Formic Acid Activation in Plants. I. Purification, Properties and Distribution of Formyltetrahydrofolate Synthetase [1, 2]

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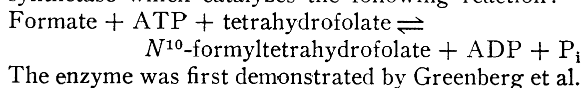
Tetrahydrofolic acid derivatives have been found to be the active cofactors in a number of reactions which involve 1-carbon moieties (9). The participation of tetrahydrofolate enzyme systems in purine and histidine biosynthesis has been well established (9). N^{10}-formyltetrahydrofolate apparently functions in purine synthesis by serving as a 1-carbon donor (2, 9). The synthesis of N^{10}-formyltetrahydrofolate is carried out by the enzyme formyltetrahydrofolate synthetase which catalyzes the following reaction:



$$\text{Formate} + \text{ATP} + \text{tetrahydrofolate} \rightleftharpoons N^{10}\text{-formyltetrahydrofolate} + \text{ADP} + P_i$$

The enzyme was first demonstrated by Greenberg et al. (4) in pigeon liver homogenates. It has been purified from acetone powders of pigeon liver (5), from human erythrocytes (1), from *Micrococcus aerogenes* (12), and from *Clostridium cylindrosporum* (10). The enzyme has been isolated in crystalline form from the latter organism. Existence of the enzyme in tissues of higher plants has not been reported.

This paper describes the purification and properties of the enzyme from tissues of higher plants. Studies of the activation of formyltetrahydrofolate synthetase by potassium and mangnesium are reported in the following paper (6).

Materials and Methods

Reagents. ATP and *dl*-tetrahydrofolate were obtained from the Nutritional Biochemicals Corporation. Other chemicals were of reagent grade and were obtained from commercial sources. Tetrahydrofolate was kept in 1.0 M mercaptoethanol and stored in an evacuated tube at −15°. Tris formate was prepared by neutralizing formic acid to pH 8.0 with solid Tris.

Standard Assay Procedures. For the assay of formyltetrahydrofolate synthetase the standard reaction mixture, in a volume of 1 ml, contained the following constituents: 100 μmoles triethanolamine buffer adjusted to pH 8.0 with HCl; 150 μmoles Tris formate; 2.5 μmoles $MgCl_2$; 200 μmoles KCl; 4 μmoles *dl*-tetrahydrofolate; 2 μmoles ATP and 20 to 60 units of enzyme. A control tube containing no ATP was carried through the procedure. The reaction mixture was incubated for 10 minutes at 30° and the reaction was stopped by the addition of 2 ml of 0.36 N HCl. After an additional 10 minutes absorbancy at 355 mμ was determined with a Beckman DU spectrophotometer. The product absorbing at 355 mμ was methenyltetrahydrofolate formed from the reaction product, formyltetrahydrofolate, by treatment with the acid. The formyltetrahydrofolate synthesized was calculated from the absorbancy using an extinction coefficient of 22×10^6 cm^2 per mole for methenyltetrahydrofolate (1). One unit of enzyme is defined as that amount which gives a synthesis of 1 mμmole of formyltetrahydrofolate under standard assay conditions. The reaction rate was proportional to enzyme concentration up to 200 units of enzyme and was linear with time for at least 30 minutes under standard assay conditions.

Tetrahydrofolate was determined by conducting the assay procedure in the presence of limiting tetrahydrofolate and excess enzyme. Under these conditions the reaction goes to completion (11). Protein was determined by the Folin-phenol method of Lowry et al. (8) using bovine albumin as the standard.

Results and Discussion

Purification of Formyltetrahydrofolate Synthetase. The enzyme was purified from both fresh spinach leaves and acetone powders of spinach leaves. Purification of a typical batch of the enzyme from an acetone powder of spinach leaves is described.

All procedures were carried out at 0 to 4°. Thirty g of spinach leaf acetone powder were stirred for 30 minutes in 500 ml of 0.05 M Tris buffer, pH 7.5, containing 0.01 M mercaptoethanol. The suspension was centrifuged for 20 minutes at 10,000 × *g* and the supernatant solution was brought to 40 % of saturation with solid ammonium sulfate. After 10 minutes the suspension was centrifuged for 20 minutes at 10,-000 × *g* and the supernatant solution was brought to 60 % of saturation with ammonium sulfate. After centrifugation the precipitate was redissolved in 40 ml of 0.05 M Tris, pH 7.5, and 10^{-3} M mercaptoethanol and dialyzed 4 hours against a solution containing 10^{-3} M Tris, pH 7.5, and 10^{-3} M mercaptoethanol.

The dialyzed extract was diluted to 10 mg protein per ml and calcium phosphate gel (3) was added at

[1] Received July 31, 1964.

[2] Contribution (Article No. 64-3-49) of the Department of Agronomy, Kentucky Agricultural Experiment Station, Lexington, and published with the approval of the Experiment Station Director.

the rate of 1 mg gel solids per mg of protein. After stirring for 20 minutes the suspension was centrifuged and the supernatant solution was discarded. The gel was washed by shaking for 20 minutes with a mechanical shaker in a 250 ml centrifuge bottle with glass beads and 100 ml of 0.02 M potassium phosphate, pH 6.8. The suspension was centrifuged and the washing procedure was repeated twice. The enzyme was then eluted from the gel by shaking the gel as described above with seven 100 ml increments of 0.06 M potassium phosphate, pH 6.8. The eluates were combined and brought to 45 % of saturation with ammonium sulfate. The suspension was centrifuged and the supernatant solution was brought to 60 % of saturation with ammonium sulfate. After centrifugation the precipitate was redissolved in 20 ml of 0.05 M Tris, pH 7.5, containing 0.01 M mercaptoethanol. A summary of the purification procedure is presented in table I.

The enzyme purified by the above procedure was used unless otherwise indicated. Further purification was obtained by chromatography of the extract on a DEAE cellulose column with gradient elution with potassium phosphate at pH 6.8. A column (2 cm × 30 cm) was filled to a height of 25 cm with DEAE cellulose and the column was equilibrated with 0.01 M potassium phosphate. One-half of the 45 to 60 % ammonium sulfate fraction from step 4 (table I) consisting of 130 mg protein was dialyzed in 10^{-3} M Tris, pH 7.5 for a period of 6 hours and added to the top of the column. The extract was washed onto the column with approximately 100 ml of 0.01 M potassium phosphate. Gradient elution was begun with the mixing chamber containing 300 ml of 0.01 potassium phosphate, pH 6.8 and the reservoir filled with 0.2 M potassium phosphate, pH 6.8. Fractions of 5 ml of the effluent were collected with a fraction collector. The elution profile, showing both the specific activity of formyltetrahydrofolate synthetase and protein concentration is given in figure 1. The specific activity of fractions 27 to 34 combined was 8600 units per mg of protein and the enzyme recovery was equivalent to 15 % of the initial activity.

Substrate Saturation. The influence of formate concentration on the velocity of the reaction is shown in figure 2. Maximum activity was produced by a formate concentration of approximately 0.2 M. Analysis of these data by the method of Lineweaver and Burk (7) yielded a K_m value with respect to formate of 3.3×10^{-2} M.

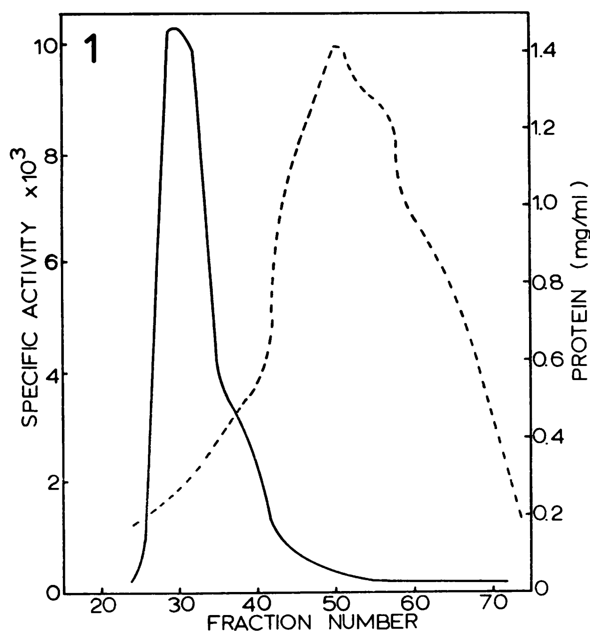


FIG. 1. Chromatography of formyltetrahydrofolate synthetase on DEAE cellulose. 130 mg of protein from step 4, table I, was chromatographed on the column. Solid line, specific activity; broken line, protein concentration.

The influence of ATP on the velocity of the reaction is shown in figure 3. Maximum enzyme activity was obtained with an ATP concentration of 2×10^{-3} M. The K_m value with respect to ATP was calculated to be 9.0×10^{-5}.

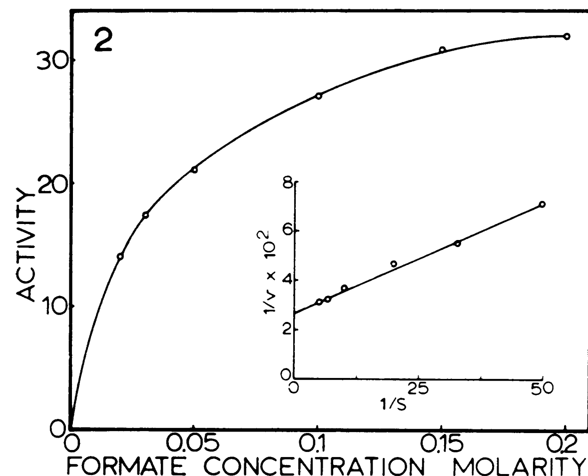
The influence of *dl*-tetrahydrofolate concentration on enzyme activity is shown in figure 4. Maximum activity was produced by a *dl*-tetrahydrofolate concentration of approximately 8×10^{-3} M. The K_m value with respect to *dl*-tetrahydrofolate was calculated to be 9.2×10^{-4} M.

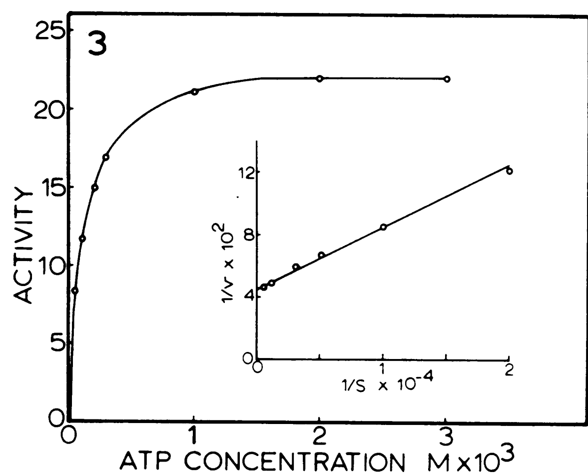
The K_m values for the enzyme from plant tissue do not differ greatly from those reported for the enzyme from other organisms.

Effect of pH on Enzyme Activity. The pH optimum for enzyme activity under standard assay conditions is shown in figure 5. Optimal enzyme activity was obtained in the pH range of 7.5 to 8.5 when triethanolamine was used as the buffer. Enzyme activity

Table I. *Purification of Formyltetrahydrofolate Synthetase from Spinach*

Step	Volume	Total protein	Specific activity	Total activity	Recovery
	ml	mg	Units/mg protein	Units	%
1. Crude extract	410	10,600	122	1,300,000	100
2. 40–60 % $(NH_4)_2SO_4$ ppt	59	4,400	300	1,310,000	101
3. Gel eluate	770	1,230	860	1,050,000	81
4. 45–60 % $(NH_4)_2SO_4$ ppt	22	265	3260	865,000	67





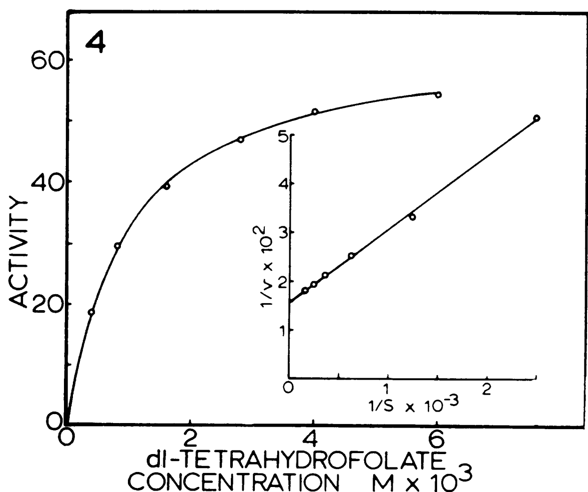


FIG. 2. Effect of formate concentration on enzyme activity. The standard assay procedure was used with purified formyltetrahydrofolate synthetase (14 μg protein) from spinach leaves.

FIG. 3. Effect of ATP concentration on enzyme activity. The standard assay procedure was used with puri-

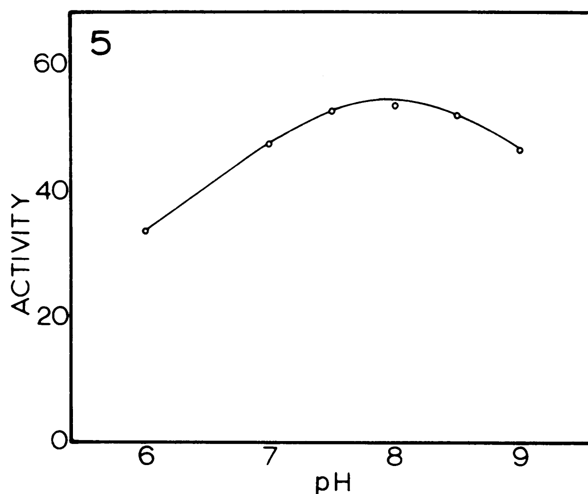


FIG. 5. pH Optimum of formyltetrahydrofolate synthetase using triethanolamine-HCl buffer. The standard assay procedure was used with purified extract (20 μg protein) from spinach leaves.

in various buffers was determined (table II). There was little difference in activity in the presence of triethanolamine, Tris, or glycyl glycine. The reaction rate in the presence of phosphate buffer was approximately one-third of that in the presence of the other buffers. The inhibition of enzyme activity in phosphate buffer was probably due to product inhibition. The enzyme from *Clostridium cylindrosporum* was inhibited by 30 % by glycyl glycine (11).

Stability. Purified formyltetrahydrofolate synthetase can be kept for several weeks without serious loss

Table II. *Activity of Formyltetrahydrofolate Synthetase in Various Buffers*

The standard assay procedure was used with 100 μmoles of the indicated buffers at pH 8.0. Purified enzyme extract from step 4 (20 μg protein) was diluted in 0.05 M Tris, pH 8.0, before use.

Buffer	Enzyme activity
	mμmoles formyltetrahydrofolate formed/10 min
Triethanolamine	90
Glycyl glycine	90
Tris	91
Potassium phosphate	31

fied formyltetrahydrofolate synthetase (10 μg protein) from spinach leaves.

FIG. 4. Effect of *dl*-tetrahydrofolate concentration on enzyme activity. The standard assay procedure was used with purified formyltetrahydrofolate synthetase (20 μg protein) from spinach leaves.

of activity if stored at − 15° at pH 7.5 to 8.0 and in the presence of 0.01 M mercaptoethanol. The enzyme appears to be more stable in the presence of ammonium sulfate. Repeated freezing and thawing destroys activity rather rapidly. At 0° the enzyme is most stable at pH 7.0 to pH 8.0 and in the presence of a reducing agent (table III). Mercaptoethanol had a very marked effect on stability at pH 8.0. While enzyme activity decreased rapidly at pH 8.0 in the absence of mercaptoethanol, little activity was lost in 20 hours in the presence of 0.01 M mercaptoethanol. Activity was lost rapidly at pH 6.0, even in the presence of mercaptoethanol. The increase in activity after 2 hours in the presence of mercaptoethanol at pH 7.0 and 8.0 (table III) was probably a result of reversal of inactivation during dialysis in the absence of mercaptoethanol.

Table III. *Stability of Formyltetrahydrofolate Synthetase*

The enzyme (step 4) was dialyzed for 4 hours against 10^{-3} M Tris, pH 7.5 immediately preceding the experiment. Extracts were then incubated with and without mercaptoethanol and at 3 different pH levels controlled with 0.2 M triethanolamine buffer before assay by the standard assay procedure.

Storage conditions		Activity remaining:	
pH	Time	Without mercaptoethanol	In 10^{-2} M mercaptoethanol
	hr	%	%
6.0	2	78	81
	5	68	75
	20	30	48
7.0	2	90	104
	5	82	95
	20	38	60
8.0	2	82	107
	5	61	93
	20	26	91

Distribution of Formyltetrahydrofolate Synthetase in Plants. Formyltetrahydrofolate synthetase is apparently widely distributed in plants. The enzyme was found in every plant tissue examined for its presence (table IV). There was no particular pattern of distribution of the enzyme in different plant organs or at different stages of growth.

An experiment was conducted to determine the distribution of the enzyme in various fractions of the cell. When plant homogenates were centrifuged for 1 hour at 144,000 × *g*, formyltetrahydrofolate synthetase remained in solution (table V), indicating that the enzyme is in the soluble phase of the cytoplasm.

Summary

The formate activating enzyme, formyltetrahydrofolate synthetase, was found in every plant tissue examined for its presence. The enzyme is associated

Table IV. *Distribution of Formyltetrahydrofolate Synthetase in Various Plant Tissues*

Plant tissues were homogenized with an equal weight of 0.05 M Tris, pH 8.0, and centrifuged at 20,000 × *g* for 15 minutes. Extracts were assayed under standard assay conditions.

Plant tissue	Units/ml extract	Protein	Specific activity
Spinach leaves	730	8.6	85
Pea leaves	1600	18.6	86
Pea roots	178	6.8	26
Bean leaves	590	34.0	17
Bean roots	695	6.0	115
Bean cotyledons	362	21.2	17
Germinating tomato seeds	410	10.8	38
Tobacco leaves	404	12.6	32
Tobacco roots	74	5.2	14
Germinating tobacco seeds	305	7.2	42
Tobacco seedlings	505	14.0	36

Table V. *Recovery of Formyltetrahydrofolate Synthetase in Supernatant Solutions after High Speed Centrifugation of Plant Homogenates*

Homogenates were prepared by grinding the tissue with an equal weight of extraction medium consisting of 0.4 M sucrose, 0.2 M Tris and 5 × 10^{-3} M EDTA at pH 7.5. Extracts were assayed by the standard assay procedure. Precipitate which formed when the homogenate assay was stopped was removed by centrifugation before absorbency reading.

Source of enzyme	Preparation of extract	Specific activity	Recovery after centrifugation
		Units/mg protein	%
Bean roots (*Phaseolus vulgaris* L.)	Homogenate	84	100
	Supernatant from centrifugation*	132	106
Tobacco leaves (*Nicotiana tabacum* L.)	Homogenate	28	100
	Supernatant from centrifugation*	42	98

* Centrifuged at 144,000 × *g* for 1 hour.

with the soluble phase of the cytoplasm. Eighty-fold purification was obtained with ammonium sulfate fractionation, calcium phosphate gel adsorption and DEAE cellulose column chromatography.

The Michaelis constant values with respect to formate, ATP, and *dl*-tetrahydrofolate were calculated to be 3.3×10^{-2} M, 9×10^{-5} M and 9.2×10^{-4} M, respectively. The optimum pH for enzyme activity was 7.5 to 8.5.

The enzyme lost activity rapidly when stored at 0° in the absence of reducing agents, but could be stored for several weeks with little loss of activity at − 15° in 0.01 M mercaptoethanol at pH 7.5 to pH 8.0.

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