5

* 1 unit = 0.1 nmole of FA equivalent formed per 30 minutes under standard assay conditions.

Table 3

Intracellular Localization of the Dihydrofolate Synthetase in Spinach Leaves

Fraction	Protein	Enz. act.	Specific act.	Ratio
	mg	units*	Units*/mg protein	%
Homogenate	908.0	150.0	0.165	100
Debris & Nuclear	66.6	3.7	0.056	2.3
Chloroplast	150.1	15.0	0.100	10.0
Mitochondrial	69.2	58.5	0.845	39.0
Soluble	592.4	59.8	0.101	39.9

* 1 unit = 0.1 nmole of FA equivalent formed per 30 minutes under standard assay conditions.

enzyme was partially stabilized in the presence of ammonium sulfate and 2-mercaptoethanol. As shown in Table 4 the enzyme activity of extracts from pea seedlings showed a 73% decrease after storage of the enzyme for 24 hours. However, the enzyme of isolated mitochondria from pea seedlings was much more stable than that of extracts from pea seedlings when intact isolated mitochondria were stored at 0°C. Furthermore the enzyme of extracts from isolated mitochondria was relatively stable, so the loss of enzyme activity was 20% after the enzyme had been stored at 0°C in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.05 M 2-mercaptoethanol and 0.2 M ammonium sulfate.

Table 4

Decrease % of Dihydrofolate Synthetase Activity in Various Storage States

Storage states	Standing time at 0°C	
	24 hr.	48 hr.
Isolated mitochondria (a)	3 %	5 %
Extracts from isolated mitochondria of pea seedlings (b)	20	27
Extracts from pea seedlings (b)	73	80

(a) Isolated mitochondria were stored as precipitates.

(b) Extracts were stored in the soluble state containing 0.05 M Tris-HCl buffer (pH 7.5), 0.05 M 2-mercaptoethanol and 0.2 M ammonium sulfate.

4. Discussion

Iwai *et al.*,[23, 30] Roos and Cossins[31] reported that folate compounds incresed during the germinating process. Banerjee *et al.*,[32] however, reported the folic acid and the citrovorum factor in some pulses diminished during the germination process. Braganca *et al.*[33] also reported a loss of folic acid during germination, which was due to the appearance of an enzyme which splits folic acid at the C-9-N-10 linkage. Okinaka and Iwai[23] reported that dihydropteroate synthase, which is a key enzyme in folate compound biosynthesis, increased with germination. The present data also support an increase in folate compounds during germination and indicate that dihydrofolate synthetase increased with germination. This suggests that folate compounds were synthesized with the increase in enzyme activity present on the biosynthetic pathway of folate compounds during germination.

Wang *et al.*[34] have shown that 10-formyl tetrahydrofolate synthetase is localized in mitochondria. Folate and dihydrofolate reductases are also contained in the mitochondria from rat livers.[24] Okinaka and Iwai[23] have demonstrated that a dihydropteroate synthase is localized in the mitochondria of plants. The present data demonstrates that a key enzyme of folate synthesis, dihydrofolate synthetase, is also localized in the mitochondria of plants, which suggests that most pteroylglutamate precursors,

including dihydropteroylglutamate, are synthesized in the mitochondria. The further reduction and addition of C_1 units is also thought to partly occur in the mitochondria.

The dihydrofolate synthetase was easily solubilized by more than 95% using osmotic shock when isolated mitochondria were suspended in 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol.

CHAPTER III

PURIFICATION AND PROPERTIES OF THE DIHYDROFOLATE SYNTHETASE FROM PEA SEEDLINGS

1. Introduction

Previous investigation[11,60] have shown that the probable intermediate in the biosynthesis of folate compounds in plants is dihydropteroic acid, which combines enzymatically with L-glutamic acid to form dihydrofolic acid and that the enzyme (dihydrofolate synthetase) which catalyzes the condensation of dihydropteroic and L-glutamic acids was partially purified from pea seedlings.[60]

Griffin and Brown[18] have reported the occurence and partial purification of the enzyme from *Escherichia coli*. The high purification of the enzyme from microorganisms and higher plants was difficult, since the enzyme was fairly labile. The distribution, intracellular localization and stability of the dihydrofolate synthetase in plants have been described in chapter II.

Thus in this chapter an attempt to extract and highly purify from the cell particles was carried out and the properties of the enzyme were investigated. The results will be described in this chapter.

2. Materials and Methods

Chemicals.

ATP, GTP, ITP, CTP, UTP, ADP, and AMP were purchased from the Sigma Chemical Company. Albumin from bovine serum, ovalbumin, chymotrypsinogen A from beef pancrease, and myoglobin from the sperm whale were from Schwarz/Mann. DEAE-cellulose was from the Brown Company, and Sephadex G-100 and Sephadex G-200 were from Pharmacia Fine Chemicals. Pteroic acid was the gift of Dr. G. Toennies and Dr. John A. King. Pteroic acid was reduced to the dihydro form with sodium dithionite by the method of Futterman,[26] and to the tetrahydro form by hydrogenation in glacial acetic acid according to the directions of O'Dell *et al.*[35]

Standard assay conditions

The reaction mixtures contained Tris-HCl buffer (100 μmoles, pH 8.8) ; magnesium sulfate (5.0 μmoles) ; L-glutamic acid (5.0 μmoles) ; 2-mercaptoethanol (50 μmoles) ; ATP (1.0 μmole) ; enzyme (1.2 μg) and dihydropteroic acid (0.05 μmole) in a final volume of 1.0 ml. The reaction was carried out at 37°C for 30 min, then was stopped by heating the whole in a boiling water bath for one min. After diluting the reaction mixture with cold water, the amount of dihydrofolate formed was determined by microbiological assay with *Lactobacillus casei* ATCC 7469 in 10 ml of an assay medium for folic acid.[28] Amounts are expressed as folate equivalents. One unit of dihydrofolate synthetase

catalyzes the formation of 0.1 mμmole of folate equivalent per 30 min under standard assay conditions. The specific activity of dihydrofolate synthetase is expressed in terms of units per mg protein.

Determination of protein.

The amount of protein was determined by the method of Lowry *et al.*[29] using crystalline bovine serum albumin as the standard.

Germination of pea seeds.

Air-dried, healthy pea seeds (*Pisum sativum* L. var. Alaska) were soaked in distilled water at 25°C for 18 hours, then were allowed to germinate at 20°C for 6 days on moist absorbent cotton in a vat covered with wet filter paper.

3. Results and Discussion

Purification of the dihydrofolate synthetase from pea seedlings.

Step I. Purification of the enzyme directly extracted from pea seedlings was difficult because of the enzyme labile as previously reported.[60] However, the enzyme extracted from particles was fairly stable, so the author used this extraction of the enzyme. The medium used to isolate the cell particles contained 0.5 M sucrose, 0.05 M Tris-HCl buffer at pH 7.5 and 50 mM 2-mercaptoethanol. Six days old seedlings (100 Kg) were homogenized with 100 liters of the isolation medium. This and all subse-

quent steps were performed at 0-5°C, except when otherwise noted. The homogenate was squeezed through cotton cloth by basket centrifugation.

Step II. After the juice (130 liters) which had stood for more than 2 hours to remove debris was decanted, the green supernatant solution was centrifuged at 20,000 x g for 30 min. The precipitate was used as the particle fraction. The precipitate was suspended in 10 liters of 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol for 20 min with mechanical stirring, then it was centrifuged at 20,000 x g for 30 min. The supernatant solution was used as the extract of the mitochondrial fraction.

Step III. Solid ammonium sulfate was added to the above extracts to give 0.10 saturation. The mixture was equilibrated with stirring for 30 min, then it was centrifuged at 20,000 x g for 30 min. Solid ammonium sulfate was then added to the supernatant solution to give 0.35 saturation. The mixture was equilibrated with stirring for 30 min, then it was centrifuged at 20,000 x g for 30 min.

Step IV. The resultant precipitate was dissolved in 2.4 liters of 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. Then the solution was divided into six parts of 400 ml each for convenient fractionation on DEAE-cellulose. Each 400 ml portion was dialyzed against 0.01 M Tris-HCl buffer

at pH 7.5 containing 0.05 M 2-mercaptoethanol. The resulting precipitate was centrifuged and discarded. Each dialysate was placed on a DEAE-cellulose column (6 x 70 cm) previously equilibrated with 0.01 M ammonium sulfate in 0.01 M Tris-HCl buffer and 0.05 M 2-mercaptoethanol. The column was washed with 2 liters of the same buffer and developed by linear gradient elution. The mixing chamber contained 2 liters of 0.01 M ammonium sulfate in 0.01 M Tris-HCl buffer at pH 7.5 and 0.05 M 2-mercaptoethanol, and the reservoir contained 2 liters of 0.2 M ammonium sulfate in the same buffer solution. The enzymatically active eluates from the DEAE-cellulose column were combined, then the precipitate with ammonium sulfate (0.60 saturation) was recovered and stored at 0°C. The remainder of the dialyzed enzyme solution was treated as above.

Step V. After the precipitate had been dissolved in 100 ml of 0.01 M Tris-HCl buffer containing 0.05 M 2-mercaptoethanol, the solution was divided into two parts of 50 ml each for convenient fractionation on Sephadex. Each 50 ml portion was applied to a Sephadex G-200 column (6 x 90 cm) equilibrated with 0.8 M ammonium sulfate in 0.1 M Tris-HCl buffer and 0.05 M 2-mercaptoethanol. The same buffer solution was allowed to flow through the column. The enzymatically active eluates were combined and their 0.60 saturation precipitate with solid ammonium sulfate was dissolved in 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M

2-mercaptoethanol.

Step VI. The solution was rechromatographed on a Sephadex G-200 column (6 x 90 cm) using the same conditions as in step V. The enzymatically active eluates were combined and their 0.60 saturation precipitate with solid ammonium sulfate was collected and dialyzed against 0.01 M potassium phosphate buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol.

Step VII. The dialyzed enzyme solution was applied to a hydroxylapatite column (4 x 15 cm) equilibrated with 0.01 M potassium phosphate buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. Elution was carried out with 0.01 and 0.05 M potassium phosphate buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. Results are shown in Figure 3. Enzyme fractions in tube number 80 to 96 were pooled, and the precipitate with ammonium sulfate (0.60 saturation) was recovered and dissolved in 3 ml of 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol.

Step VIII. The solution was chromatographed on a Sephadex G-200 column (2.5 x 90 cm) under the conditions used for step V on the the Sephadex G-200 column. The elution pattern is shown in Figure 4. The enzymatically active eluates were combined and their 0.60 saturation precipitate with ammonium sulfate was dissolved in 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. Solid ammonium sulfate was added to the solution (0.60 saturation) and the whole was stored as the puri-

fied enzyme precipitation at 0°C. Purification steps and the yields of dihydrofolate synthetase from pea seedlings are summarized in Table 5.

Table 5

Summary of the Purification of the Dihydrofolate Synthetase from Pea Seedlings

Fraction	Total protein	Total activity	Specific activity	Purification	Yield
	mg	units*	units*/mg	ratio	%
I. Juice	4,128,000	477,300	0.116	1.0	100
II. Mitoch. fr. Extracts	698,000	321,556	0.460	4.0	67.3
III. Am_2SO_4 ppt.	129,000	162,540	1.26	10.9	34.1
IV. DEAE-cellulose column chromatography	4,930	32,045	6.50	56.1	6.7
V. 1st Chromatography on Sephadex G-200	727	9,742	13.4	115.5	2.0
VI. 2nd Chromatography on Sephadex G-200	100	8,560	85.6	738.0	1.8
VII. Hydroxyapatite column chromatography	45	8,190	182	1569.0	1.7
VIII. 3rd Chromatography on Sephadex G-200	30	6,840	228	1965.6	1.4

* 1 unit = 0.1 mμmole of FA equivalent formed per 30 min under standard assay conditions.

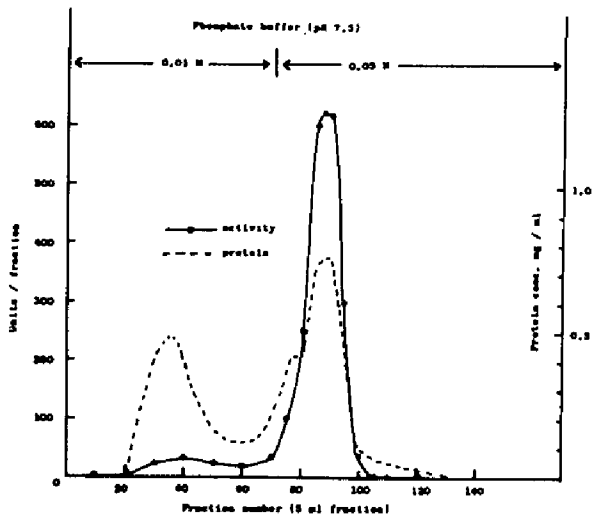


Fig. 3. Elution Pattern of the Dihydrofolate Synthetase from a Hydroxylapatite Column

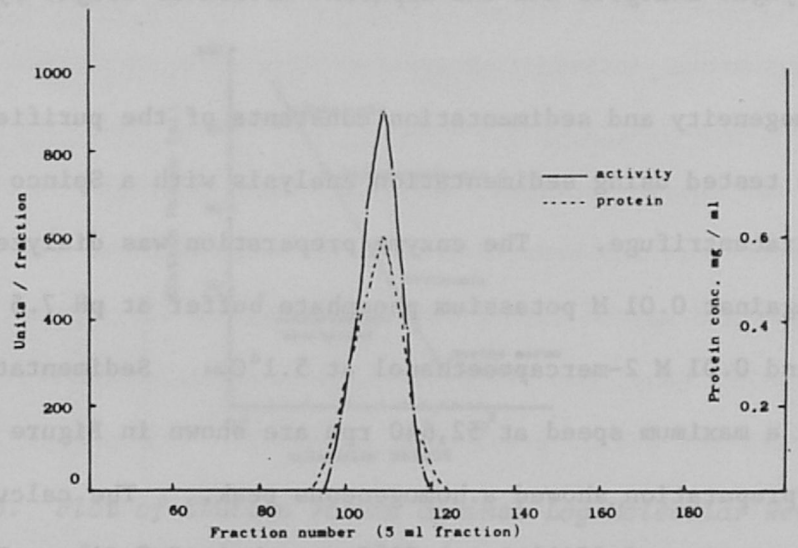


Fig. 4. Third Chromatography of Dihydrofolate Synthetase on Sephadex G-200

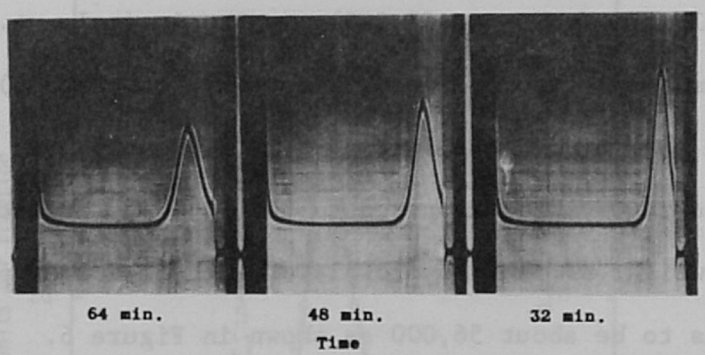


Fig. 5. Sedimentation Patterns of the Dihydrofolate Synthetase from Pea Seedlings

The sample contained 1.2% enzyme, o.1 M KCl, 0.01 M 2-mercaptoethanol, and 0.01 M potassium phosphate buffer at pH 7.5.

The determination was carried out at 5.1°C and 52,640 rpm.

Ultracentrifugal analysis and the apparent molecular weight of the enzyme.

The homogeneity and sedimentation constants of the purified enzyme were tested using sedimentation analysis with a Spinco Model E ultracentrifuge. The enzyme preparation was dialyzed overnight against 0.01 M potassium phosphate buffer at pH 7.5 in 0.1 M KCl and 0.01 M 2-mercaptoethanol at 5.1°C. Sedimentation patterns at a maximum speed at 52,640 rpm are shown in Figure 5. The enzyme preparation showed a homogeneous peak. The calculated sedimentation coefficient at 20°C was about 3.9 S*. The apparent molecular weight of the enzyme was determined according to the method of Andrews[36] using Sephadex G-100 (1.5 x 70 cm). Bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000), chymotrypsinogen A from beef pancrease (mol. wt. 25,000), and myoglobin from the sperm whale (mol. wt. 17,800) were used as the reference standard. When compared to the reference standards, the molecular weight of the dihydrofolate synthetase from pea seedlings seems to be about 56,000 as shown in Figure 6. After the enzyme preparation had been stored at 0°C for 3 months in 0.01 M Tris-HCl buffer containing 2 M ammonium sulfate and 0.05 M 2-mercaptoethanol, it was applied to Sephadex G-100 column.

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* In a previous report,[b] the sedimentation coefficient was reprorted to be 7.5 S. Further repeated experiments, however, indicated that it is 3.9 S.

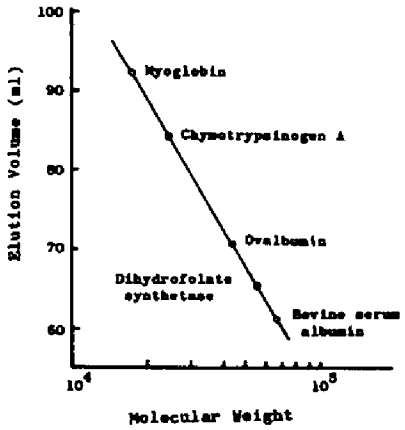

Fig. 6. Plot of Elution Volume against Log Molecular Weight for Proteins on a Sephadex G-100 column

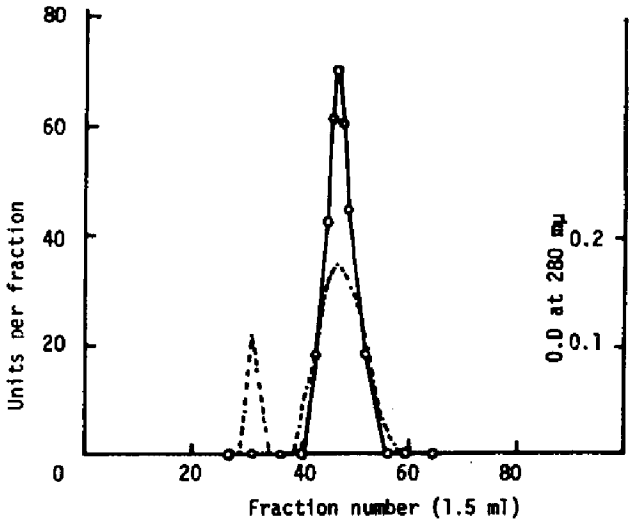

Fig. 7. Chromatography of Purified Dihydrofolate Synthetase on Sephadex G-100

----- O.D. at 280 mμ o——o activity

The elution pattern is shown in Figure 7. The new protein peak which had no enzyme activity appeared in fractions 29 to 34. The apparent molecular weight was about 120,000. The main peak was active for enzyme activity, and its molecular weight 56,000. This indicates that the nonactive peak may be a dimer of the active enzyme and may be formed during storage of the enzyme.

On the other hand, the value of the sedimentation coefficient (7.5 S), which the author previously reported[61], was thought to be the value of the nonactive dimer.

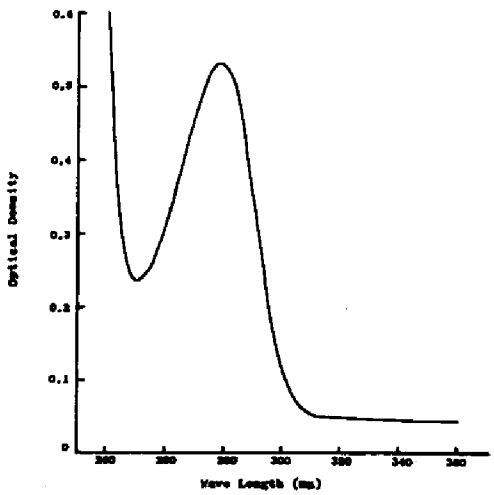

Fig. 8. Absorption Spectrum of the Dihydrofolate Synthetase Purified from Pea Seedlings

Protein concentration was 0.485 mg per ml.

Ultraviolet absorption spectrum of the enzyme.

The ultraviolet absorption spectrum of the enzyme in 0.01 M Tris-HCl buffer at pH 7.5 containing 0.005 M 2-mercaptoethanol

is presented in Figure 8. The enzyme shows a single absorption peak with a maximum at 278 mμ and a minimum at 250 mμ. The extinction coefficient ($E^{1\%}_{1\ cm}$) value of the enzyme at 280 mμ was 10.8.

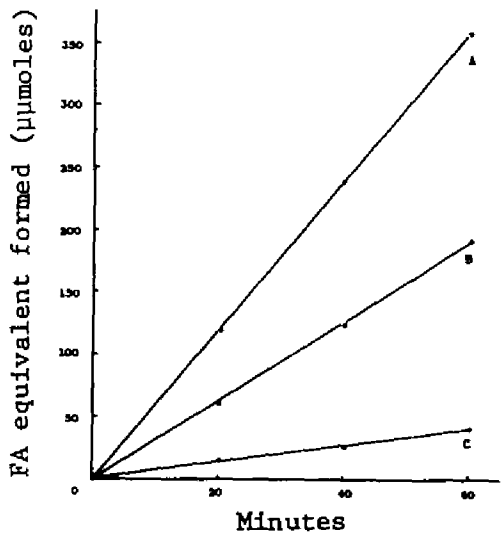

Fig. 9. Relationship between the Time Course of the Reaction and Enzyme concentration

The standard assay procedure was used, except where altered reaction time and amounts of enzyme are indicated.

A 2.4 μg. B 1.2 μg. C 0.24 μg.

Time course of the reaction.

The time course of the reaction was tested with various concentrations of the enzyme. The reaction proceeded linearly and the amounts of FA equivalent formed were proportional to the amounts of enzyme preparation used, as shown in Figure 9.

Effect of pH on enzyme activity.

The effect of pH on enzyme activity is illustrated in Figure 10. Tris-HCl and glycine-NaOH buffers were used at final concentrations of 0.1 M. Maximum activity was obtained at pH 8.8. This value is in agreement with the optimum pH obtained in a previous experiment using a crude enzyme preparation.[60)]

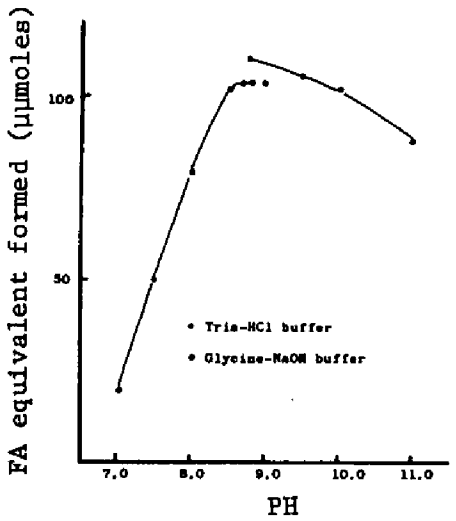

Fig. 10. Effect of PH on Enzyme Activity

Assay conditions were the same as those described in "Materials and Methods", except that various buffers at the indicated pH values were used at final concentration of 0.1 M.

Component study of the reaction.

As shown in Table 6, the reaction was dependent on dihydropteoate, L-glutamate, ATP, Mg^{2+}, and the enzyme. Omission of K^{+} or

2-mercaptoethanol from the complete system resulted in a decrease in the reaction velocity. This enzyme was specific for dihydropteroic acid as the substrate. Pteroic and tetrahydropteroic acids were not used as substrates. ATP was not replaceable with any othernucleotides, as shown in Table 7.

Table 6

Component Study for Dihydrofolate Synthetase (I)

Omission	FA equivalent formed
	μμ moles
None	108
Dihydropteroate	0
L-Glutamate	0
ATP	0
Mg^{++}	0
K^{+}	23
2-Mercaptoethanol	74
Enzyme	0

The standard assay method was used except for the omission of the indicated substances. The enzyme already contained 5 μmoles of ammonium sulfate.

Table 7

Component Study for Dihydrofolate Synthetase (II)

Omission	Addition	FA equivalent formed
None	None	119 μμ moles
Dihydropteroate	None	0
Dihydropteroate	Pteroate	0
Dihydropteroate	*Tetrahydropteroate	0
ATP	None	0
ATP	ADP	0
ATP	AMP	0
ATP	GTP	0
ATP	ITP	0
ATP	CTP	0
ATP	UTP	0

* Incubation was performed at 37°C for 30 minutes in an argon atmosphere.

Griffin and Brown[18] reported that in the partially purified enzyme from *E. coli*, ITP, GTP, and ADP could be utilized 60, 35, and 10%, respectively, as effectively as ATP. Km values for dihydropteroate, L-glutamate, and ATP were calculated as 1.0×10^{-6}, 1.5×10^{-3} and 1.0×10^{-4} M, respectively. These Km values are summarized in Table 8.

Table 8

Michaelis-Menten Constant (Km Value) for the Binding of the Substrate

Substrate	Km
Dihydropteroate	1.0×10^{-6} M
L-Glutamate	1.5×10^{-3} M
ATP	1.0×10^{-4} M
$MgSO_4$	1.1×10^{-3} M
$MnSO_4$	6.3×10^{-5} M

Divalent cation requirements.

The effector of various divalent cations on the enzyme activity was investigated using their sulfate forms. Results are shown in Table 9. Mn^{2+} was more effective than Mg^{2+} in promoting enzyme activity, and this effect could be replaced by Fe^{2+} to a lesser extent. Mn^{2+} was, however, only 10 to 15% as effective as Mg^{2+} in the partially purified enzyme from *E. coli*. Km values for Mg^{2+} and Mn^{2+} were calculated as 1.1×10^{-3} and 6.3×10^{-5} M, respectively, in the highly purified enzyme from pea seedlings. The Km value of the highly purified enzyme from pea seedlings had about a 5 fold decrease in comparison with that from the partially purified enzyme from pea seedlings for Mn^{2+}. This presents the possibility that the enzyme activity might require Mn^{2+} rather than Mg^{2+} as the divalent cation *in vivo*.

Table 9

Divalent Cation Requirements for Dihydrofolate Synthetase

Divalent cations	Conc.	FA equivalent formed
	mM	µµ moles
None	0	0
Mg^{2+}	1.0	53
	5.0*	98
Mn^{2+}	0.5*	124
	1.0	77
Fe^{2+}	1.0	16
Co^{2+}	1.0	6
Zn^{2+}	1.0	4
Cu^{2+}	1.0	0
Ni^{2+}	1.0	0
Ca^{2+}	1.0	0

Each metal was used in the form of its sulfate.

* Optimal conc.

Univalent cation requirement.

The enzyme, dialyzed for 18 hours against 0.01 M Tris-HCl buffer at pH 7.5 containing 0.01 M 2-mercaptoethanol in the cold, was used for the following experiments.

The effect of various univalent cations in their chloride forms on the enzyme activity was investigated at a final concentration of 100 mM. Results are shown in Table 10. An absolute

univalent cation requirement for enzyme activity was observed. K^+ was the most effective, and was replaceable by NH_4^+, and Rb^+. These results agree with those obtained in a previous experiments using the partially purified enzyme preparation[60].

Table 10

Univalent Cation Requirement for Dihydrofolate Synthetase

Addition	FA equivalent formed
	μμ moles
None	0
K^+	108
NH_4^+	95
Rb^+	89
Na^+	8
Cs^+	5
Li^+	3

The standard assay was used except that the enzyme was dialyzed against 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M of 2-mercaptoethanol.

100 μmoles of each metal was used in the form of the chloride.

Inhibition of the enzymatic reaction by ADP.

The enzymatic reaction was inhibited by the addition of ADP, but not AMP, as shown in Table 11. Furthermore, another experiment detected ADP formed enzymatically from ATP using chromatographic procedures. This indicates that the product from ATP in the reaction was composed of ADP and Pi, as shown in following equation :

$$H_2Pte + L\text{-}Glu + ATP \longrightarrow H_2FA + ADP + Pi$$

The systematic name for dihydrofolate synthetase should be 7,8-dihydropteroate : L-glutamate ligase (ADP).

Table 11

Effect of ADP and AMP on the Enzymatic Formation of Dihydrofolate

Addition	Conc. mM	FA equivalent μμ moles
None		118
ADP	2.5	88
	5.0	64
AMP	2.5	112
	5.0	110

The standard assay method was used except for the addition of the indicated substances.

CHAPTER IV

PURIFICATION AND PROPERTIES OF THE DIHYDROFOLATE SYNTHETASE FROM *Serratia indica*

1. Introduction

Since an enzyme which catalyzes the formation of dihydrofolate from L-glutamate and dihydropteroate was found in extracts of *E. coli*,[6] the significance of dihydrofolate synthetase to the pathway which passes dihydropteroic acid as an intermediate in the biosynthesis of folic acid compounds has been confirmed[6,11,18,60]

Dihydrofolate synthetase was partially purified from *E. coli*[18] and was highly purified from pea seedlings.

In previous chapter, the author described studies on the purification of and the properties of the dihydrofolate synthetase from pea seedling. However the enzyme could not be stored for long time, so the author could not study the reaction mechanism.

In this chapter, the author found that the enzyme from *S. indica* was more stable than the one from pea seedlings and a good yield was observed in contrast to the enzyme from pea seedlings : thus, the author tried to purify the dihydrofolate synthetase from *S. indica*. The purification of and some properties of the dihydrofolate synthetase from *S. indica* are described in this chapter.

2. Materials and Methods

Chemicals.

ATP, GTP, ITP, CTP, UTP, ADP and AMP were purchased from the Sigma Chemical Company. L-Glutamic acid, folic acid, 2-mercaptoethanol, ascorbic acid and dihydrostreptmycin were from commercial sources. DEAE-cellulose was obtained from the Brown Company. Sephadex G-100, Sephadex G-200 and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals. L-γ-Glutamyl-L-glutamic acid was the gift of Dr. T. Hata. Pteroic acid was kindly provided by Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company.

Reduction of pteridine compounds.

Pteroic acid was reduced to the dihydro form by treatment with sodium dithionite, as described by Futterman[26], and to the tetrahydro form by hydrogenation in glacial acetic acid according to O'Dell *et al.*[35] Folic acid was reduced to the dihydro form with sodium dithionite by the method of Futterman.[26]

Determination of protein.

The amount of protein was determined by the method of Lowry *et al.*[29] using crystalline bovine serum albumin as the standard.

Standard assay conditions of the enzyme reaction.

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 ; 50 μmoles of 2-mer-

captoethanol ; 5.0 μmoles of ATP ; 1.5 μg of enzyme and 0.05 μmole of dihydropteroic acid in a final volume of 1.0 ml. The reaction was carried out 37°C for 30 min, and was stopped by heating the whole in a boiling water bath for 1 min.

Microbiological assay.

When standard assay conditions were used, the dihydrofolate formed during the reaction was determined by a microbiological assay procedure with *L. casei* ATCC 7469.

The reaction mixture were diluted to the proper concentrations for assay with cold water. An aliquot of the diluted sample was added to the assay tube containing the assay medium for folic acid in a final volume of 10 ml. In this assay, a uniform medium available for the microbiological assay of several kinds of B vitamins,[28] which had been certified in our laboratory, was used. After an 18 hour-incubation at 37°C, the growth of the microorganism was measured turbidimetrically with a Coleman Universal Spectrophotometer (Model 14) at 675 mμ. For convenience, folic acid was used as the standard. The amounts of folate compounds produced enzymatically were expressed as "folate equivalents".

When the enzyme reaction was performed under an atmosphere of argon to determine the stoichiometry of the enzymatic reaction, the dihydrofolate formed by the enzymatic reaction was determined by an aseptic microbiological assay procedure[6] with *L. casei*. The reaction mixtures were diluted to the proper concentration

for assay with sterile water containing 6 mg of sodium ascorbate per ml. An aliquot of the diluted sample was added aseptically to the assay tube which contained the previously autoclaved and cooled assay medium for folic acid in a final volume of 10 ml. In this assay, as described above a uniform medium available for microbiological assay of several kinds of B vitamins was used. After an 18 hour-incubation at 37°C the growth of the microorganism was measured turbidimetrically with a Coleman Universal Spectrophotometer at 675 mμ. In this case dihydrofolate was used as the reference substance.

Bioautography.

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 ; 50 μmoles of 2-mercaptoethanol ; 0.1 mg of enzyme and 1.0 μmole of dihydropteroic acid in a final volume of 1.0 ml under an argon atmosphere.

After the reaction was carried out at 37°C for 1 hour, an aliquot of the reaction mixture was spotted on a 22 x 40 cm sheet of Whatman No. 1 filter paper, which had been soaked in a solution containing sodium ascorbate(6 mg per ml, pH 6.8) and dried according to Brown *et al.*[6] to prevent destruction of the reduced forms of folate compounds. The chromatogram was developed in 0.1 M potassium phosphate buffer at pH 6.8, containing sodium ascorbate

(6 mg per ml, pH 6.8) in an argon atmosphere using an ascending technique in the dark. Dihydropteroic, dihydrofolic and folic acids were developed in parallel as the reference substances. After development, the wet chromatogram was placed quickly on the surface of the solidified folic acid assay medium with 1.5 % agar in sterile glass dish (25 x 34.5 cm), which had been seeded with a washed culture of *L. casei*. After 10 min the paper was removed and the tray, covered by a glass plate, was incubated at 37°C for 18 hours.

Determination of inorganic phosphate.

The inorganic phosphate cleaved from ATP-γ-^{32}P was determined by a slight modification of the method of Y. Takahashi.[37] The reaction mixture contained 1.0 μmole of dihydropteroic acid ; 5.0 μmoles of L-glutamic acid ; 2.5 μmoles of ATP (containing 409,310 dpm of ATP-γ-^{32}P) ; 5.0 μmoles of magnesium sulfate ; 50 μmoles of potassium sulfate ; 50 μmoles of 2-mercaptoethanol and 0.1 mg of enzyme in a total volume of 1.0 ml of 0.05 M Tris-HCl buffer (pH 9.0). The reaction mixture was incubated for 1 hour at 37°C under an argon atmosphere. An aliquot of the reaction mixture (0.1 ml) was diluted with 4.9 ml of a sterile cold solution containing sodium ascorbate (6 mg per ml) to determine the dihydrofolate formed. This was dispensed asceptically to assay tubes as decribed above.

One tenth ml of 60% PCA was added to the residual reaction

mixture (0.9 ml) and the whole was centrifuged at 10,000 x g for 10 min. An aliquot of the supernatant (0.75 ml) was added to a solution containing 1.0 ml of 2% sodium molybdate, 1.0 ml of 1.5 N sulfuric acid and 4 ml of isobutanol, and the whole was shaken vigorously for 10 seconds. After standing for 1 min, an aliquot of the upper solution was transfered to a vial containing 10 ml of scintillator. The radioactivity was assayed with a Packard Tri-Carb Liquid Scintillation Spectrometer. The amounts of inorganic phosphate formed in the reaction were determined from the radioactivity values.

Determination of ADP-U-^{14}C

Reaction mixtures, which contained 5.0 µmoles of ATP (containing 521,000 dpm of ATP-U-^{14}C) ; 0.1 µmole of dihydropteroic acid ; 5.0 µmoles of L-glutamic acid ; 50 µmoles of 2-mercaptoethanol ; 50 µmoles of potassium sulfate ; 5.0 µmoles of magnesium sulfate and 0.1 mg of enzyme in a total volume of 1.0 ml of 0.1 M Tris-HCl buffer (pH 9.0), were incubated for 1 hour at 37°C under an argon atmosphere. At the same time, reaction mixtures to which magnesium sulfate had not been added were used as the reference control. Ten µmoles of ADP and 10 µmoles of AMP mixtures (0.1 ml) were added as carriers to an aliquot of the reaction mixtures (0.9 ml) , then the mixtures were placed on a Dowex 1 x 2, formate form, column (1 x 22 cm) equilibrated with 0.2 M ammonium formate. The column was washed with 100 ml of equili-

brating solution, and elution was carried out by linearly increasing the concentration of ammonium formate from 0.2 to 1.5 M. Fractions were collected at a rate of 5 ml per tube per 10 min. The amounts of ADP enzymatically formed were determined from the counts of the ADP fractions. The residual reaction mixtures (0.1 ml) were used to assay the dihydrofolate formed enzymatically as described above.

Microorganism and conditions of culture.

Serratia indica IFO 3759, obtained from the Institute for Fermentation, Osaka, was grown in a medium composed of 4% sucrose ; 1% ammonium sulfate ; 0.2% disodium phosphate ; 0.07% malic acid ; 0.25% disodium carbonate ; 0.04% $MgSO_4.7H_2O$ and 0.001% calcium carbonate. The pH was adjusted to 7.5 with 2 N NaOH.

The cultures were carried out in a 25-liter jar fermentor containing 20 liters of the medium at 30°C for 18 hours under aeration. Cells were harvested by centrifugation, and washed twice with 0.9% NaCl. Washed cells were stored frozen at -30°C until use.

3. Results

Culturing time and enzyme activity.

Changes in dihydrofolate synthetase activity during growth were investigated with *S. indica*. After the maximum specific activity was obtained at 18 hours, the activity decreased with time , as shown in Figure 11.

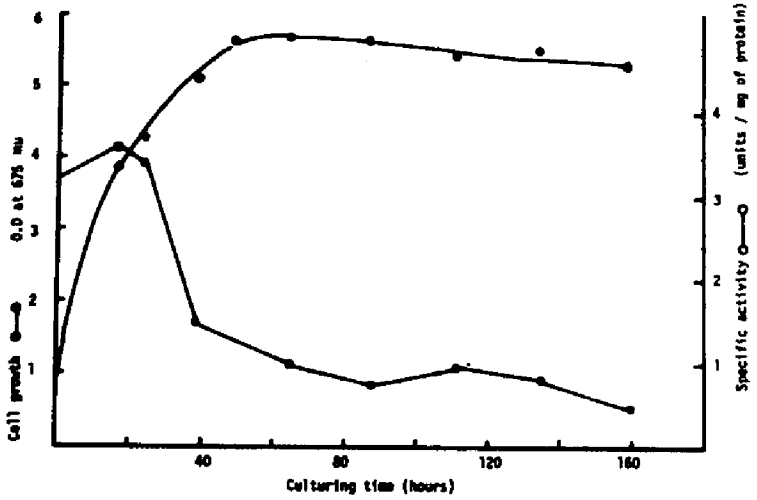

Fig. 11. Change of Dihydrofolate Synthetase Activity during Cell Growth

Stability of the crude dihydrofolate synthetase.

The effect of pH on enzyme stability was determined. The crude enzyme, which was only extracted from washed cells, was

left at 4°C for 42 hours in 0.05 M Tris-HCl buffer containing 0.05 M 2-mercaptoethanol. The enzyme was relatively stable at about pH 8.0 as shown in Figure 12. Furthermore, the effect of ammonium sulfate on enzyme stability was determined with the crude enzyme preparation (Figure 13). The enzyme was stabilized with an increase in the ammonium sulfate concentration from 0.001 to 0.2 M. These conditions were used in the following purification processes.

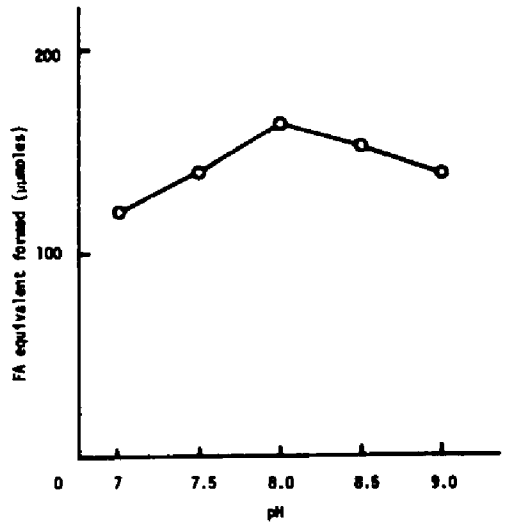

Fig. 12. Stability of Dihydrofolate Synthetase at Various PH Values

The standard assay was used, except that the enzyme in step II which had been left at 4°C at the indicated pH for 42 hours, was used.

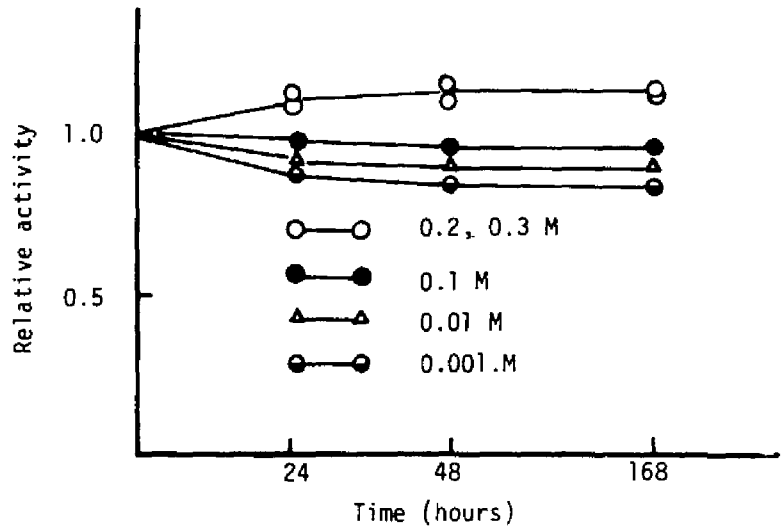

Fig. 13. Effect of Ammonium Sulfate on the Stability of Dihydrofolate Synthetase

Purification of the dihydrofolate synthetase from S. indica.

Step I. Preparation of cell-free extracts

Washed cells (about 700 g wet weight) were suspended in 2 liters of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol. The suspension was divided into 500 ml portions. Each portion was subjected to the action of Kaijo Denki ultrasonic oscillator (20 Kc) for 30 min. Cells and debris were removed by centrifugation at 20,000 x g for 20 min.

Step II. Streptomycin treatment and ammonium sulfate precipitation

A one tenth volume of a 6% dihydrostreptomycin solution

was added to 2.3 liters of the extract and the precipitate formed was removed by centrifugation at 20,000 x g for 20 min. Solid ammonium sulfate was added to 2.5 liters of the supernatant solution to 0.8 saturation. The mixture was equilibrated by stirring it for 30 min then it was centrifuged at 20,000 x g for 20 min. The resulting precipitate was dissolved in the buffer solution (0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol) and this solution was dialized for 36 hours against three changes of the buffer solution.

Step III. DEAE-Sephadex column chromatography

After the resulting precipitate had been removed by centrifugation, the supernatant solution (1150 ml) was subjected to DEAE-Sephadex column chromatography. DEAE-Sephadex A-50 was packed in a column (4.3 x 52 cm) and equilibrated with 0.01 M Tris-HCl buffer containing 0.05 M 2-mercaptoethanol and 0.05 M ammonium sulfate. The solution was passed through the column which was then washed with 3 liters of the equilibrating buffer solution, which removed much of the inactive protein. The enzyme was subsequently eluted with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol and 0.1 M ammonium sulfate, at a flow rate 1 ml per min in fractions of 20 ml. Elution of the protein was followed by measurement with Lowry's *et al.* method, as well as by the determination of enzyme activity. Active fractions were combined to give 3320 ml, which was concent-

rated by the addition of solid ammonium sulfate to 0.80 saturation. The precipitate obtained by centrifugation was dissolved in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol, then dialyzed for 48 hours against four changes of 5 liters of the same buffer.

Step IV. The second DEAE-Sephadex column chromatography

The dialyzed enzyme solution was subjected to a second DEAE-Sephadex column chromatography (Figure 4). DEAE-Sephadex A-50 was packed in a column (2.5 x 75 cm) and equilibrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol and 0.01 M Ammonium sulfate. The enzyme solution was placed on the column which was then washed with 1 liter of the same buffer solution. The enzyme was subsequently eluted by linear gradient elution. The mixing chamber contained 2 liters of 0.01 M Tris-HCl buffer, pH 8.0, with 0.05 M 2-mercaptoethanol and 0.01 M ammonium sulfate. The reservoir contained 2 liters of 0.01 M Tris-HCl buffer, pH 8.0, with 0.05 M 2-mercaptoethanol and 0.2 M ammonium sulfate. The enzymatically active eluates from the DEAE-Sephadex column were then combined to give 420 ml. These were concentrated by the addition of solid ammonium sulfate to 0.80 saturation. The precipitate was collected by centrifugation at 20,000 x g for 20 min, then it was dissolved in a minimum amount of 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol.

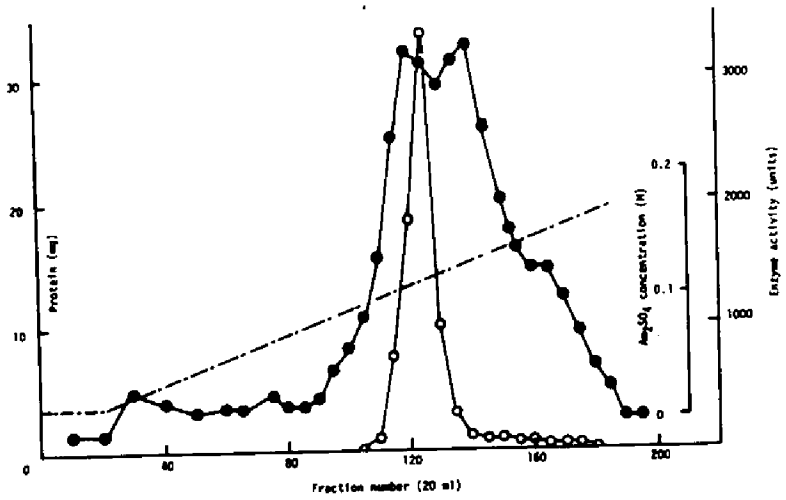

Fig. 14. Second Chromatography on a DEAE-Sephadex A-50 Column (2.5 x 95 cm)

•——• protein; ○——○ enzyme activity; –·–·–·– ammonium sulfate concentration

Step V. First Sephadex G-200 gel filtration

The enzyme solution (26 ml) was subjected to Sephadex G-200 gel filtration , as shown in Figure 15. Sephadex G-200 was packed in a column (5 x 200 cm) and equilibrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol and 0.2 M ammonium sulfate. The enzyme solution was then introduced into the column and the buffer was allowed to flow at a rate of 20 ml per hour. Twenty milileter fractions were collected. The contents of tubes number 113 to 133 were combined, and the

precipitate with ammonium sulfate (0.80 saturation) was recovered and dissolved in a minimum amount of 0.01 M Tris-HCl buffer, pH 8.0, containg 0.05 M 2-mercaptoethanol.

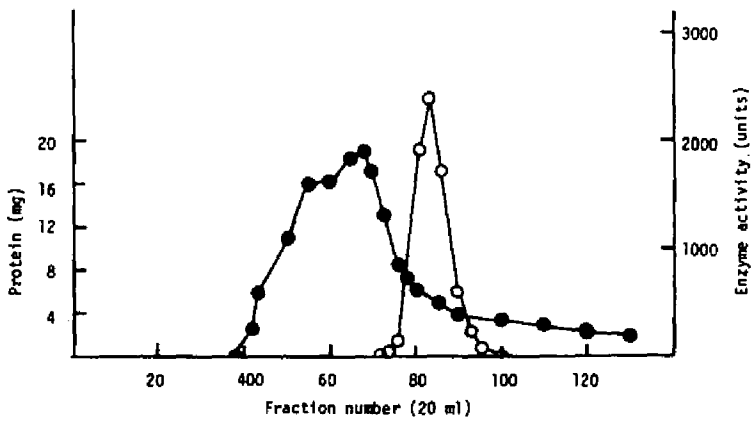

Fig. 15. Third Chromatography on a Sephadex G-200 Column

•——• protein ; o——o enzyme activity

Step VI. Second Sephadex G-200 gel filtration

The enzyme solution (5.4 ml) was subjected to Sephadex G-200 gel filtration as described in step V, except that the Sephadex G-200 was packed in a column (2.5 x 75 cm). Ten mililiter fractions were collected. The elution pattern of the enzyme is shown in Figure 16. Filtration gave a sigle, symmetric protein peak and the enzyme activity was entirely associated with this peak. The contents of tubes number 57 to 65 were combined,

and the precipitate with ammonium sulfate (0.80 saturation) was recovered and dialyzed for 48 hours against four changes of 2 liters of the buffer solution.

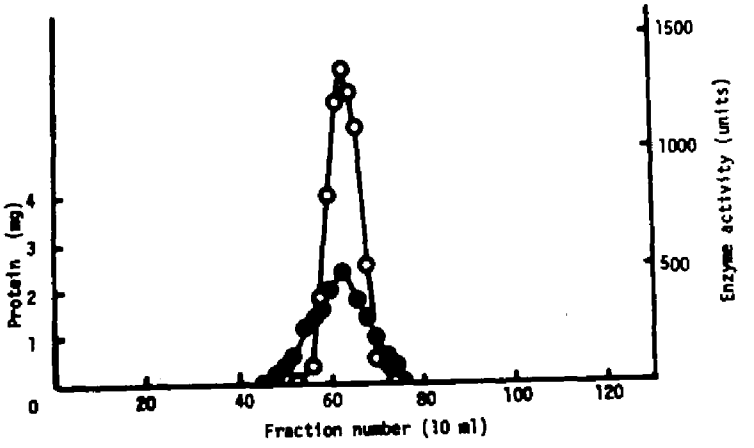

Fig. 16. Fourth Chromatography on a Sephadex G-200 Column

●——● protein ; o——o enzyme activity.

Step VII. DEAE-cellulose column chromatography

The dialyzed enzyme solution was subjected to DEAE-cellulose column chromatography. DEAE-cellulose was packed in a column (1.5 x 37 cm) and equilibrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol and 0.01 M ammonium sulfate. The enzyme solution was placed on the column which was then washed with 200 ml of the equilibrating buffer solution. The column was developed by linear gradient elution.

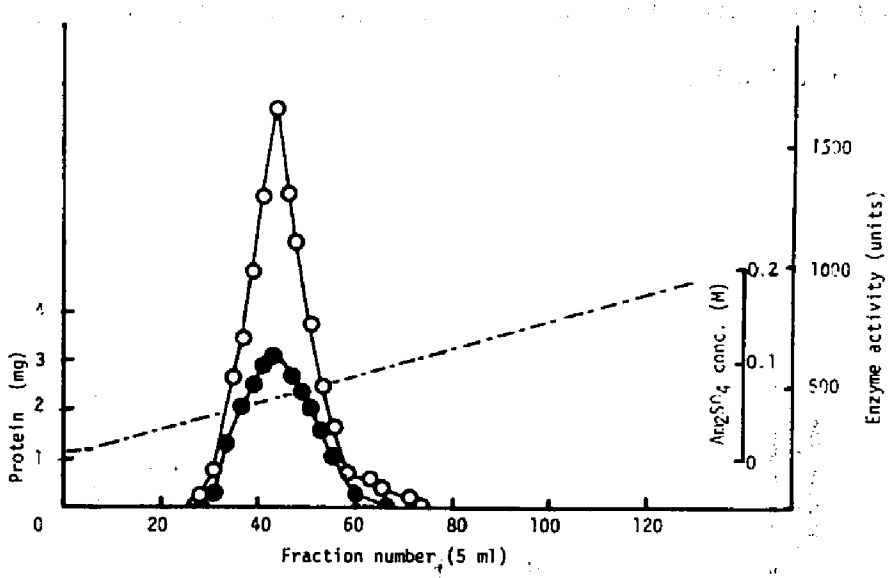

Fig. 17. Fifth Chromatography on a DEAE-cellulose Column

●—●, protein ; o—o, enzyme activity ; , ammonium sulfate concentration.

The mixing chamber contained 350 ml of the equilibrating buffer solution and the reservoir contained 350 ml of 0.01 M Tris-HCl buffer, pH 8.0, with 0.05 M 2-mercaptoethanol and 0.2 M ammonium sulfate. Five mililiter fractions were collected. The elution pattern of the enzyme is shown in Figure 17. The pattern produced a single, symmetric peak and the enzyme activity was entirely associated with this peak. The contents of tubes number 35 to 54 were combined, and the precipitate with ammonium sulfate (0.80 saturation) was stored as the purified enzyme

preparation at 0°C. The typical purification procedure is summarized in Table 12.

Activity in solution was almost completely lost by freezing or heating the purified enzyme at 70°C for 30 min. However, the enzyme in the form of ammonium sulfate precipitate was stable for 3 months in an ice bath.

Table 12

Summary of the Purification of the Dihydrofolate Synthetase from S. indica

Fraction	Total protein	Total activity	Specific activity	Purification	Yield
	mg	units*	units*/mg	ratio	%
I. Extracts	54,310	196,000	3.6	1.0	100
II. Am_2SO_4 ppt.	22,140	124,500	5.6	1.6	63.5
III. 1st DEAE-Sephadex column	2,592	56,370	21.7	6.1	28.8
IV. 2nd DEAE-Sephadex column	688	33,800	49.3	13.8	17.2
V. 1st Sephadex G-200 column	63	23,590	374.4	103.7	12.0
VI. 2nd Sephadex G-200 column	45	18,270	406.0	112.5	9.3
VII. DEAE-cellulose column	31	14,448	466.0	129.1	7.4

*1 unit = 0.1 mμmole of FA equivalent formed per 30 min under standard assay conditions.

Ultracentrifugal analysis.

The highly purified enzyme sedimented as a single symmetric peak in the ultracentrifuge in 0.01 M potassium phosphate buffer, pH 8.0, containing 0.1 M KC and 0.01 M 2-mercaptoethanol, as shown in Figure 18. The sedimentation coefficient in water at 20°C,

when the protein concentration was varied from 0.28 to 3.15 mg per ml.

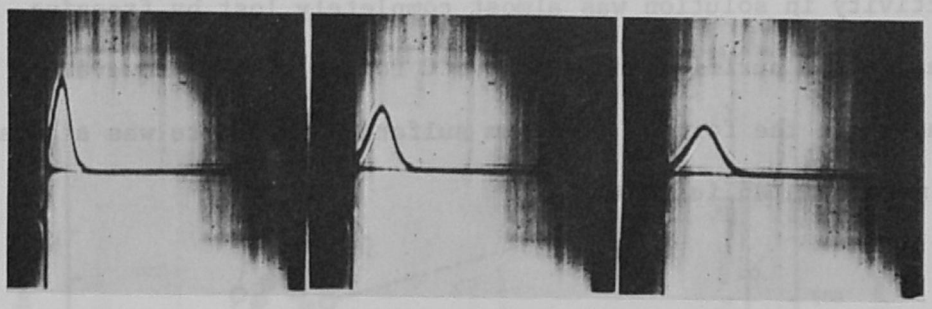

Fig. 18. Sedimentation Pattern of the Dihydrofolate Synthetase from S. indica

The sample contained 0.42% of the enzyme ; 0.1 M of KCl ; 0.01 M of 2-mercaptoethanol and 0.01 M of Tris-HCl buffer at pH 8.0.

The determination was made at 5°C and 60,000 rpm.

Estimation of molecular weight.

The molecular weight of the dihydrofolate synthetase from *S. indica*, as calculated by the method of Andrews[36] using gel filtration on a Sephadex G-100 column, was about 47,000, as shown in Figure 19.

Ultraviolet absorption spectrum.

The ultraviolet absorption spectrum of the dihydrofolate synthetase was determined in 0.01 M Tris-HCl buffer, pH 7.5,

containing 0.01 M 2-mercaptoethanol with a Hitachi Model 124 Spectrophotometer. A typical absorption spectrum of a simple protein with a maximum at 277 mμ, shown in Figure 20, was obtained.

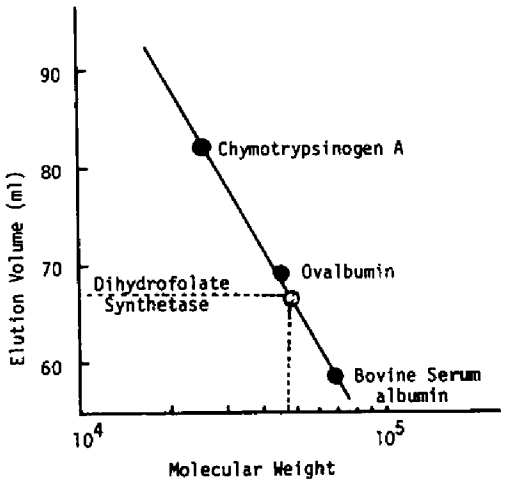

Fig. 19. Plot of Elution Volume against the Log Molecular Weight for Proteins on Sephadex G-100 Column

Time course of the reaction.

The time course of the reaction was tested at various enzyme concentrations. The reaction proceeded linearly (Figure 21) and the amount of folate equivalent formed was proportional to the amount of enzyme (Figure 22).

Effect of pH on enzyme activity.

The effect of pH on the enzyme activity is illustrated in Figure 23. Tris-HCl and glycine-NaOH buffers were used at final concentrations of 0.1 M. Maximum activity was obtained at pH 9.

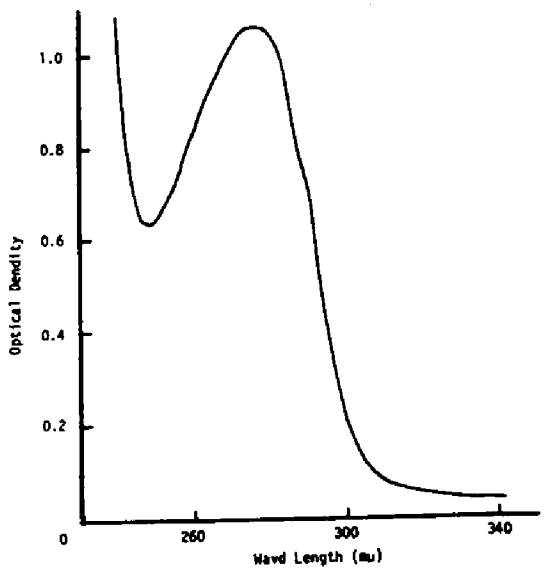

Fig. 20. Absorption Spectrum of the Dihydrofolate Synthetase Purified from S. indica

Substrate specificity and cofactor requirements.

As shown in Table 13, the reaction depended on dihydropteroic acid, ATP, L-glutamic acid, magnesium ion and the enzyme. The activity was not affected by the addition of potassium ion since the enzyme used already contained about 0.06 M ammonium sulfate. The effects of divalent and univalent cations on enzyme activity are described in detail elsewhere. Dihydropteroate was not replaceable by pteroate or tetrahydropteroate. L-Glutamate

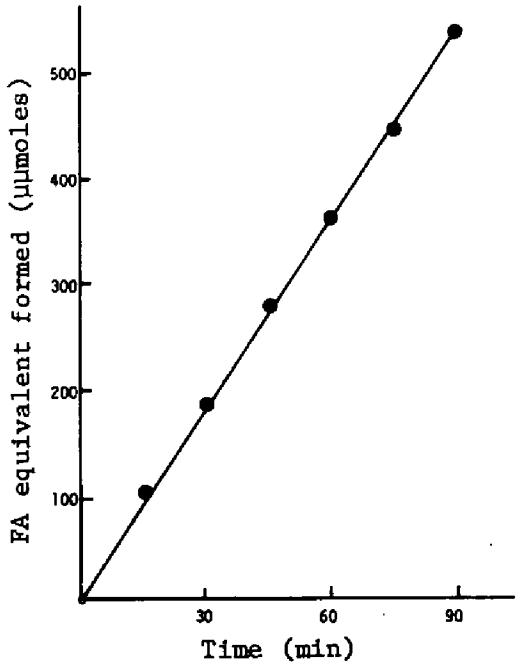

Fig. 21. Time Course of the Reaction

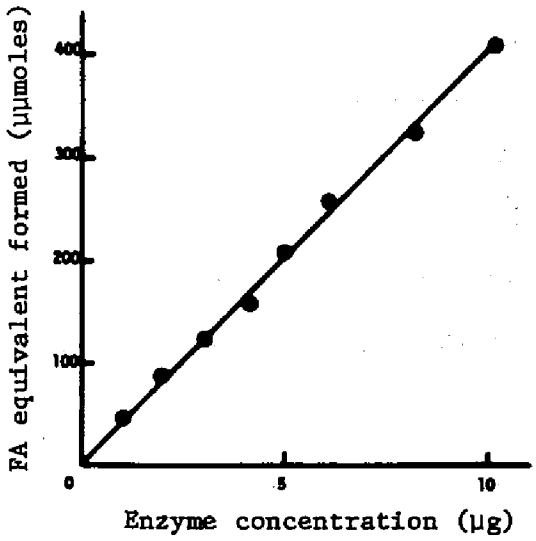

Fig. 22. Relationship between Enzyme Concentration and Dihydrofolate Synthesis

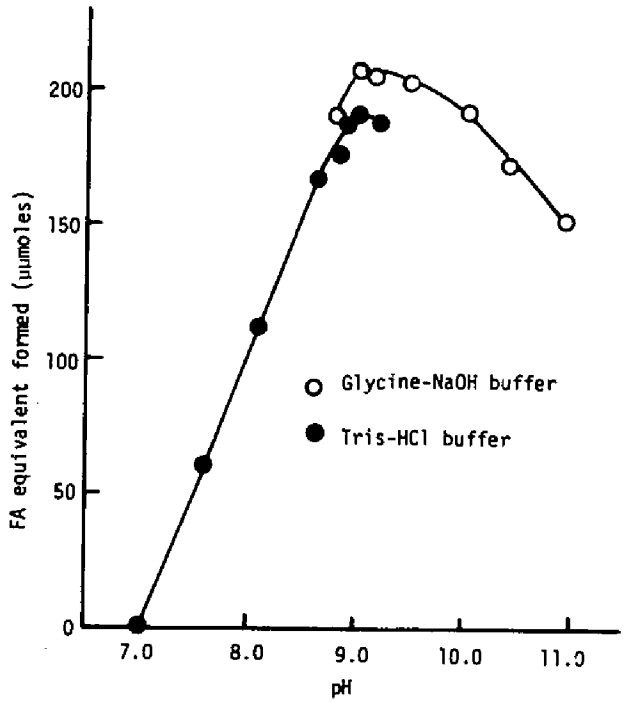

Fig. 23. Effect of PH on Dihydrofolate Synthetase Activity

was not replaceable with L-γ-glutamyl-L-glutamate. ATP was partially replaceable with ITP or GTP. ITP and GTP showed approximately 59 % and 37 % reactivity, respectively, under the conditions used, in comparison with ATP. CTP, UTP, ADP, and and AMP were not active on the enzyme. Results are summarized in Table 14.

Table 13

Component Study for Dihydrofolate Synthetase (I)

Omission	FA equivalent formed (μμmoles)
None	195
Dihydropteroate	0
L-Glutamate	0
ATP	0
Magnesium sulfate	0
Potassium sulfate	190
Enzyme	0

The standard assay method was used, except for the omission of indicated substances.

The enzyme contained about 10 mM of ammonium sulfate.

Table 14

Component Study for Dihydrofolate Synthetase (II)

Omission	Addition	FA equivalent formed (μμmoles)
None	None	195
Dihydropteroate	None	0
Dihydropteroate	Pteroate	0
Dihydropteroate	Tetrahydropteroate*	0
L-Glutamate	None	0
L-Glutamate	γ-L-glutamylglutamate	0
ATP	None	0
ATP	GTP	72
ATP	ITP	115
ATP	CTP	3
ATP	UTP	3
ATP	ADP	0
ATP	AMP	0

The standard assay method was used, except for the omission and addition of indicated substances of the same molarity with their counterparts in the standard assay.

*Incubation was performed at 37°C for 30 min under an argon atmosphere.

Reaction product.

Confirmation of the reaction product from dihydropteroate and L-glutamate was made by a bioautographic procedure with *L. casei* as the test microorganism. In this experiment, all processes involving the enzyme reaction were carried out under an argon atmosphere. The enzymatic reaction product was found to be dihydrofolic acid, as shown in Figure 24.

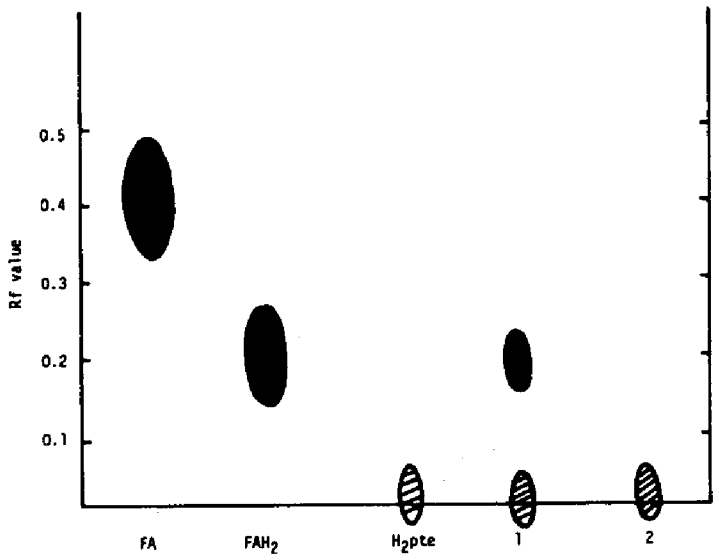

Fig. 24. Bioautogram of the Reaction Products

The numbers indicate spots as follows : No. 1, reaction mixture ; No.2, reaction mixture without enzyme. The diagonal zone indicates the fluorecence and the black zone indicates the growth of *L.ccasei*.

Incubation was made at 37°C for 2 hours under an argon atmosphere. The bioautogram was prepared with *L. casei* according to directions in Methods.

Stoichiometry of the reaction product.

To determine whether ATP as the substrate give 'ADP + Pi' or 'AMP + PPi' by the enzyme reaction, ATP-U-^{14}C and ATP-γ-^{32}P were used. After the reaction, the radioactive adenine-containing compounds were separated on a Dowex 1 x 2 formate column. The amounts of ATP, ADP and AMP formed enzymatically were estimated from the counts of the radioactivity of each fraction. The reference experiment was performed without Mg^{2+} in the reaction mixture. When dihydropteroic acid was left out of the reaction mixtures, almost no ADP was formed. ADP (3.1 mμmoles) and dihydrofolate (2.7 mμmoles) were formed enzymatically, and no significant amounts of AMP were detected.

In addition, the amounts of orthophosphate and dihydrofolate formed during the enzyme reaction were assayed using ATP-γ-^{32}P. Two and five tenth mμmoles of orthophosphate and 2.3 mμmoles of dihydrofolate were estimated. Results are shown in Table 15. They suggest that one mole of ATP was cleaved to give one mole each of ADP and orthophosphate, and that one mole of dihydrofolate was simultaneously formed by the enzymatic reaction.

Inhibition of the enzymatic reaction by ADP.

The enzymatic reaction was inhibited by the addition of ADP, but not by AMP, as shown in Table 16.

Table 15

Stoichiometry of the Reaction Products

Substrate	FAH_2	ADP	Pi
	mμmole	mμmole	mμmole
ATP-U-^{14}C	2.7	3.1	—
ATP-γ-^{32}P	2.3	—	2.5

The reaction was carried out under an argon atmosphere and standard assay conditions were used, except that ATP-U-^{14}C or ATP-γ-^{32}P was added.

Table 16

Effects of ADP and AMP on the Enzymatic Formation of Dihydrofolate

Addition	Conc.	FA equivalent formed	Inhibition
		μμmoles	%
None		195	0
ADP	2.5	114	41.5
	5.0	90	58.3
	7.5	71	63.6
AMP	2.5	194	0.5
	5.0	196	0
	7.5	190	2.6

The standard assay was used, except that the ATP concentration was 2.5 mM and the nucleotides indicated were added.

Inhibition of the enzymatic reaction by PCMB

In this experiment, the enzyme which had been dialyzed against 0.01 M Tris-HCl buffer at pH 8.0 to remove the 2-mercaptoethanol contained in the enzyme preparation, was used. Results are shown in Table 17. The enzymatic activity was strongly inhibited by the addition of PCMB. These results suggest that the sulfhydryl group of this enzyme is colsely related to the enzyme activity.

Table 17

Inhibition of Dihydrofolate Synthetase Activity by PCMB

Concentration	Inhibition
M	%
10^{-5}	15
$5x10^{-5}$	23
10^{-4}	36
$5x10^{-4}$	67
10^{-3}	98

The standard assay was used, except that the PCMB and the dialyzed enzyme were added.

4. Discussion

In chapter II, the author reported that the enzyme activity of extracts from the isolated mitochondria of pea seedlings showed a 27% decrease during storage of the enzyme for 24 hours under favourable conditions, and that the stability of extracts from isolated mitochondria was better than that of extracts from the homogenate of pea seedlings. In this study, the author found that the dihydrofolate synthetase activity from *S. indica* was very stable and there was no decrease in the percent of enzyme activity observed under favourable storage conditions for 7 days. This indicates that the enzyme from *S. indica* should be favourable for purification, so the author tried to purify it.

The enzyme from *S. indica* obtained in a 7.4% yield, though the enzyme from pea seedlings had been obtained in a 1.4% yield[61]. Similar specific activities were obtained for the purified enzymes from *S. indica* and pea seedlings. Consequently the yield of the enzyme from *S. indica* was 2 fold better in comparison with the enzyme from pea seedlings.

The purified enzyme from *S. indica* was homogeneous on DEAE-cellulose column chromatography and ultracentrifugation, but not on disc electrophoresis.

The general properties of the enzyme purified from *S. indica* resemble those prepared from other sources.[18,61] As described

in 'Results', the apparent pH optimum of the *S. indica* enzyme was about 9.0 (the same as the *E. coli* enzyme), whereas that of the pea seedling enzyme was 8.8.

The dihydrofolate synthetase activity was dependent on dihydropteroic acid, L-glutamic acid, ATP and Mg^{2+} (Table 12). The dihydropteroic acid could not be replaced by pteroic acid or tetrahydropteroic acid (Table 13). Since no inhibition of dihydrofolate synthetase activity by the addition of D-glutamic acid was observed under the standard assay conditions, the enzyme might be specific for L-glutamic acid. Moreover, γ-L-glutamyl-L-glutamic acid could not replace L-glutamic acid as the substrate to form dihydropteroyldiglutamic acid. This supports the position that the formation of 'conjugate', i.e. di- and triglutamates, take place only with tetrahydrofolic acid as the substrate.[18] ATP was partially replaced by ITP (59% active) or GTP (37% active) in the *S. indica* enzyme (Table 14) as well as in the *E. coli* enzyme, but in the pea seedling enzyme ATP was not replaced by any other nucleotide triphosphate.[61]

The product of the enzyme reaction was found to be dihydrofolic acid using the bioautographic procedure shown in Figure 24.

The enzymatic reaction was inhibited by the addition of ADP, but not AMP (Table 16). One mole of dihydropteroic acid, L-glutamic acid and ATP formed 1 mole of dihydrofolic acid, orthophosphate and ADP by the following enzymatic reaction.

Dihydropteroic acid + L-glutamic acid + ATP

$\longrightarrow$ Dihydrofolic acid + ADP + Pi

These results suggest that the systematic name for dihydrofolate synthetase should be 7,8-dihydropteroate : L-glutamate ligase (ADP).

The enzymatic reaction was not inhibited by 10^{-3} M sulfanilamide under standard assay conditions.

Inhibition of the dihydrofolate synthetase from *E. coli*[18] by PCMB has been reported. In this study, inhibition of enyzme activity by PCMB was observed (Table 17). This indicates that the sulfhydryl group of the enzyme is closely related to enzyme activity.

CHAPTER V

SOME CHARACTERISTICS OF THE DIHYDROFOLATE SYNTHETASE FROM *Serratia indica*

1. Introduction

The action mechanism of magnesium in biological catalysis is reasonably well understood. Most transphosphorylation reactions involving ATP require magnesium ions and it is generally assumed that the reactive species is a magnesium complex of ATP.

Knowledge of the action mechanism of potassium in metabolism, however, is limited. In recent years a number of enzyme catalyzed reactions have been shown to require potassium, ammonium or rubidium ions for their activity.[38-40] No detailed study of K^+ requirement was made.

Homopteroic acid, an intermediate in the synthesis of homofolate, and its tetrahydro derivative have been reported to be potent growth inhibitors of *Streptococcus faecalis* R, a folate dependent organism.[41] Kisliuk, *et al.*, has also reported that tetrahydrohomopteroic acid displays activity against a pyrimethamine-resistant strain of *Plasmodium cynomolgi* in monkeys and they suggested that this area should be studied further in hopes of developing a new type of antimalarial agent.[42]

The distribution, purification and properties of the dihydrofolate

synthetase from *S. indica* were described in chapter IV. Studies of the activation of the enzyme by magnesium and univalent cations are described in this chapter. The inhibition of dihydrofolate synthetase by reduced forms of homopteroic acid is also discussed.

2. Materials and Methods

Chemicals.

Disodium ATP and the Tris-salts of ATP were purchased from the Sigma Chemical Company. L-Glutamic acid, 2-mercaptoethanol and folic acid were from Nakarai Chemicals, Ltd., Kyoto. Pteroic acid was kindly provided from the Lederle Laboratories Division, American Cyanamid Company. Homopteroate and homofolate were kindly provided by the Cancer Chemotherapy National Service Center of the U. S. Public Health Service, through the coutesy of Dr. R. L. Kisliuk, Department of Biochemistry, Tufts University.

Reduction of pteridine compounds.

Pteroic acid was reduced to the dihydro form by treatment with sodium dithionite as described by Futterman.[26] Homopteroic and homofolic acids were reduced to their dihydro forms by treatment with sodium dithionite as described by M. Friedkin, *et al.*[43]

Tetrahydrohomoptic acid was prepared by the hydrogenation of homopteroic acid according to the direction of R. L. Kisliuk.[44]

Purification of the dihydrofolate synthetase from S. indica.

The enzyme used in these studies was purified from *S. indica* by the procedure reported in chapter IV.

Standard assay conditions.

Dihydrofolate synthetase activity was assayed as previously described in chapter IV using a microbiological assay with *L. casei*.

Activity is expressed as 0.1 millimicromole of folate equivalent formed per 30 min. The Tris-salts of all compounds were used in order to eliminate potassium and sodium ions from the assay mixture in experiments in which the potassium ion content was varied.

Protein concentration was determined by the method of Lowry *et al.*[29]

Activation by divalent cation.

The ffects of Mg^{2+}, Mn^{2+} and Fe^{2+} on the activity of the dihydrofolate synthetase from *S. indica* are shown in Figure 25. The concentration of Mg^{2+} producing maximum enzyme activity was 5×10^{-3} M. A concentration of Mg^{2+} greater than the optimum resulted in inhibition of enzyme activity.

Mn^{2+}, Fe^{2+} and Ca^{2+} also activated the enzyme (Table 18), but none of these was as effective as Mg^{2+}.

It is generally agreed that $MgATP^{2+}$ is the reactive species in most reactions where ATP serves as a phosphate donor (45). As expected, the reaction catalyzed by dihydrofolate synthetase exhibited an absolute dependence on magnesium, it was also shown that the required concentration of magnesium was in excess of that required to form a complex with ATP. The effect of ATP concentration on enzyme activity is shown in Figure 31. Data from the activation curve were plotted according to the method of Lineweaver and Burk (46) in Figure 31 (inset) and the Michaelis constant (Km) for ATP was determined to be 2.2×10^{-4} M $MgATP^{2+}$ appears to be the required substrate.

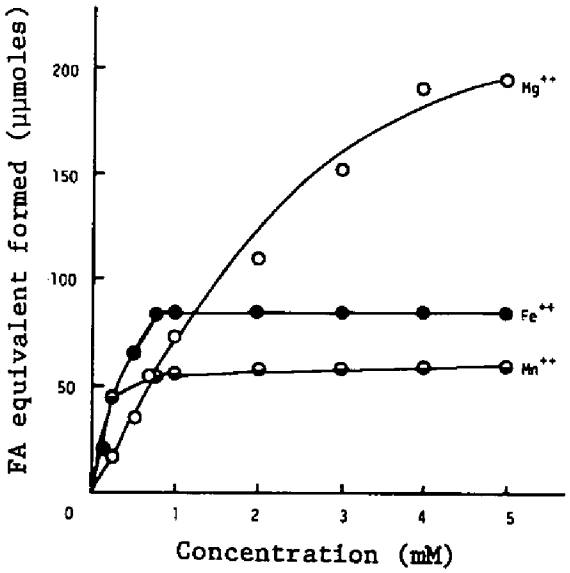

Fig. 25. Effects of Divalent Cation Concentrations on Dihydrofolate Synthetase Activity.

Divalent cations were used in the form of sulfate.

Table 18

Effects of Divalent Cations on Dihydrofolate Synthetase Activity

Divalent cations	Conc. (mM)	FA equivalent formed (μμmoles)
Mg^{2+}	5.0	195
Mg^{2+}	1.0	68
Mn^{2+}	5.0	69
Mn^{2+}	1.0	61
Fe^{2+}	5.0	79
Fe^{2+}	1.0	77
Co^{2+}	1.0	0
Ni^{2+}	5.0	0
Zn^{2+}	5.0	0
Ca^{2+}	5.0	6
Cd^{2+}	5.0	0
Cu^{2+}	5.0	0

The standard assay method was used, except that the divalent cations indicated were added.

Activation by univalent cations.

The effects of K^+, NH_4^+ and Rb^+ on the activity of the dihydrofolate synthetase from *S. indica* are shown in Figure 26. The optimum concentration for K^+ and Rb^+ was 50 to 100 mM. However, 70% of the maximum activity was produced by a K^+ concentration of 10 mM. The optimum concentration of NH_4^+ was 20 to 30 mM and a concentration of NH_4^+ greater than the optimum resulted in a slight inhibition of activity. The apparent Michaelis constants (km) from Figure 26, for K^+, NH_4^+ and Rb^+ were determined to be about 7×10^{-3}, 3.5×10^{-3} and 13×10^{-3} M, respectively. The effect of K^+ was partly replaceable by Tl^+ and Cs^+.

In contrast, Na^+ and Li^+ were ineffective. Results are shown in Table 19.

Interestingly, there is a correlation between the ionic radii of these univalent cations and their abilities to activate the enzyme, *i. e.* K^+, NH_4^+, and Cs^+ have shorter or longer ionic radii than these of effective univalent cations.

Effect of K^+ on the kinetic constants of the enzyme reaction.

To investigate the effect of K^+, the kinetic constants of the enzyme reaction for substrates (ATP, dihydropteroate, or L-glutamate) were determined at K^+ concentrations of 7.5 and 100 mM

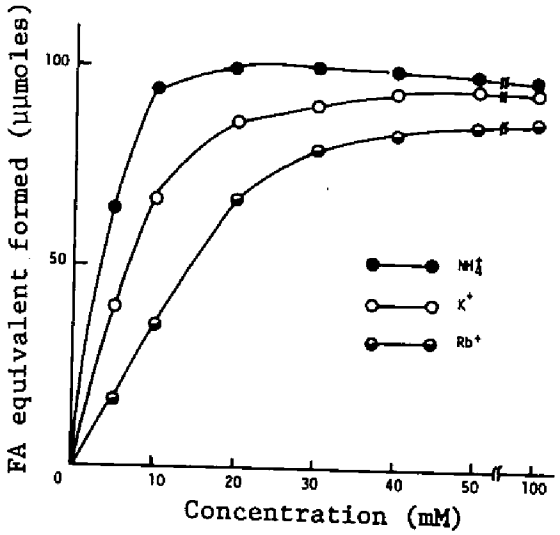

Fig. 26. Effects of Univalent Cation Concentrations on Dihydrofolate Synthetase Activity

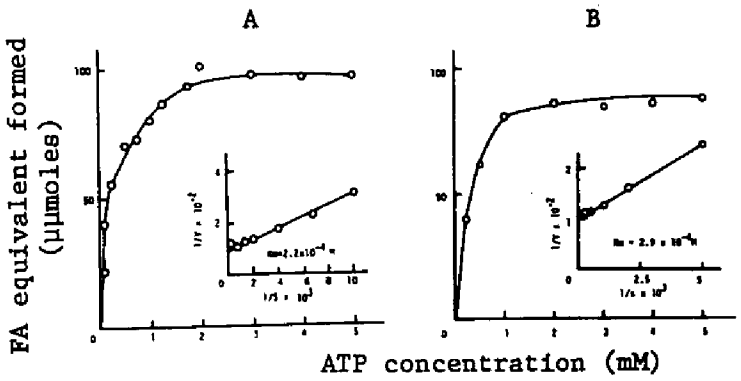

Fig. 27. Effect of the ATP Concentration on Dihydrofolate Synthetase Activity

(A). The standard assay was used except that dialyzed enzyme was used.

(B). The standard assay was used except that dialyzed enzyme with a 7.5 mM concentration of K^+ was used.

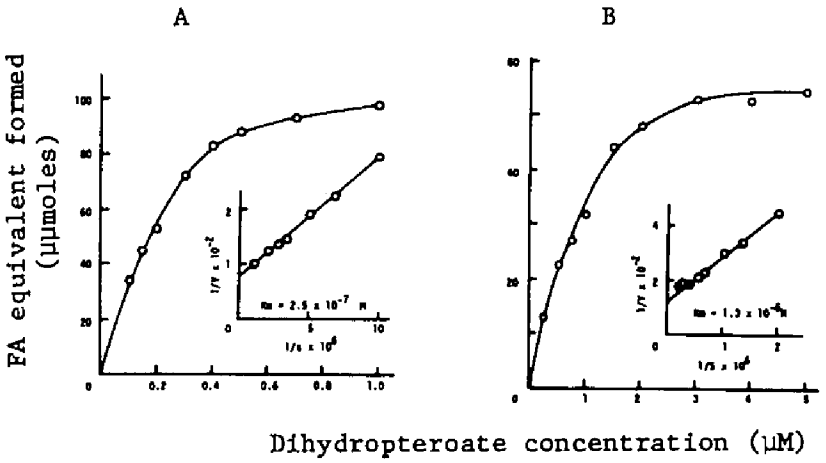

Fig. 28. Effect of the Dihydropteroate Concentration on Dihydrofolate Synthetase Activity

(A). The standard assay was used except that dialyzed enzyme was used.

(B). The standard assay was used except that dialyzed enzyme with a 7.5 mM concentration of K^+ was used.

(Figures 27-29). The standard assay was used except that enzyme dialyzed for 48 hours against 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol was used. Results are shown in Table 20.

The Km values for dihydropteroate and L-glutamate were greatly changed by changing the K^+ concentration from 100 to 7.5 mM. The Km value for ATP, however, was little changed by decreasing the K^+ concentration in the assay mixture from 100 to 7.5 mM, but K^+ at a low concentration (7.5 mM) decreased the Vmax for ATP.

Table 19

Km and Maximum Velocity Values for Various Cations

Addition	Optimum conc.	Km	Vmax.
	mM	mM	µµmoles
None			0
NH_4^+	25	3.5	101
K^+	100	7	95
Rb^+	100	13	88
Tl^+	100	—	46
Cs^+	100	—	14
Na^+	100	—	0
Li^+	100	—	0

The standard assay was used except that enzyme dialyzed against 0.01 M Tris-HCl buffer (pH 8.0) containing 0.05 M of 2-mercaptoethanol was used.

Table 20

Effect of K^+ on the Kinetic Constants of the Reaction

Substrate	K^+ Conc.	Km	Relative V
	mM	M	
ATP	100	2.2×10^{-4}	1.0
	7.5	2.9×10^{-4}	0.85
Dihydropteroate	100	2.5×10^{-7}	1.0
	7.5	1.3×10^{-6}	0.63
L-Glutamate	100	2.5×10^{-4}	1.0
	7.5	9.1×10^{-3}	0.97

The standard assay was used except that enzyme dialyzed for 48 hours against 0.01 M Tris-HCl buffer (pH 8.0) containing 0.05 M of 2-mercaptoethanol was used.

The Vmax for dihydropteroate was also decreased greatly by decreasing the K^+ concentration from 100 to 7.5 mM, but the Vmax for L-glutamate was not changed. These results suggest that K^+ effects the affinity between the enzyme and substrates.

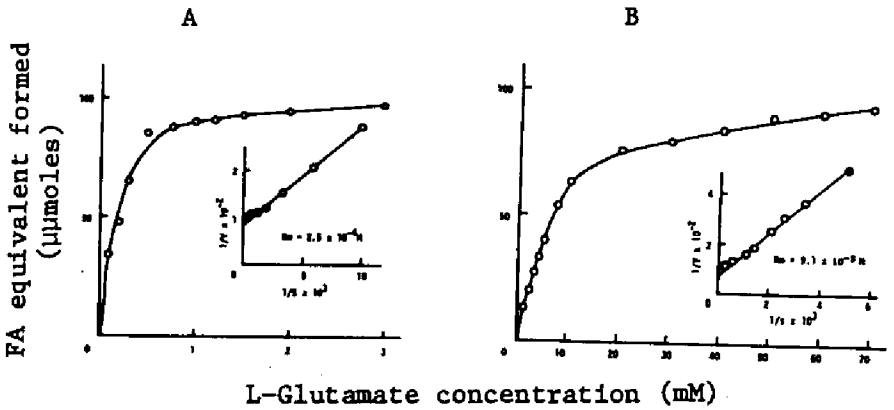

Fig. 29. Effect of the L-Glutamate Concentration on Dihydrofolate Synthetase Activity

(A). The standard assay was used except that dialyzed enzyme was used.

(B). The standard assay was used except that dialyzed enzyme with a 7.5 mM concentration of K^+ was used.

Inhibition of dihydrofolate synthetase activity by reduced forms of homopteroate.

The effects of reduced compounds on the growth of *L. casei* were investigated in a preliminary experiment (Figure 30). The growth of *L. casei* was not inhibited by the addition of 10^{-7} M

homopteroate or the reduced compounds. Table 21 shows that the enzyme activity was highly inhibited by the addition of dihydro-homopteroate, and slightly by tetrahydropteroate. Since the enzyme reaction was performed in air as in standard assay conditions, except for the addition of the reduced compounds, the inhibition of activity by tetrahydrohomopteroate may be thought to be due to oxidation of the tetrahydro form to the dihydro form.

Table 21

Inhibition of Dihydrofolate Synthetase Activity by Homopteroate and Its Reduced Compounds

Addition	Conc. M	FA equivalent formed μμmoles	Inhibition %
Homopteroate	0	198	0
	10^{-5}	210	0
	10^{-4}	205	0
Dihydrohomopteroate	10^{-7}	192	3.1
	10^{-6}	172	13.3
	$5x10^{-6}$	158	20.4
	10^{-5}	139	29.8
	$5x10^{-5}$	86	56.7
	10^{-4}	50	74.8
Tetrahydrohomopteroate	$5x10^{-6}$	201	0
	10^{-5}	192	3.0
	$5x10^{-5}$	172	13.3
	10^{-4}	130	35.4

The standard assay method was used, except for the addition of indicated substances.

4. Discussion

In common with other enzymes involving ATP, dihydrofolate synthetase requires magnesium ion as an activator. The requirement for magnesium ion was partially replaceable by manganese or ferrous ions. It has been reported that in the dihydrofolate synthetase from pea seedlings manganese ion is more effective than magnesium ion in stimulating enzyme activity and that higher concentrations of manganese ion (5 mM) markedly inhibited enzyme activity.[60]

With the dihydrofolate synthetase from *S. indica*, we observed that no other divalent cation was as effective as the magnesium ion and that high concentrations of manganese ion (ca. 5 mM) did not inhibit enzyme activity as much as it did with the enzyme from *E. coli*.[18]

In addition K^+, or a related univalent cation, is essential ; a K^+ concentration of 0.1 M being required for maximum activity.

Increasing the K^+ concentration of the assay mixture from 7.5 to 100 mM results in a marked decrease of the Km with respect to dihydropteroate and L-glutamate, and also results in an increase in the Vmax with respect to ATP and dihydropteroate. These data indicate that K^+ increase the apparent affinity of the enzyme for dihydropteroate and L-glutamate, which suggests that K^+ is required to bind dihydropteroate and L-glutamate to the enzyme.

Kinetic investigations of some enzymes which require univalent cations for their activity suggested that the conformation of the enzyme protein is changed in the presence or absence of univalent cations.[47-51] Recently, it has been reported that formyltetrahydrofolate synthetase was dissociated into four subunits in the absence of univalent cations such as the potassium ion.[50,51]

It has also been reported that certain univalent cations which have ionic radii near that of the potassium ion are effective in the complex formation between the apopropanediol dehydratase and coenzyme B_{12}, and their effectiveness was colsely related to the catalytic activity in the propanediol dehydratase reaction.

To detect possible changes in the fourth structure of dihydrofolate synthetase, in the presence or absence of potassium ion, the S value was measured by ultracentrifugation analysis, but no changes were detected.

Homopteroic acid is an intermediate in the synthesis of homofolic acid and occurs as a contaminant in certain commercial preparations of homofolic acid. Kisliuk *et al.* reported that tetrahydrohomopteroate displayed activity against a pyrimethamine-resistant strain of *Plasmodium cynomolgi* in monkey. Pyrimethamine is believed to exert its antimalarial effect by inhibiting dihydrofolate reductase.[52] Sulfonamides are believed to have antimalarial activity because they inhibit the incorporation of *p*-aminobenzoic acid into dihydrofolic acid.[52]

Since tetrahydrohomopteroic acid has a *p*-aminobenzoic acid moiety it might act at the same site as the sulfonamides. The presence of the pteridine moiety in the new drug introduces the additional possibility that the enzymes in dihydrofolate biosynthesis which involve pteridine but not *p*-aminobenzoic acid may be inhibited. Two examples of enzymes in the category are (i) the enzyme that catalyzes the pyrophosphorylation of 6-hydroxymethyldihydropterin[53] and (ii) the enzyme that catalyzes the addition of glutamate to dihydropteroate.[18] However, the action site on the dihydrofolate biosynthetic pathway is obscure. The author found that dihydrohomopteroate inhibits dihydrofolate synthetase activity. Since the enzyme reaction was performed in air as is the standard assay, except for the addition of the compounds, inhibition of the enzyme activity by tetrahydrohomopteroate may be due to the oxidation of the tetrahydro form to the dihydro form.

The dose of tetrahydrohomopteroate *in vivo* may be oxidized to dihydrohomopteroate. If so, the inhibition of dihydrofolate synthetase activity by dihydrohomopteroate indicates that the enzyme is the true action site.

CHAPTER VI

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

1. Introduction

Vitamins are substances required by animal organisms in minute amounts for the maintenance of normal growth. As animal organisms can not biosynthesize vitamins, they must be taken from food for growth. One of vitamin, folate compounds, exists in most organisms and is one of the most powerfuk catalysts of several metabolic reactions though it is needed only in minute amounts. It is generally accepted that organisms which can not biosynthesize folate compounds *in vivo* and require them for growth lack the enzyme on the folate biosynthetic pathway. Animal organisms and one group of lactic acid bacteria are known to have nutritional requirements for folate compounds. *Streptococcus faecalis* R can replace pteroylglutamic acid with pteroic acid.[54] *Lactobacillus casei* requires pteroylglutamic acid.[54] *Pediococcus cerevisiae* requires cofactor forms of pteroylglutamic acid.[55]

This suggests that nutritional requirements for folate compounds might result in lack of the enzyme on the folate biosynthetic pathway. The author tried to confirm this possibility by

detecting the enzymes, dihydropteroate synthase and dihydrofolate synthetase, which are thought to be the key enzymes on the folate biosynthetic pathway.

2. Materials and Methods

Materials.

Hydroxymethylpterin was prepared by the method of Waller *et al.*[56] For use as a substrate in the enzymatic reaction, hydroxymethylpterin was reduced, by treatment with sodium borohydride [57] to the dihydro derivative. *p*-Aminobenzoic acid labeled with ^{14}C 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 from commercial sources. Pteroic acid was provided from the Lederle Laboratories Division, American Cyanamid Company and was reduced to dihydropteroic acid by treatment with sodium dithionite as described by Futterman.[26]

Measurement of dihydropteroate synthase activity.

A radioassay[59] for the enzyme using ^{14}C-labeled *p*-aminobenzoic acid as the substrate was made. The reaction mixture contained 2 mμmoles of *p*-aminobenzoic acid-$^{14}COOH$ (8,000 cpm) ; 10

mμmoles of hydroxymethyldihydropterin ; 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 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 at pH 7.0 as the developter. 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 synthetse activity was determined by a microbiological assay.[58] 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 ou at 37°C for 30 min, then it was stopped by heating the whole 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.[28] These are expressed as folate equivalents.

Determination of protein.

The amount of protein was determined by the method of Lowry *et al.*[29] using crystalline bovine serum albumin as the 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 hours in submerged cultures. The growth medium for the microorganisms contained 1% yeast extract, 0.5% peptone, 1% glucose and 0.5% Na-acetate and KH_2PO_4.

Saccharomyces carlsbergensis 4228, ATCC 9080, and *Saccharomyces cerevisiae* (baker's yeast) were grown at 30°C for 24 hours 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 hours in shaking cultures in modified Massen medium (glucose, 3% ; D,L-malic acid, 0.07% ; asparagine, 1% ; K_2HPO_4, 0.25% ; $MgSO_4.7H_2O$, 0.04% ; Na_2CO_3, 0.25% ; $CaCl_2$, 0.001% ; 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 (sucrose, 3% ; $NaNO_3$, 0.2% ; K_2HPO_4, 0.1% ;

$MgSO_4.7H_2O$, 0.05% ; $FeSO_4.7H_2O$, 0.001% ; yeast extract, 0.05% ; 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 blendor. The homogenate was squeezed through two layers of gauze, then centrifuged at 15,000 x 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 with sea sand in a porcelain mortar, then were extracted with the isolating medium. The extracts were centrifuged at 15,000 x g for 20 min in the cold.

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

3. Results

Distribution of dihydrofolate synthetase in microorganisms and mushrooms.

Dihydrofolate synthetase activities in the extracts of various organisms and mushrooms were measured by microbiological assays with *L. casei*. Results are shown in Tables 22 and 23. Data in Table 23 are expressed as values per one gram of fresh weight. The enzyme activity was widely distributed in microorganisms and mushrooms. High enzyme activity was detected in *Escherichia* strains such as *E. coli*, *S. indica*, *B. cereus* and *Pseudomonas riboflavina*.

Table 22

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 riboflavina IFO 3140	3.65
Neurospora crassa IFO 6068	2.25

* 1 unit = 0.1 mμmole of FA equivalent formed per 30 min under standard assay conditions.

Table 23

Distribution of Dihydrofolate Synthetase Activity in Mushrooms

Mushrooms	Protein	Enzyme activity	Specific activity
	mg*	units*	units/mg of protein
Flammulina velutipes (Enokitake)	5.11	3.25	0.64
Lentinus edodes (Pileus) (Shiitake)	4.07	1.73	0.43
Lentinus edodes (Stalk) (Shiitake)	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

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

* 1 unit = 0.1 mμmole of FA equivalent formed per 30 min under standard assay conditions.

Nutritional requirements for folate compounds and enzymatic activities on the folate biosynthetic pathway.

The relationship between nutritional requirements for folate compounds and enzymatic activities on the folate biosynthetic pathway was tested using rat liver, chicken liver, *S. faecalis* R, *L. casei* and *P. cerevisiae* and the nutritional requirements for folate compounds were confirmed. Low dihydropteroate synthase activity was detected in *S. faecalis* R, in comparison with *L. casei* and *P. cerevisiae*.

In contrast, high dihydrofolate synthetase activity was detected in *S. faecalis* R, but not in *L. casei*, *P. cerevisiae*, rat liver or chicken liver. Results are shown in Table 24. They suggest that the occurrence of dihydrofolate synthetase is indis-

pensable in organisms which don't require pteroylglutamic acid for growth. These observations support the position that dihydropteroate is a true intermediate in the biosynthesis of folate compounds and the pathway through dihydropteroic acid as an intermediate is the main route in the biosynthesis of folate compounds.

Table 24

Requirement for Folate Compounds and Some Enzyme Activities Involved in Folate Biosynthesis

Organisms	Dihydropteroate Synthetase activity	Dihydrofolate Synthetase activity	Comparative Growth-promoting Activity*		
			Pteroic acid	Folic acid	H_4 folic acid
	Specific Activity unit[a]/ug of protein	Specific Activity unit[b]/mg of protein			
Streptococcus faecalis R ATCC 8043	0.045	1.45	+++	+++	+++
Lactobacillus casei ATCC 7469	0.091	trace	–	+++	+++
Pediococcus cerevisiae ATCC 8081	0.247	trace	–	–	+++
Rat liver	———	trace	–	+++	+++
Chicken liver	———	trace	–	+++	+++

a 1 unit = 0.25 μμmole of dihydropteroate formed per 60 min under standard assay conditions.

b 1 unit = 0.1 mμmole of FA equivalent formed per 30 min under standard assay conditions.

* Cited from *Biochemistry of B Vitamins*.[54]

4. Discussion

It has been reported that dihydropteroate synthase is widely distributed in various organisms, i.e. bacteria and plants.[23,57] In previous chaper, the author reported that dihydrofolate synthetase activity is widely distributed in various plants.[60] As shown in Tables 22 and 23, dihydrofolate synthetase is also distributed in bacteria, yeasts, molds and mushrooms. *S. indica*, *E. coli*, *B. cereus*, *Pseudomonas riboflavina* and *N. crassa* showed high specific activity in comparison with other microorganisms, mushrooms and plants.

In *S. faecalis* R which requires pteroic acid, folic acid or the tetrahydro form of folic acid as a nutrient for growth, low dihydropteroate synthase activity and high dihydrofolate synthetase activity were detected (Table 24).

In *L. casei*, which requires folic acid or the tetrahydro form of folic acid, low dihydropteroate synthase activity was detected but no dihydrofolate synthetase activity was detected.

Furthermore, in *P. cerevisiae* which requires the tetrahydro form of folic acid as a nutrient for growth, dihydropteroate synthase activity was detected, but no dihydrofolate synthetase activity was detected. In contrast, in animals such as rat and chicken which require folic acid or the tetrahydro form of folic acid, no dihydrofolate synthetase acti-

vity was detected in their livers. These results are shown in Table 24.

They suggest that the occurrence of dihydrofolate synthetase is indispensable in organisms which don't require folic acid compounds for growth, and support the position that dihydropteroate is a true intermediate in the biosynthesis of folate compounds.

SUMMARY

Dihydrofolate synthetase (EC 6.3.2.12), which catalyzes the formation of dihydrofolate from dihydropteroate and L-glutamate, has been found in various plant tissues and microorganisms. The enzyme was firstly purified in a homogeneous state from pea seedlings and some properties were investigated. Dihydrofolate synthetase was also isolated in a homogeneous state from *Serratia indica* and some characteristics of the enzyme were compared with those of the enzyme from pea seedlings. These results are summarized as follows.

CHAPTER II : Dihydrofolate synthetase was widely distributed in various plants.

The amount of folate compounds rapidly increased during germination of the pea. An especially high increase was observed 2days after sowing during germination. Dihydrofolate synthetase activity also increased with the increase in the amount of folate compounds. The dihydrofolate synthetase was localized mostly in the mitochondrial fraction and it was easily extracted from the cell particle by osmotic shock. The enzyme which was extracted from the isolated mitochondria was relatively stable in comparison with that extracted from whole cells.

CHAPTER III : Dihydrofolate synthetase was extracted from the cell particles of pea seedlings and purified about 2000 fold by ammonium sulfate fraction, DEAE-cellulose column chromatography, Sephadex G-200

gel filtration, and hydroxylapatite column chromatography. The enzyme preparation obtained was confirmed ultracentrifugationally to be in the homogeneous state. The sedimentation coefficient of this enzyme was calculated as 3.9 S. The apparent molecular weight of the enzyme was determined to be about 56,000.

Optimum pH for the reaction was 8.8. The enzymatic reaction required dihydropteroate, L-glutamate and ATP as substrates, and divalent (Mg^{2+} or Mn^{2+}) and univalent (K^+, NH_4^+ or Rb^+) cations as cofactors. The enzyme was specific for dihydropteroic acid as the substrate. ATP was not replaceable with any other nucleotides. Km values for dihydropteroate, L-glutamate, ATP, Mg^{2+} and Mn^{2+} were 1.0×10^{-6}; 1.5×10^{-3}; 1.0×10^{-4}; 1.1×10^{-3} and 6.3×10^{-5} M, respectively. The enzymatic reaction inhibited by the addition of ADP, but not by AMP. This suggests that the product from ATP in the reaction is composed of ADP + Pi.

CHAPTER IV : The dihydrofolate synthetase was purified from extracts of *S. indica* about 130 fold by ammonium sulfate fractionation, DEAE-Sephadex column chromatography, Sephadex G-200 gel filtrations, and DEAE-cellulose column chromatography. The enzyme preparation obtained was confirmed to be in the homogeneous state by DEAE-cellulose column chromatography and ultracentrifugation. The sedimentation coefficient of this enzyme was calculated as 3.9 S. The apparent molecular weight of the enzyme was determined to be about 47,000. The optimum pH for the reaction was 9.0. L-Glutamate was not replace-

able with L-γ-glutamyl-L-glutamate. Neither pteroic acid nor tetrahydropteroic acid could be used as the substrate. ATP was partially replaced by ITP or GTP. The enzymatic reaction was inhibited by the addition of ADP, but not by AMP. Each one mole of ADP, Pi and dihydrofolate was produced from each one mole of dihydropteroic acid, L-glutamic acid and of ATP by the following equation:

$$\text{Dihydropteroic acid} + \text{L-Glutamic acid} + \text{ATP} \xrightarrow[Mg^{2+},\ K^{+}]{\text{Dihydrofolate synthetase}} \text{Dihydrofolic acid} + \text{ADP} + \text{Pi}$$

These results suggest that the systematic name for dihydrofolate synthetase should be 7,8-dihydropteroate: L-glutamate Ligase (ADP).

CHAPTER V: The dihydrofolate synthetase from *S. indica* was shown to require a divalent cation and a univalent cation for its activity. The divalent cation requirement was satisfied by magnesium ion, manganese ion or ferrous ion. The maximum activity was obtained with 5 mM of magnesium ion. Manganese ion, which was the most effective in activating the dihydrofolate synthetase from pea seedlings, was less effective in activating the *Serratia* enzyme. The univalent cation requirement was satisfied by potassium ion, ammonium ion or rubidium ion, and the maximum activity was obtained with about 100 mM of these univalent cations.

Increasing the potassium concentration in the assay medium decreased the Km values with respect to dihydropteroate and L-glutamate, and increased the Vmax with respect to ATP and dihydro-

pteroate. These results suggest that potassium ion may function in binding dihydropteroate and L-glutamate to the enzyme.

The potassium ion concentration had little effect on the Km value with respect to ATP.

Dihydrofolate synthetase was inhibited by the addition of reduced forms of homopteroic acid. Strong inhibition by dihydrohomopteroate was observed in comparison to that by tetrahydrohomopteroate.

Properties of dihydrofolate synthetase from pea seedlings and *S. indica* are summarized in Table 25.

CHAPTER VI: Dihydrofolate synthetase was widely distributed in various mushrooms and microorganisms. Animals and microorganisms which essentially require pteroylglutamic acid as a nutrient for growth, i.e. the rat and chicken, *L. casei* and *P. cerevisiae* had no detectable dihydrofolate synthetase activity in their livers and in the cells. *S. faecalis* R, which can replace pteroylglutamic acid with pteroic acid as a nutrient for growth, had little dihydropteroate synthase activity but showed normal dihydrofolate synthetase activity. This suggests that the nutritional requirements for folate compounds shown in various organisms *in vivo* will be able to explain by the detection of dihydropteroate synthase activity and dihydrofolate synthetase activities *in vitro*.

These results, showing the wide distribution of dihydrofolate synthetase in plants and microorganisms, and the close relationship between the nutritional requirements for folate compounds *in vivo*

in each organism and its enzyme activity *in vitro*, suggest that this enzyme is a key enzyme in the biosynthetic pathway of folic acid in nature, and also an important enzyme managing the nutritional requirements for folate compounds in organisms.

Table 25

Properties of Dihydrofolate synthetase

Properties	Pea seedlings	*S. indica*
Sedimentation coefficient	3.9 S	3.9 S
Molecular weight	56,000	47,000
Stable pH	7.5	8.0
Km value for dihydropteroate	1.0×10^{-6} M	1.9×10^{-7} M
L-glutamate	1.5×10^{-3} M	2.7×10^{-4} M
ATP	1.0×10^{-4} M	2.2×10^{-4} M
Requirement for nucleotide	ATP	ATP, ITP, GTP
Requirement for divalent cation	$Mn^{2+} > Mg^{2+} > Fe^{2+}$	$Mg^{2+} > Fe^{2+} > Mn^{2+}$
Requirement for univalent cation	$K^{+} > NH_4^{+} > Rb^{+}$	$NH_4^{+} > K^{+} > Rb^{+} > Tl^{+} > Cs^{+}$
Inhibitor	PCMB, Ag^{+}	PCMB

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. Kazuo Iwai, Professor of Kyoto University, for his kind advices and guidance throughout the course of this work.

The author is also indebted to Mr. Fujino, S. for his collaborations in this experimental work.

Thanks are also due to the members of the Research Institute for Food Science, Kyoto University, for their helpful discussions and encouragement.

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