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Purification and Regulatory Properties of Mung Bean (*Vigna Radiata* L.) Serine Hydroxymethyltransferase[1]

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ABSTRACT

Serine hydroxymethyltransferase, the first enzyme in the pathway for interconversion of C_1 fragments, was purified to homogeneity for the first time from any plant source. The enzyme from 72-h mung bean (*Vigna radiata* L.) seedlings was isolated using Blue Sepharose CL-6B and folate-AH-Sepharose-4B affinity matrices and had the highest specific activity (1.33 micromoles of HCHO formed per minute per milligram protein) reported hitherto.

The enzyme preparation was extremely stable in the presence of folate or L-serine. Pyridoxal 5′-phosphate, ethylenediaminetetraacetate and 2-mercaptoethanol prevented the inactivation of the enzyme during purification. The enzyme functioned optimally at pH 8.5 and had two temperature maxima at 35 and 55°C. The K_m values for serine were 1.25 and 68 millimolar, corresponding to V_{max} values of 1.8 and 5.4 micromoles of HCHO formed per minute per milligram protein, respectively. The $K_{0.5}$ value for L-tetrahydrofolate (H_4folate) was 0.98 millimolar. Glycine, the product of the reaction and D-cycloserine, a structural analog of D-alanine, were linear competitive inhibitors with respect to L-serine with K_i values of 2.30 and 2.02 millimolar, respectively. Dichloromethotrexate, a substrate analog of H_4folate was a competitive inhibitor when H_4folate was the varied substrate. Results presented in this paper suggested that pyridoxal 5′-phosphate may not be essential for catalysis.

The sigmoid saturation pattern of H_4folate (n_H = 2.0), one of the substrates, the abolition of sigmoidicity by NADH, an allosteric positive effector (n_H = 1.0) and the increase in sigmoidicity by NAD^+ and adenine nucleotides, negative allosteric effectors (n_H = 2.4) clearly established that this key enzyme in the folate metabolism was an allosteric protein. Further support for this conclusion were the observations that (a) serine saturation exhibited an intermediary plateau region; (b) partial inhibition by methotrexate, aminopterin, *O*-phosphoserine, DL-α-methylserine and DL-*O*-methylserine; (c) subunit nature of the enzyme; and (d) decrease in the n_H value from 2.0 for H_4folate to 1.5 in presence of L-serine.

These results highlight the regulatory nature of mung bean serine hydroxymethyltransferase and its possible involvement in the modulation of the interconversion of folate coenzymes.

The major storage form of folate coenzymes in plants is N^5-methyltetrahydrofolate (21). Although this compound is present in abundant quantities the metabolism of C_1 compounds and the regulation of the enzymes involved in the biosynthesis of these folate compounds is incompletely understood (2). The first enzyme in the pathway for the interconversion of C_1 compounds is serine hydroxymethyltransferase (5,10-methylenetetrahydrofolate-glycine hydroxymethyltransferase, EC 2.1.2.1) which catalyzes the transfer of the hydroxymethyl group of serine to H_4folate[3] to give N^5,N^{10}-methylene H_4folate and glycine. In addition to its reduction to N^5-CH_3-H_4folate, the N^5,N^{10}-methylenetetrahydrofolate is converted via a series of reactions, to purines, thymidylate, etc. The N^5-CH_3-H_4folate in turn donates its methyl group to homocysteine to form methionine whose central role in methyltransfer reactions via *S*-adenosylmethionine to form important biological compounds like Chl, plastoquinones, lignin, and pectin is well established (6). Thus, a systematic investigation of the regulation of the enzymes of the folate pathway is essential for the understanding of the modulation of biosynthesis of the above mentioned plant metabolites.

Early investigations (5, 14) revealed that glycolate and glycine infiltrated into higher plants were readily converted to serine. Later investigations, with cell-free preparations from several plant materials (4), led to the characterization of this reaction as a folate dependent one. Detailed investigation on serine hydroxymethyltransferase showed that this enzyme from bacterial and mammalian sources (18, 22) was a pyridoxal phosphate dependent enzyme and catalyzed the following reaction:

$$\text{L-Serine} + H_4\text{folate} \rightleftharpoons N^5,N^{10}\text{-}CH_2\text{-}H_4\text{folate} + \text{glycine}.$$

Recent work in our laboratory established that this enzyme is a regulatory protein exhibiting homotropic and heterotropic interactions (7, 16, 18). It was therefore of interest, in spite of a large number of unsuccessful attempts by earlier workers (13, 15, 28), to isolate the enzyme from a plant source and examine its interactions with various metabolites.

MATERIALS AND METHODS

Chemicals. The following biochemicals were obtained from Sigma: L-serine, DL-dithiothreitol, 2-mercaptoethanol, pyridoxal 5′-phosphate, EDTA (disodium salt), D-cycloserine, glycine, Tris, ammonium persulfate, Blue dextran, SDS, 3-(3-dimethylaminopropyl) 1-ethylcarbodiimide, Folin-Ciocalteu phenol reagent, Coomassie Brilliant Blue G, folic acid, ovalbumin, Cyt *c*, α-chymotrypsinogen, ferritin, catalase (EC 1.11.1.6), human IgG, conalbumin, NAD^+, NADH, NADP, AMP, ATP, TMP, spermine tetrahydrochloride, spermidine trihydrochloride, acrylamide, N,N^1-methylenebis acrylamide, Coomassie Brilliant Blue R, L-cysteine, PPO, D-alanine, L-lysine, L-methionine, L-threonine, DL-α-methylserine, *O*-phosphoserine, and DL-*O*-methylserine.

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[3] Abbreviations: H_4folate (±), DL-tetrahydrofolate; N^5-CH_3-H_4folate, N^5-methyltetrahydrofolate; N^5,N^{10}-CH_2-H_4folate: N^5,N^{10}-methylenetetrahydrofolate; Dimedon: 5,5′-dimethylcyclohexane-1,3-dione.

DEAE-Sephadex A-50, Blue Sepharose CL-6B, Sephacryl S-200 (super fine grade) and AH-Sepharose were from Pharmacia. Ultrogel AcA-34 was a gift from LKB. Aquacide II-A C-grade was purchased from Calbiochem. H_4folate prepared by the method of Hatefi *et al.* (8) was a gift from Dr. John H. Mangum, Brigham Young University. Methotrexate, dichloromethotrexate, and aminopterin were kindly given by Dr. Robert Silber, New York University Medical Center, New York. L[3-^{14}C]Serine (58.5 mCi/mmol) was purchased from the Radiochemical Center, Amersham. All other chemicals were of analytical grade. Organic solvents were distilled before use.

Germination of Mung Bean (*Vigna radiata* L.) Seeds. Mung bean seeds, purchased from the local market, were surface sterilized by washing with 0.02% $HgCl_2$ solution, washed free of $HgCl_2$, and soaked in distilled H_2O for 8 h. The soaked seeds were spread on moist filter paper placed on a wet cotton bed in a tray. The seeds were allowed to germinate for 72 h at 37°C in the dark.

Assay of Serine Hydroxymethyltransferase. The assay mixture (0.1 ml) contained 20 mM K-phosphate (pH 7.4), 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM pyridoxal 5'-phosphate, 1.8 mM H_4 folate, 3.6 mM L-[3-^{14}C]serine (~60,000 counts), 1.8 mM DTT, and an appropriate amount of the enzyme. The mixture was preincubated for 5 min at 37°C. The reaction was started by the addition of L-serine and stopped by the addition of 0.1 ml dimedon (0.4 M in 50% [v/v] ethanol). The mixture was heated for 5 min at 100°C, [^{14}C]formaldehyde-dimedon adduct extracted into toluene, and the radioactivity determined in a Beckman LS-100C liquid scintillation counter (16). One unit of the enzyme activity was defined as the amount that catalyzed the formation of 1 μmol formaldehyde/min at 37°C at pH 7.4. Specific activity was expressed as units/mg protein. Protein concentration was determined by the method of Lowry *et al.* (11), with BSA as the standard.

Preparation of Tissue Homogenate and Supernatant Fractions. The following buffers were used in this study: buffer A, 300 mM mannitol, 1 mM EDTA, 0.01% BSA, and 1 mM 2-mercaptoethanol in 1 liter of deionized H_2O (pH 7.2), and was used for grinding tissues. buffer B, the same as above without 2-mercaptoethanol to wash the 6,780*g*- and 105,500*g*-pellets.

The chilled 72-h mung bean seedlings were minced and homogenized for 30 s in buffer A and the homogenate was passed through two layers of cheese cloth. The resulting filtrate was then centrifuged for 15 min at 1,000*g*. The supernatant was spun at 10,800*g* for 15 min.

The pellet obtained was suspended in the buffer B and centrifuged at 270*g* for 10 min. The supernatant thus obtained was centrifuged at 6,780*g* for 15 min and the pellet obtained suspended in buffer B. This fraction was referred to as the 6,780*g* pellet. The 10,800*g* supernatant was centrifuged at 17,300*g* for 15 min and the resultant supernatant centrifuged at 105,500*g* for 60 min in a Beckman model LS-50 preparative ultracentrifuge. This supernatant was referred to as the cytosol and the pellet obtained suspended in buffer B was referred to as the 105,500*g* pellet.

Coupling of Folic Acid to AH-Sepharose. To a mixture of AH-Sepharose (5 g) and folic acid (330 mg) in 40 ml of deionized H_2O, 5 ml 0.8 M 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide was added. The pH was maintained at 5.5 by the addition of 0.1 N NaOH. After stirring overnight at 4°C in the dark, the gel was washed successively with 1 liter each of 1 N NaOH containing 1 M NaCl, deionized H_2O, 1 N HCl containing 1 M NaCl, and finally with 2 liters of deionized H_2O. Folate-AH Sepharose was stored in 1 M K-phosphate (pH 7.4), containing 1 M NaCl and 10 mM 2-mercaptoethanol in test tubes under N_2, in the dark at 4°C (18).

Blue Sepharose CL-6B. Freeze-dried powder of Blue Sepharose CL-6B was washed free of dextran with 0.1 M NaCl and then with deionized H_2O. The gel was stored in 10 mM K-phosphate (pH 7.4).

Purification of Mung Bean Serine Hydroxymethyltransferase. K-phosphate (pH 7.4, 50 mM), containing 1 mM 2-mercaptoethanol, 1.0 mM pyridoxal 5'-phosphate, and 1.0 mM EDTA (hereafter referred to as buffer C) was used for homogenizing the seedlings. K-phosphate (pH 7.4, 10 mM), containing 1 mM 2-mercaptoethanol, 1.0 mM pyridoxal 5'-phosphate, and 1.0 mM EDTA (hereafter referred to as buffer D) was used for dialysis and for equilibration of chromatographic columns, etc. All the purification steps were carried out at 0 to 4°C and all centrifugations at 17,300*g* for 15 min in a Sorvall RC 5B centrifuge.

Step 1: Preparation of Crude Extract. Mung bean seedlings (72-h old, 250 g) were homogenized in a precooled Waring Blendor for 2 min with 80 ml buffer C. The homogenate was passed through two layers of cheese cloth and the filtrate was centrifuged.

Step 2: Ammonium Sulfate Fractionation. Solid ammonium sulfate (22.6 g/100 ml) was added to the supernatant solution to obtain 0.40 of saturation. After 10 min, the precipitate was removed by centrifugation. The supernatant fraction was raised to 0.8 of saturation by a further addition of solid ammonium sulfate (25.8 g/100 ml) and the precipitate was dissolved in a small volume (about 5 ml) of buffer C. This solution was dialyzed for 40 h against six changes of 1 liter of buffer D.

Step 3: Negative Adsorption on DEAE-Sephadex A-50. The clear supernatant obtained after centrifugation of the dialyzed enzyme from the previous step was added to 30 ml packed volume of DEAE-Sephadex A50 equilibrated with buffer D and stirred gently for 10 min. The supernatant solution obtained on centrifugation at 3,020*g* for 5 min was designated as DEAE-Sephadex supernatant.

Step 4: Group-Specific Affinity Chromatography on Blue Sepharose CL-6B. Blue Sepharose CL-6B was packed into a column (1 × 20 cm) and equilibrated with buffer D. The enzyme fraction from the previous step was loaded on to the affinity gel column at a flow rate of 10 ml/h. The column was then washed with buffer D at a flow rate of 20 ml/h, until the eluate had an *A* of 0.02 at 280 nm. The enzyme was desorbed from the Blue Sepharose gel with buffer D containing 1 M KCl. Fractions (2 ml) containing serine hydroxymethyltransferase activity were pooled and concentrated using Aquacide II-A C-grade.

Step 5: Molecular Exclusion Chromatography on Sephacryl S-200. The concentrated enzyme (2 ml) was chromatographed on a Sephacryl S-200 column (bed volume, 190 ml), equilibrated with buffer D containing 0.1 M KCl at a flow rate of 18 ml/h. Fractions (2 ml) with specific activity greater than 0.50 were pooled and dialyzed against 2 liters of buffer D.

Step 6: Affinity Chromatography on Folate-AH-Sepharose-4B. The dialyzed enzyme obtained from the previous step was loaded on to the folate-AH-Sepharose matrix. It was left on the column for 30 min before washing the column with buffer D. The washing was continued until the eluate had an *A* of 0.02 at 280 nm. The enzyme was desorbed from the folate-AH-Sepharose gel with buffer D containing 10 mM folic acid adjusted to pH 7.4. Five fractions of 1.5 ml containing the enzyme activity were pooled and used in this study. A typical purification chart is shown in Table I.

Regeneration of DEAE-Sephadex A-50, Blue Sepharose CL-6B, and Folate-AH-Sepharose-4B Matrices. The columns were regenerated for repeated use by washing the gel with 3 bed volumes of buffer D containing 3 M KCl, followed by 40 bed volumes of deionized H_2O. The gels were stored in buffer D containing 0.02% sodium azide. The azide was washed off before use.

Analytical Polyacrylamide Gel Disc Electrophoresis. Polyacrylamide gel disc electrophoresis at 4°C with 0.5 M Tris-0.39 M glycine buffer (pH 8.6), in 7.5% gels was conducted at a current of 4 mamp/gel. The gel was stained for protein either with 0.02% Coomassie Brilliant Blue G in 3.5% (v/v) $HClO_4$ at room temperature for 2 h or with Coomassie Brilliant Blue R. Destaining of

Table I. *Purification of Serine Hydroxymethyltransferase from Mung Bean Seedlings*

One unit is defined as 1 μmol of HCHO formed/min at 37°C (pH 7.4).

Step	Total Activity	Specific Activity	Recovery	Purification
	units	*units/mg protein* $\times 10^{-3}$	*%*	*-fold*
Crude	6.60	2.8	100	1
$(NH_4)_2SO_4$ (40–80%)	3.86	4.6	59	2
DEAE Sephadex A-50	4.23	25.0	64	9
Blue Sepharose CL-6B	2.49	128	38	45
Sephacryl S-200	0.75	742	11	265
Folate sepharose	0.58	1,330	8	475

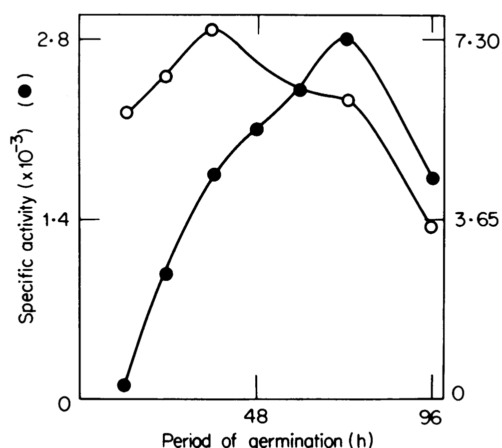

FIG. 1. Changes in serine hydroxymethyltransferase activity during germination of mung bean (*Vigna radiata* L.). Mung bean seeds were germinated in separate batches for different intervals indicated in the figure at 37°C. In each group, 20 g wet weight of seedlings were homogenized with 10 ml buffer C (pH 7.4). The homogenate was passed through two layers of cheese cloth and the filtrate was centrifuged at 12,000*g* for 10 min. The supernatant fraction was assayed for enzyme activity (16).

Coomassie Brilliant Blue G-stained gels was not necessary (20) and in the case of Coomassie Brilliant Blue R, destaining of the gels was carried out using the solvent system acetic acid-methanol-water (2:1:20, v/v/v). Attempts at locating the enzyme on the gel by activity staining or sequentially slicing the gel and eluting the enzyme were unsuccessful.

Molecular Weight Determination. The mol wt of serine hydroxymethyltransferase was determined by gel filtration (1) using Ultrogel AcA-34 equilibrated with buffer D containing 0.1 M KCl. The following proteins of known mol wt served as markers: horse heart ferritin (480,000), bovine liver catalase (232,000), human IgG (156,000), and horse heart Cyt *c* (12,384). The mol wt was calculated from a plot of log mol wt against elution volume. The subunit mol wt of mung bean serine hydroxymethyltransferase was determined by SDS/polyacrylamide gel disc electrophoresis in the presence and absence of 2-mercaptoethanol by the method of Weber and Osborn (25).

RESULTS

Changes in the Activity of Mung Bean Serine Hydroxymethyltransferase During Germination. Mung bean seeds were germinated in separate batches for different intervals of time (Fig. 1). It is evident from the figure that the highest specific activity of the enzyme was attained after 72 h of germination and thereafter it decreased. The total activity, however, was high to start with and decreased only after 36 h. As the specific activity of the 72-h germinated seedlings was high, these seedlings were used as the starting material for the purification of the enzyme.

Subcellular Distribution of Serine Hydroxymethyltransferase in Mung Bean. The cytosol fraction contained the maximum activity (~3 units), whereas the 6,780*g* pellet and the 105,500*g* pellet had only 2% of the total activity present in the homogenate. The cytosolic fraction had the highest specific activity. The recovery was 134% and the high recovery was probably due to removal of some naturally occurring inhibitor(s) during fractionation.

Enzyme Purification. The final enzyme preparation on polyacrylamide gel disc electrophoresis (Fig. 2) revealed a single protein band. The specific activity of the pooled fractions from this affinity column was 1.33 units/mg protein (step 6, Table I). The specific activities of several preparations of the enzyme were in the range of 1.10 to 1.50 units/mg protein. All these preparations gave a single band on electrophoresis.

Molecular Weight of Mung Bean Serine Hydroxymethyltransferase. The mol wt of the folate enzyme (step 6, Table I) was estimated to be 205,000 ± 5,000 by using a calibrated Ultrogel AcA-34 gel column.

SDS/polyacrylamide gel disc electrophoresis of the purified enzyme revealed a single protein band. The mol wt of this polypeptide chain was estimated to be 50,000 ± 1,400.

Stability of the Purified Mung Bean Serine Hydroxymethyltransferase. When the enzyme from the folate affinity matrix (step 6, Table I) was dialyzed against buffer D for 24 to 30 h with six changes of 500 ml buffer each time, it was inactivated (28%, Table II). This enzyme could not be reactivated by the addition of folate (1-20 mM). The enzyme could also be eluted from the folate affinity matrix with 1 M KCl, but it was rapidly inactivated (results not given). These experiments indicate that folate protects the

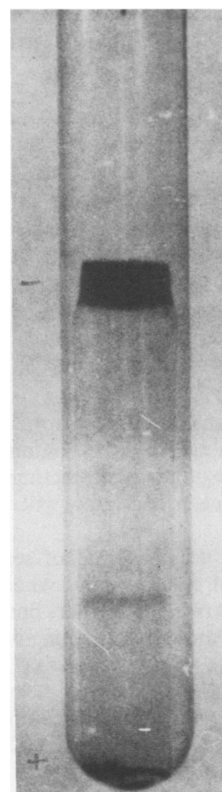

FIG. 2. Polyacrylamide gel disc electrophoresis of mung bean serine hydroxymethyltransferase. The final enzyme preparation (105 μg, step 6, Table I) was electrophoresed in 7.5% polyacrylamide gels at pH 8.3 in Tris-glycine buffer and a current of 4 mamp/tube. The gel was stained for protein with Coomassie Brilliant Blue R and destained.

Table II. *Stability of Mung Bean Serine Hydroxymethyltransferase*

(A) Enzyme (step 6, Table I). (B) The enzyme (100 μg) was dialyzed against 1 liter buffer D for 24 h at 4°C. (C) The enzyme was dialyzed against 2 liters of buffer D containing 3.6 mM L-serine for 36 h at 4°C. (D) The enzyme was dialyzed against 1 liter 50 mM K-phosphate (pH 7.4) containing 3.6 mM L-serine for 36 h at 4°C. (E) One ml enzyme was dialyzed against 1 liter 50 mM K-phosphate (pH 7.4) containing 3.6 mM L-serine for 36 h at 4°C. To different aliquots of this enzyme were added: (a) 1 mM 2-mercaptoethanol; (b) 1 mM EDTA; (c) 0.1 mM pyridoxal 5'-phosphate; and (d) all the above components together.

Serial No.	Component Omitted	Specific Activity
		units/mg protein $\times 10^{-3}$
A	None	1.22
B	Folic acid	0.34
C	Folic acid, added 3.6 mM L-serine	1.26
D	2-Mercaptoethanol, EDTA, pyridoxal 5'-phosphate, added 3.6 mM L-serine	0.60
E	(a) To D added 1 mM 2-mercaptoethanol	0.62
	(b) To D added 1 mM EDTA	0.58
	(c) To D added 0.1 mM pyridoxal 5'-phosphate	0.59
	(d) To D added 1 mM 2-mercaptoethanol, 1 mM EDTA and 0.1 mM pyridoxal 5'-phosphate	0.57

Table III. *Requirement of Pyridoxal 5'-Phosphate for Catalysis*

(F) The enzyme was dialyzed against buffer D containing 3.6 mM L-serine for 40 h with four 500-ml changes. The enzyme was designated as the 'Serine-enzyme.' (G) To the serine-enzyme, 10 mM L-cysteine were added and incubated for 20 min at 4°C. (H) The cysteine-treated serine-enzyme was dialyzed against buffer D containing 3.6 mM L-serine but without 0.1 mM pyridoxal 5'-phosphate. (I) To the enzyme obtained from the above treatment, 0.1 mM pyridoxal 5'-phosphate was added.

Serial No.	Enzyme	Specific Activity
		units/mg protein $\times 10^{-3}$
F	Serine-enzyme	1.26
G	Serine-enzyme plus 10 mM L-cysteine	0.19
H	Enzyme G dialyzed against buffer D minus 0.1 mM pyridoxal 5'-phosphate but containing 3.6 mM L-serine	1.29
I	Enzyme H plus 0.1 mM pyridoxal 5'-phosphate	1.17

enzyme from inactivation. It is evident from Table III that dialysis of the folate enzyme against buffer D containing 3.6 mM L-serine, the second substrate, resulted in protecting the enzyme against inactivation. This enzyme preparation will be hereafter referred to as serine-enzyme.

It was observed that the mammalian serine hydroxymethyltransferases (18, 22) lost their activity when 2-mercaptoethanol and EDTA were omitted from the buffers both during purification and dialysis. These enzymes could be reactivated by the addition of these components. The mung bean serine hydroxymethyltransferase was partially inactivated when 2-mercaptoethanol and EDTA were omitted from buffer D and, unlike in the mammalian enzyme, the activity was not regained upon addition of these compounds (Table II).

Requirement of Pyridoxal 5'-Phosphate for Catalysis. The serine-enzyme was treated with 10 mM L-cysteine for 20 min at 4°C and dialyzed against buffer D without pyridoxal 5'-phosphate but containing 3.6 mM L-serine. It can be seen from Table III that there was no significant loss of activity. Similar results were obtained when the folate-enzyme was treated with 10 mM, L-cysteine and dialyzed against buffer D containing 10 mM folic acid but not pyridoxal 5'-phosphate (results not given). The spectrum of the enzyme obtained after cysteine treatment and dialysis had no absorbance in the visible region (figure not shown). Omission of pyridoxal 5'-phosphate in the buffer during purification of the enzyme resulted in rapid inactivation.

Catalytic Properties of Mung Bean Serine Hydroxymethyltransferase. The purified enzyme showed: (a) A linear relationship between initial reaction rate and enzyme concentration up to 40 μg protein/ml reaction mixture (Fig. 3A). (b) A linear rate of formaldehyde formation for 20 min when 3.6 mM L-serine, 1.8 mM H_4folate; and 40 μg protein/ml reaction mixture were used (Fig. 3B). (c) Optimum enzyme activity was observed at pH 8.5 at 37°C when 3.6 mM L-serine and 1.8 mM H_4folate were used (Fig. 3C). (d) Optimum enzyme activity was observed at 35 and 55°C at pH 7.4 when saturating concentrations of 3.6 mM L-serine and 1.8 mM H_4folate were used (Fig. 3D).

Regulatory Properties of Mung Bean Serine Hydroxymethyltransferase. A sigmoidal saturation pattern was observed when the activity of mung bean serine hydroxymethyltransferase was assayed in the presence of increasing concentrations of H_4folate and at 3.6 mM L-serine (Fig. 4). The same data plotted as a double reciprocal plot gave a concave upward curvilinear line (inset A, Fig. 4). The $K_{0.5}$ and n_H values of 0.98 mM and 2.0, respectively, were calculated (from inset B, Fig. 4) using the Hill equation (23).

The prevalence of cooperative interactions with H_4folate indicated in Figure 3 was confirmed by determining the saturation pattern of H_4folate in the presence of NADH and NAD^+, two

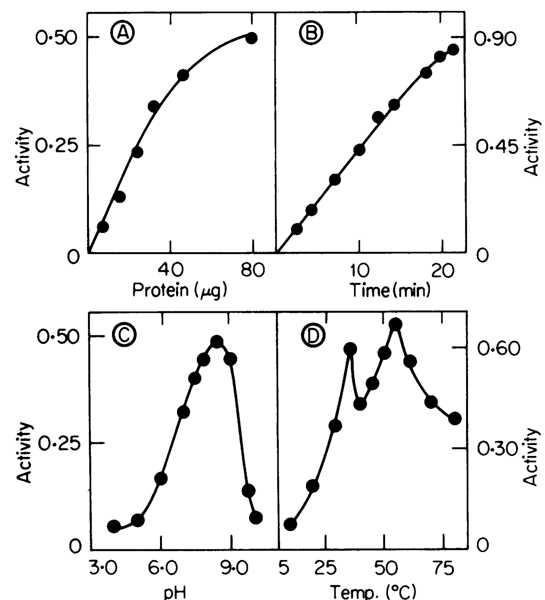

FIG. 3. Catalytic properties of mung bean serine hydroxymethyltransferase. A, effect of enzyme concentration: Varying amounts of purified enzyme as indicated in the figure were incubated with 1.8 mM H_4folate and 3.6 mM L-serine and enzyme activity assayed (16). B, time course of the reaction: The reaction mixture was scaled up to 1.5 ml and contained 400 μg of the enzyme. Aliquots (0.1 ml) were withdrawn at time intervals indicated in the figure and the amount of formaldehyde was estimated (16). C, pH optima of the enzyme: The enzyme (40 μg/ml reaction mixture) was incubated with different buffers (pH 4.0–10.5) as indicated in the figure and assayed for enzyme activity (16). Range of buffers used: 0.8 M sodium acetate-acetic acid buffer (pH 3.6–5.6); 0.8 M K-phosphate (pH 5.7–8.0); 0.8 M Tris-HCl buffer (pH 7.2–9.0), and 0.8 M sodium carbonate bicarbonate buffer (pH 9.2–10.7). Buffer ions had no effect on the enzyme activity. D, temperature optima of the enzyme: The enzyme (40 μg/ml reaction mixture) was assayed (16) at different temperatures as indicated in the figure in presence of saturating amounts of substrates.

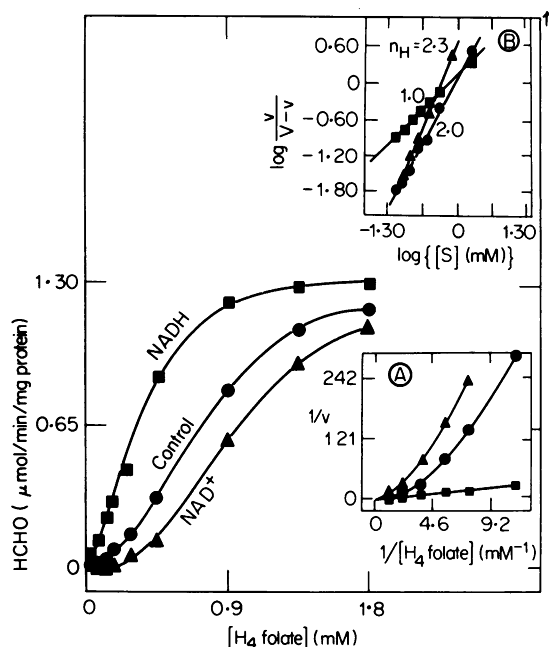

FIG. 4. Cooperative interactions of H_4folate and the effects of nicotinamide nucleotides on these interactions with mung bean serine hydroxymethyltransferase. The enzyme was preincubated with H_4folate (concentrations indicated in the figure) for 5 min at 37°C. The reaction was started by the addition of 3.6 mM L-[3-^{14}C]serine and the assay mixtures were incubated for 15 min at 37°C and [^{14}C]formaldehyde estimated (16). Preincubated with H_4folate (●). The effects of 10 mM NADH (■) and 10 mM NAD^+ (▲) were studied by incubating the enzyme already preincubated with H_4folate with NADH or NAD^+ for 5 min at 37°C and assayed for activity (16). Care was taken to ensure that solutions of all reaction components were adjusted to pH 7.4. (Inset A), Lineweaver-Burk plots in the presence and absence of the added nucleotides; (●), no effector; (■), 10 mM NADH; (▲), 10 mM, NAD^+. (Inset B), Hill plots of the saturation data obtained.

allosteric effectors. In the presence of 10 mM NADH, the sigmoid pattern of H_4folate saturation was converted to a hyperbolic one, whereas in the presence of 1 mM NAD^+, the pattern was more sigmoid. Analysis of this data by a double reciprocal plot gave a linear and more curved line for NADH and NAD^+, respectively. Hill plots of the above data gave n_H values of 1.0 and 2.3 for NADH and NAD^+, respectively (insets A and B, Fig. 4). Both these metabolites had no significant effect at saturating concentrations of H_4folate indicating that they may be functioning as allosteric effectors. Both NADH and NAD^+ were not consumed in the reaction. The effects of NADPH and NADH were the same. These allosteric interactions were present at all stages of purification of the enzyme indicating that these observations were not artifacts of purification but were inherent properties of the enzyme.

In addition to the nucleotides, several end products of C_1 metabolism as well as related compounds were tested for their effect on the activity and on H_4folate saturation. AMP and ATP were negative allosteric effectors as they increased the sigmoidicity of the H_4folate saturation reflected by the increase in the n_H and $K_{0.5}$ values from 2.0 to 2.4 and from 0.9 to 1.28 mM, respectively (Table IV). NADH, which is a substrate for the next enzyme in the folate pathway N^5,N^{10}-CH_2-H_4folate reductase, was a positive allosteric effector as evidenced by the decrease in n_H values with increasing concentrations of NADH. TMP, the product of thymidylate synthetase which utilizes N^5,N^{10}-CH_2-H_4folate, decreased the n_H value from 2.0 to 1.5 (Table IV).

Lysine was reported to inhibit the enzyme from *Lemna minor* in view of the common intermediate in the biosynthesis of lysine

Table IV. *Regulation of Mung Bean Serine Hydroxymethyltransferase by Nucleotides*

The folate enzyme was preincubated with the various effectors mentioned and with 1.8 mM H_4folate for 5 min at 37°C and the enzyme activity was determined (16). The n_H and $K_{0.5}$ values were calculated from the Hill plots of the saturation data.

Effector Added	n_H	$K_{0.5}$
mM		*mM*
None	2.00	0.98
NADH, 1	1.63	0.80
NADH, 2	1.50	0.70
NADH, 5	1.30	0.63
NADH, 10	1.00	0.48
NAD^+, 1	2.10	1.02
NAD^+, 5	2.25	1.10
NAD^+, 10	2.30	1.15
NADP, 5	2.20	1.07
AMP, 1	2.42	1.28
ATP, 1	2.40	1.28
TMP, 1	1.68	0.82
TMP, 10	1.53	0.75

Table V. *Regulation of Mung Bean Serine Hydroxymethyltransferase by Amino Acids and Polyamines*

The folate enzyme was preincubated with 1.8 mM, H_4folate and various amino acids and polyamines mentioned for 5 min at 37°C and enzyme activity was assayed (16). The n_H and $K_{0.5}$ values were calculated from the Hill plots of the saturation data.

Effector Added	n_H	$K_{0.5}$	V_{max}
mM			*units × 10^3*
None	2.00	0.98	1,330
L-Lysine, 1	1.96	0.98	1,536
L-Lysine, 10	1.96	0.96	1,626
L-Methionine, 10	2.10	1.10	1,400
L-Threonine, 10	2.10	1.19	1,285
L-Serine, 3.6	1.50	0.70	1,342
Spermidine, 1	1.916	1.00	1,620
Spermine, 1	1.98	0.90	1,332

and methionine (26). It was, therefore of interest to examine the effects of these amino acids on mung bean serine hydroxymethyltransferase activity. Lysine and methionine were simple activators without any significant effect on n_H values (Table V). Threonine, which was reported to be an alternate substrate for the enzyme from mammalian sources was also without any effect on the substrate saturation pattern of H_4folate, suggesting that the compound may not be a substrate for the plant enzyme. Polyamines have been implicated as growth factors and play an important role in cell division and it was of interest to examine the effect of polyamines on mung bean serine hydroxymethyltransferase activity. Spermidine and spermine had only a marginal effect on the n_H value (Table V).

A hyperbolic saturation pattern was obtained with serine at saturating concentrations of H_4folate (Fig. 5) with an intermediary saturation region between 12 and 40 mM. Increasing the serine concentrations up to 200 mM resulted in a further increase in the velocity and an apparent maximum value was obtained at 200 mM L-serine. Two straight lines with different slopes corresponding to K_m values of 1.25 and 68 mM, respectively were obtained in the double reciprocal plot of the same data (inset, Fig. 5).

Effect of Antifolate Compounds on Mung Bean Serine Hydroxymethyltransferase. Inhibition of serine hydroxymethyltransferase activity by methotrexate, aminopterin, and dichloromethotrexate followed different patterns (Fig. 6). The concentrations required for 50% inhibition were 2.6 mM (dichloromethotrexate), 17.4 mM

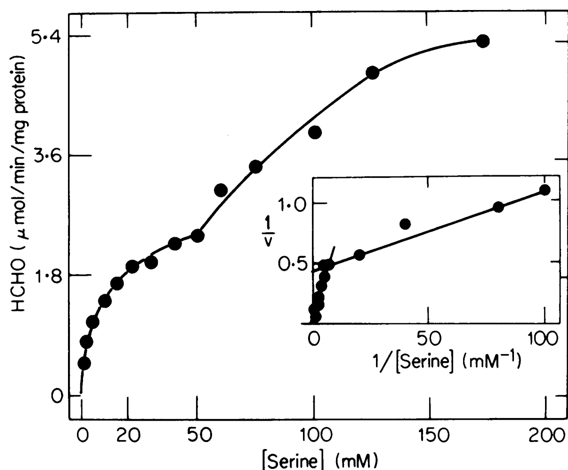

FIG. 5. Serine saturation of mung bean serine hydroxymethyltransferase. The enzyme was preincubated with L-serine (concentrations indicated in the figure) for 5 min at 37°C. The reaction was started by the addition of 1.8 mM H_4folate and the enzyme activity assayed (16). (Inset), Lineweaver-Burk plot.

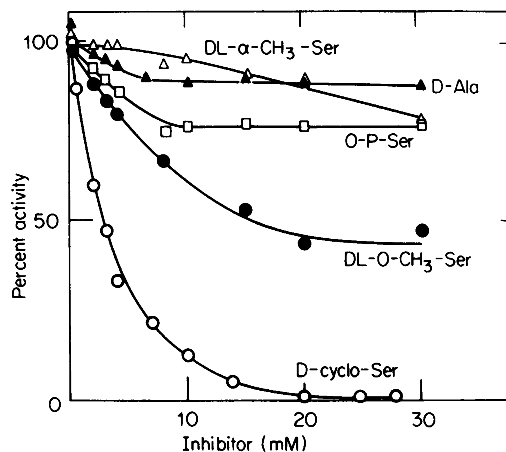

FIG. 7. Inhibition of mung bean serine hydroxymethyltransferase by serine analogs. The enzyme was preincubated successively for 5 min at 37° with 1.8 mM H_4folate and D-alanine (▲) or DL-α-methylserine (△) or O-phosphoserine (□) or DL-O-methylserine (●) or D-cycloserine (○) and the enzyme activity was assayed (16). The enzyme activity in the absence of inhibitor was normalized to 100.

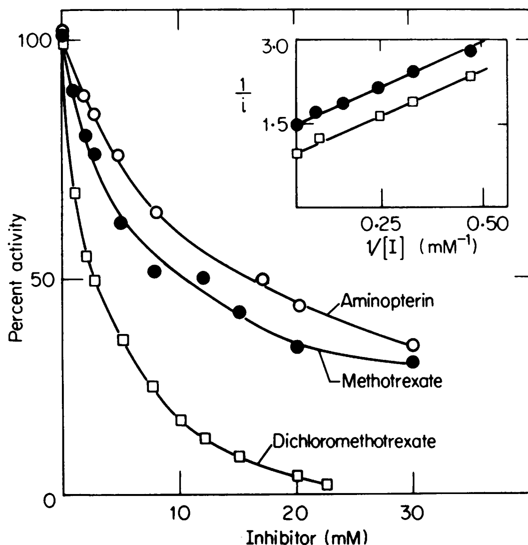

FIG. 6. Inhibition of mung bean serine hydroxymethyltransferase by antifolate compounds. The enzyme was preincubated successively for 5 min at 37°C with 1.8 mM H_4folate and methotrexate (●) or aminopterin (○) or dichloromethotrexate (□) and the enzyme activity assayed (16). The enzyme activity in the absence of inhibitor was normalized to 100. (Inset), plot of reciprocal of fractional inhibition (i) *versus* the reciprocal of inhibitor concentration. Aminopterin also gave a similar result as methotrexate (not shown in figure).

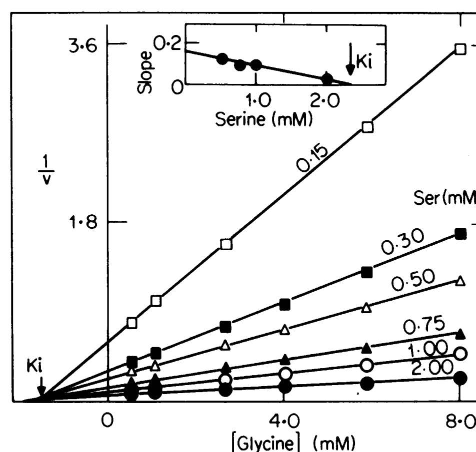

FIG. 8. Competitive inhibition of mung bean serine hydroxymethyltransferase activity by glycine. Dixon plot: The enzyme was preincubated with 1.8 mM H_4folate and varying concentrations of L-serine: 0.150 mM (□), 0.30 mM (■), 0.50 mM (△), 0.75 mM (▲), 1.0 mM (○), and 2.0 mM (●) at different fixed concentrations of glycine (0–8 mM) as indicated in the figure. (Inset), replot of slopes obtained from the Dixon plot *versus* serine concentration as indicated in the figure.

(aminopterin), and 10 mM (methotrexate). In partial inhibition analysis (23, 24), an ordinate intercept value greater than 1 (inset, Fig. 6) was obtained when $1/i$ was plotted against $1/[I]$ which suggested that methotrexate was a partial inhibitor. Aminopterin was also a partial inhibitor (details not shown in inset, Fig. 6). Dichloromethotrexate completely inhibited the enzyme activity as indicated by the intercept value of 1 (inset, Fig. 6).

Inhibition of Mung Bean Serine Hydroxymethyltransferase by Serine Analogs. DL-α-Methylserine, O-phosphoserine, and DL-O-methylserine inhibited the mung bean serine hydroxymethyltransferase activity partially whereas D-cycloserine, a rigid cyclic analog of D-alanine inhibited the enzyme activity completely (Fig. 7).

Determination of Inhibition Constants (K_i) for Glycine and D-Cycloserine. The product of the reaction, glycine (0-8 mM) competitively inhibited the enzyme when L-serine was the varied substrate at a saturating concentration of H_4folate. A Dixon plot of $1/v$ *versus* glycine concentration gave a K_i value of 2.30 mM (Fig. 8, inset).

D-Cycloserine which inhibited the enzyme activity completely was also a competitive inhibitor (Fig. 9) with respect to L-serine and a K_i value of 2.02 mM as revealed by the Dixon plot and the replot of slope *versus* D-cycloserine concentration (Fig. 9, insets A and B).

DISCUSSION

Our earlier work (7, 18) had clearly demonstrated that serine hydroxymethyltransferase, the first enzyme of the pathway for the metabolism of folate coenzymes was regulated in mammalian systems by allosteric interactions with metabolites. In order to demonstrate that the folate metabolism in plants could also be regulated by such allosteric interactions, it was necessary to purify

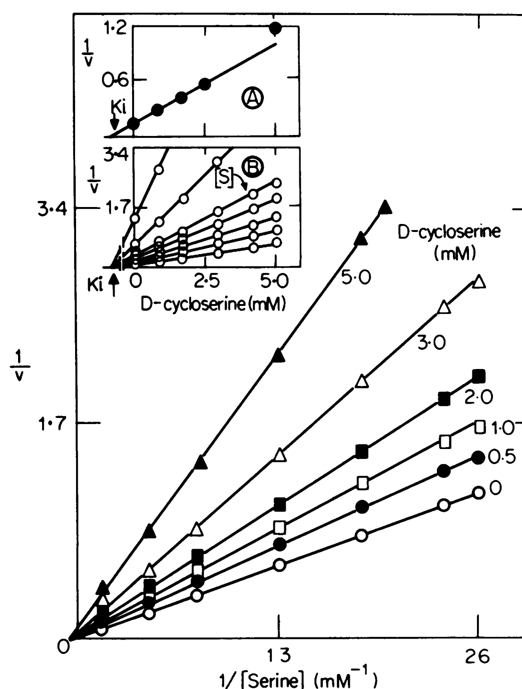

FIG. 9. Competitive inhibition of mung bean serine hydroxymethyltransferase activity by D-cycloserine. Lineweaver-Burk plot: The enzyme was preincubated with 1.8 mM H_4folate and varying concentrations of L-serine as indicated in the figure at different fixed concentrations of D-cycloserine: No D-cycloserine (○), 0.5 mM D-cycloserine (●), 1.0 mM D-cycloserine (□), 2.0 mM D-cycloserine (■), 3.0 mM D-cycloserine (△) and 5.0 mM D-cycloserine (▲). (Inset A), replot of slopes obtained from the main figure *versus* D-cycloserine concentration as indicated in the figure. The intercept on the *x*-axis gave a K_i value of 2.02 mM. (Inset B), Dixon plot of the saturation data: The intercept on the x-axis gave a K_i value of 1.98 mM.

this enzyme from a plant source. Previous attempts to isolate this enzyme had resulted in only partial purification due to the reported instability of this enzyme from cauliflower buds (13) and tobacco roots (15). The use of pyridoxal 5′-phosphate, 2-mercaptoethanol, and EDTA prevented the inactivation of this enzyme during purification (16). An interesting feature of the purification procedure described here was the use of Blue Sepharose, a group specific affinity matrix which probably binds by interaction at the folate site of the enzyme (17). In addition, use of folate AH-Sepharose enabled the isolation of the enzyme in homogeneous form as evidenced by polacrylamide gel electrophoresis (Fig. 2). The V_{max} value for the mung bean enzyme was 1.33 μmol formaldehyde formed/min·mg protein compared to the value of 11 × 10^{-6} μmol formaldehyde formed/min·mg protein in the case of *L. minor* (26).

Unlike the earlier preparations (13, 15) the mung bean enzyme was extremely stable (at least 6 weeks at 4°C without appreciable loss of activity) in the presence of either folate or L-serine, in addition to 2-mercaptoethanol and EDTA (Table II). The protection of enzymes against inactivation by substrates or substrate analogs has been well documented (3). The mammalian serine hydroxymethyltransferase was protected against thermal inactivation by 3.6 mM L-serine and in fact a major step in the purification of the rabbit enzyme (22) involved heating the enzyme at 70°C in presence of L-serine. We have scrupulously avoided the use of heat denaturation during our purification procedures in order to ensure that the regulatory properties were not lost by such a treatment.

The mol wt of the mung bean enzyme was 205,000 ± 5,000, comparable to values in the range of 220,000 ± 5,000 seen in the case of the bacterial and mammalian enzymes (12). The mung bean enzyme was composed of four identical subunits of mol wt 50,000 ± 1,400.

It has been well established that serine hydroxymethyltransferase from mammalian (18) and bacterial sources (12) required pyridoxal 5′-phosphate for the dealdolization mechanism. On the other hand, there is some doubt on the requirement of pyridoxal 5′-phosphate for catalysis for some of the plant serine hydroxymethyltransferases (13). As most of the early work was carried out with partially purified preparations, it was difficult to draw unequivocal conclusions. The absence of absorbance in the visible region and complete activity in preparations in which the bound pyridoxal 5′-phosphate was removed by treatment with 10 mM L-cysteine and the absence of inhibition by D-alanine suggested that pyridoxal 5′-phosphate may not be involved in the reaction catalyzed by this enzyme. It would be extremely worthwhile to probe into the active site of this enzyme and determine the catalytic mechanism.

The saturation pattern of the enzyme with L-serine, the second substrate was biphasic with an intermediary plateau region (Fig. 5). A replot of the data gave K_m values of 1.25 and 68 mM (Fig. 5, inset). These results indicate that there may be two distinct classes of binding sites, one set with a high affinity for serine, being saturated at low concentrations of serine and another set with low affinity saturating at higher concentrations of serine. The high affinity site has a lower turnover number (369) compared to the lower affinity sites (1,107).

The following mechanisms could be postulated to explain the biphasic saturation patterns (a) heterogeneity of enzyme active sites, (b) certain complex mechanisms involving branched reaction pathways, as in hysteretic enzyme mechanisms, (c) coupling between active sites on different polypeptide chains, and (d) coupling between an effector site and the active site. One obvious explanation for a saturation pattern of this type is the presence of isoenzymes; this appears improbable in case of mung bean serine hydroxymethyltransferase because of the observation of a single band on polyacrylamide gel disc electrophoresis and in SDS/polyacrylamide gel disc electrophoresis.

Glycine, which is a product of the reaction, inhibited the mung bean enzyme in a linear competitive manner when L-serine was the varied substrate, suggesting that glycine and serine were interacting with the same form of the enzyme. D-Cycloserine, a structural analog of alanine, also competitively inhibited the enzyme activity. Other serine analogs such as *O*-phosphoserine, DL-α-methylserine, and DL-*O*-methylserine were all partial inhibitors, suggesting that there may exist on the enzyme multiple sites of interaction for these inhibitors and possibly for the substrate serine.

The anitfolate compounds bind very strongly to dihydrofolate reductase from mammalian sources (10) and also from soybean (19). It was, therefore, of interest to examine the effect of these compounds on mung bean serine hydroxymethyltransferase activity. Surprisingly, some of the folate analogs, such as methotrexate and aminopterin used in cancer chemotherapy to inhibit dihydrofolate reductase, were partial inhibitors of mung bean serine hydroxymethyltransferase reaction. This would suggest that the folate pathway, except probably thymidylate synthetase, which requires stoichiometric amounts of H_4folate, could operate albeit less efficiently even at higher concentrations of these compounds. The theoretical basis for the partial inhibition analysis (23, 24) is as follows:

When [*I*] is a partial competitive inhibitor, the following equation can be derived:

$$\frac{1}{i} = \frac{\alpha K_i(K_m + [S])}{K_m(\alpha - 1)} \frac{1}{[I]} + \frac{\alpha K_m + [S]}{K_m(\alpha - 1)} \quad (1)$$

where $i = 1 - v_i/v$, v_i being the velocity of the reaction at a fixed

substrate concentration in the presence of the inhibitor and v being the velocity of the reaction at the same substrate concentration in the absence of the inhibitor.

The intercept on the ordinate of a $1/i$ *versus* $1/[I]$ plot is given by

$$\frac{\alpha K_m + [S]}{K_m(\alpha - 1)} \qquad (2)$$

whose value is always greater than 1 because α is greater than 1. When $\alpha = \infty$, the equation for a complete competitive inhibitor is given by:

$$\frac{1}{i} = [K_i(1 + [S]/K_m)]\frac{1}{[I]} + 1 \qquad (3)$$

The ordinate intercept value is always equal to 1. α in equation (1) and in expression (2) represents the factor by which K_i and K_m are altered when EI and ES react with the substrate or an inhibitor to give EIS and ESI complexes which are catalytically active. Partial inhibition could be explained by assuming that the inhibitor binds to different sites on the enzyme, binding to the inhibitor site leads to inhibition, whereas binding to the noninhibitory site prevents the binding to the inhibitor site and thus results in the abolition of inhibition.

The most important property of the mung bean serine hydroxymethyltransferase was its sigmoid saturation pattern with H_4folate. This pattern was characteristic of homotropic cooperative interactions (Fig. 4). The abolition of homotropic cooperative interactions by NADH and TMP, as evidenced by the hyperbolic saturation pattern (Fig. 4), and the increased sigmoidicity in the presence of NAD^+ (Fig. 4) and other end products, such as adenine nucleotides (Table IV), suggested that the metabolic state of the cell could modulate the activity of this enzyme to meet the demands of the germinated seedlings.

Several metabolic pathways are known to be closely integrated so that alterations in one sequence results in changes in the levels of the products of related pathways. This is referred to as interpathway regulation or metabolic interlock (9). Regulation of this type occurs in microorganisms and in higher plants (27). In higher plants, the highly branched pathway of methionine-lysine biosynthesis could be regulated by an allosteric mechanism. Methionine is one of the end products of folate metabolism and also serves as a methyl donor for a wide variety of biologically important compounds via the formation of *S*-adenosylmethionine and *S*-methylmethionine. The biosynthesis of lysine and methionine are interrelated through homoserine (27). Both these amino acids were simple activators of the mung bean serine hydroxymethyltransferase (Table V). It was reported earlier that lysine was an inhibitor of the *L. minor* serine hydroxymethyltransferase (26). A more detailed examination of the effects of other end products of C_1 metabolism, such as Chl, would provide a deeper insight into the intricate control mechanisms operating in plants.

The results presented in this paper clearly highlight the regulatory nature of mung bean serine hydroxymethyltransferase, and its possible involvement in the modulation of the interconversion of folate coenzymes.

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