Biochem. J. (1980) **187**, 623–636
Printed in Great Britain

Purification and Physicochemical, Kinetic and Immunological Properties of Allosteric Serine Hydroxymethyltransferase from Monkey Liver

Kashi S. RAMESH and N. APPAJI RAO
Department of Biochemistry, Indian Institute of Science, Bangalore-560012, India

(*Received 17 September 1979*)

1. The homogeneous serine hydroxymethyltransferase purified from monkey liver, by the use of Blue Sepharose affinity chromatography, exhibited positive homotropic co-operative interactions ($h = 2.5$) with tetrahydrofolate and heterotropic interactions with L-serine and nicotinamide nucleotides. 2. The enzyme had an unusually high temperature optimum of 60°C and was protected against thermal inactivation by L-serine. The allosteric effects were abolished when the monkey liver enzyme was purified by using a heat-denaturation step in the presence of L-serine, a procedure adopted by earlier workers for the purification of this enzyme from mammalian and bacterial sources. 3. The enzyme activity was inhibited completely by N^5-methyltetrahydrofolate, N^5-formyltetrahydrofolate, dichloromethotrexate, aminopterin and D-cycloserine, whereas methotrexate and dihydrofolate were partial inhibitors. 4. The insoluble monkey liver enzyme–antibody complex was catalytically active and failed to show positive homotropic co-operative interactions with tetrahydrofolate ($h = 1$) and heterotropic interactions with NAD^+. The enzyme showed a higher heat-stability in a complex with its antibody than as the free enzyme. 5. These results highlight the pitfalls in using a heat-denaturation step in the purification of allosteric enzymes.

Serine hydroxymethyltransferase (5,10-methylenetetrahydrofolate–glycine hydroxymethyltransferase, EC 2.1.2.1) has been purified from several sources [rat liver (Nakano *et al.*, 1968); rabbit liver (Fujioka, 1969; Schirch, 1971); *Escherichia coli* (Mansouri *et al.*, 1972); ox liver (Jones & Priest, 1976); lamb liver (Ulevitch & Kallen, 1977)]. No evidence for co-operative interactions of substrates or effectors with the enzyme was obtained by these workers. It was shown, however, that the partially purified enzyme from pig kidney and monkey liver exhibited co-operative interactions with H_4folate (Harish Kumar *et al.*, 1976). In addition, the monkey liver enzyme exhibited heterotropic interactions with nicotinamide nucleotides (Ramesh & Appaji Rao, 1978). In view of the apparent contradictions between our results and those of earlier workers, it was necessary to evolve a mild purification procedure for the isolation of an allosteric serine hydroxymethyltransferase.

Abbreviations used: H_4folate, (±)-L-tetrahydrofolate; H_2folate, dihydrofolate; 5-CH_3-H_4folate, N^5-methyltetrahydrofolate; 5-CHO-H_4folate, N^5-formyltetrahydrofolate; SDS, sodium dodecyl sulphate; dimedone, 5,5-dimethylcyclohexane-1,3-dione; IgG, immunoglobulin G.

In the present paper we report a procedure for the isolation of the allosteric serine hydroxymethyltransferase from monkey liver and its physicochemical, kinetic and immunological properties.

Experimental

Materials

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: L-serine, DL-dithiothreitol, 2-mercaptoethanol, pyridoxal 5'-phosphate, EDTA (disodium salt), DL-β-phenylserine (*threo* form), 2,4-dinitrophenylhydrazine, D-cycloserine, glycine, Tris, ammonium persulphate, SDS, Blue Dextran, 3-(3-dimethylaminopropyl) 1-ethylcarbodi-imide, Folin–Ciocalteu phenol reagent, Coomassie Brilliant Blue G, folic acid, H_2folic acid, DL-5-CH_3-H_4-folate (barium salt), ovalbumin, cytochrome *c*, α-chymotrypsinogen, ferritin, haemoglobin, catalase (EC 1.11.1.6), human IgG and conalbumin. Acrylamide and *NN'*-methylenebisacrylamide were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. CM-Sephadex C-50, Blue Sepharose CL-6B, Sephacryl S-200 (superfine grade) and AH-Sepharose were from Pharmacia Fine Chemicals, Uppsala, Sweden.

0306-3275/80/060623-14$01.50/1

Freund's complete adjuvant and agar were from Difco Laboratories, Detroit, MI, U.S.A. DE-52 DEAE-cellulose (microgranular) was purchased from Whatman, Maidstone, Kent, U.K. Coomassie Brilliant Blue R-250 was a product of Bio-Rad Laboratories, Richmond, CA, U.S.A. H_4folate, prepared by the method of Hatefi *et al.* (1960), was a gift from Dr. John H. Mangum, Brigham Young University, Provo, UT, U.S.A. 5-CHO-H_4folate (folinic acid) was a product of ICN Pharmaceuticals, Plainview, NY, U.S.A. Methotrexate, dichloromethotrexate and aminopterin were kindly given by Dr. Robert Silber, New York University Medical Center, New York, NY, U.S.A. Ultrogel AcA-34 was a product of Industrie Biologique Française and was a gift from Mr. Henrik Perlmutter, LKB-Produkter AB, Stockholm, Sweden. DL-[3-^{14}C]Serine (specific radioactivity 48.5 mCi/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A.

Animals

Adult bonnet monkeys (*Macaca radiata*) maintained under standard experimental conditions of light, temperature and diet and procured from the Central Animal Facility, Indian Institute of Science, Bangalore, were used for the present study. The animals were killed by air-embolism.

Assay of serine hydroxymethyltransferase

The assay mixture (0.1 ml) contained 0.4 M-potassium phosphate buffer, pH 7.4, 1 mM-2-mercaptoethanol, 10 mM-EDTA, 0.2 mM-pyridoxal 5'-phosphate, 1.8 mM-H_4folate, 3.6 mM-L-[3-^{14}C]serine, 1.8 mM-dithiothreitol and an appropriate amount of the enzyme (0.5–2.0 μg). The mixture was preincubated for 5 min at 37°C and the reaction was stopped by the addition of 0.1 ml of dimedone [0.4 M in 50% (v/v) ethanol]. The mixture was heated for 5 min at 100°C and [^{14}C]formaldehyde–dimedone adduct was determined in a Beckman LS-100C liquid-scintillation spectrometer. One unit of enzyme activity was defined as the amount that catalysed the formation of 1 μmol of formaldehyde/min at 37°C at pH 7.4. Specific activity was expressed as units/mg of protein. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Preparation of tissue homogenates and soluble fractions

The following buffer was used for the preparation of the soluble fractions from various organs: 50 mM-potassium phosphate buffer (pH 7.4)/0.25 M-sucrose / 20 mM - EDTA / 1 mM - mercaptoethanol / 0.05 mM-pyridoxal 5'-phosphate. Heart, lung and liver, removed immediately after the monkey had been killed by air-embolism, were rapidly perfused with ice-cold 0.25 M-sucrose to remove blood, blotted and immediately homogenized. All the other organs were washed three times with ice-cold 0.25 M-sucrose before homogenization. Except for skeletal muscle, all the other organs were minced and then homogenized in 5 vol. of the buffer by using six strokes in a motor-driven Potter–Elvehjem homogenizer with a tight-fitting Teflon pestle. Skeletal muscle was ground with glass/sand and 5 vol. of the homogenizing buffer in an ice-cold mortar. Tissue homogenates were centrifuged at 10500 *g* for 10 min in a Sorvall RC-5B Automatic Superspeed refrigerated centrifuge (DuPont Instruments) followed by centrifugation at 105500 *g* for 60 min in a Beckman model L5-50 preparative ultracentrifuge. The supernatant was dialysed for 24 h against buffer without sucrose.

Isolation of cell organelles from monkey liver

Nuclei were isolated by the method described by Blobel & Potter (1966). Microsomal fraction, mitochondria and cytosol were prepared by the procedure described by Conn & Stumpf (1972).

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 water, 5 ml of 0.8 M-3-(3-dimethylaminopropyl)-1-ethylcarbodiimide was added. The pH was maintained at 5.5 by the addition of 1 M-NaOH. After being stirred overnight at 4°C in the dark, the gel was washed successively with 1 litre each of 1 M-NaOH/1 M-NaCl, deionized water, 1 M-HCl/1 M-NaCl and finally with 2 litres of deionized water. Folate–AH-Sepharose was stored in 1 M-potassium phosphate buffer, pH 7.4, containing 1 M-NaCl and 10 mM-2-mercaptoethanol in test tubes under N_2 in the dark at 4°C.

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 water. The gel was stored in 50 mM-potassium phosphate buffer, pH 7.4.

Purification of serine hydroxymethyltransferase from monkey liver

Potassium phosphate buffer, pH 7.4 (50 mM), containing 1 mM-2-mercaptoethanol, 0.05 mM-pyridoxal 5'-phosphate and 20 mM-EDTA was used throughout. All the purification steps were carried out at 0–3°C.

Step 1: preparation of crude extract. Liver (75–80 g) removed immediately after the animal had been killed by air-embolism was trimmed of excess fat, connective tissue and gall-bladder. It was diced and homogenized with 220 ml of 50 mM-potassium phosphate buffer, pH 7.4, in a precooled industrial

Waring Blendor at high speed for 2min. The homogenate was centrifuged at 27000*g* for 20min, and the supernatant designated as the crude enzyme extract.

Step 2: precipitation with ammonium sulphate. Solid $(NH_4)_2SO_4$ (enzyme grade) was added to the crude extract to give 25% saturation (13.4g/100ml). After equilibration for 15min, the extract was centrifuged at 27000*g* for 20min. To the supernatant solution solid $(NH_4)_2SO_4$ was added to raise the saturation to 50% (14.6g/100ml). The pellet obtained after centrifugation (27000*g* for 20min) was dissolved in 100ml of 50mM buffer and was dialysed for 48h against eight changes of 2 litres of the same buffer.

Step 3: CM-Sephadex C-50 ion-exchange chromatography. The clear supernatant obtained after centrifugation of the dialysed enzyme was applied to a CM-Sephadex C-50 column (2.5cm × 36cm) equilibrated with 50mM-potassium phosphate buffer, pH7.4, at a flow rate of 12ml/h. The column was washed with 10 bed volumes of the equilibrating buffer, until the absorbance of the eluate at 280nm was less than 0.05. The enzyme was eluted with a linear 0.05–0.5M-potassium phosphate buffer gradient (250ml of each). Fractions (5ml) containing significant amounts of the enzyme (specific activity >1.8 units/mg) were pooled, and concentrated by $(NH_4)_2SO_4$ precipitation (50% saturation). The precipitate was dissolved in 2ml of 50mM buffer and dialysed against 2 litres of the 50mM-potassium phosphate buffer for 24h.

Step 4: molecular-exclusion chromatography on Ultrogel AcA-34. The enzyme from the previous step was chromatographed on an Ultrogel AcA-34 column (2.2cm × 115cm), pre-equilibrated with 50mM-potassium phosphate buffer, pH7.4, containing 0.2M-KCl at a flow rate of 15ml/h. Fractions (2.4ml) with specific activity more than 2.9 were pooled and dialysed against 5 litres of 50mM buffer, pH7.4.

Step 5: affinity chromatography on Blue Sepharose CL-6B (a) or folate–AH-Sepharose 4B (b) columns. Blue Sepharose CL-6B was packed in a column (1cm × 20cm) and equilibrated with 50mM buffer. The enzyme fraction from the previous step was loaded on to the affinity-gel column at a flow rate of 1 column volume/h. The column was then washed with the equilibrating buffer at a flow rate of 2 column volumes/h, until the eluate had an absorbance of 0.02 at 280nm. The enzyme was desorbed from the Blue Sepharose gel with the buffer containing 1M-KCl. Fractions (2ml) containing serine hydroxymethyltransferase activity were pooled and dialysed against 20vol. of 50mM-potassium phosphate buffer, pH7.4.

Alternatively, folate–AH-Sepharose-4B could be used for the enzyme purification. Application of the sample, washing of the affinity matrix and desorption of the enzyme from the affinity gel were similar to the procedure described for Blue Sepharose CL-6B.

Regeneration of Blue Sepharose CL-6B and folate–AH-Sepharose-4B matrices. The regeneration process includes washing the gel with 3 bed volumes of 50mM-potassium phosphate buffer, pH7.4, containing 3M-KCl, followed by 40 bed volumes of deionised water. The gel was stored in phosphate buffer containing 0.02% NaN_3.

Purification of serine hydroxymethyltransferase from sheep and rabbit livers

The enzyme was purified by following the procedure described above for isolating the enzyme from monkey liver. The enzymes were homogeneous as determined by polyacrylamide-gel disc electrophoresis.

Analytical polyacrylamide-gel disc electrophoresis

Polyacrylamide-gel electrophoresis at 4°C, with 0.5M-Tris/0.39M-glycine buffer, pH8.6, in 7% gels was conducted at a current of 5mA/gel. Gels were either stained for protein [with 0.02%, Coomassie Brilliant Blue G in 3.5% (v/v) $HClO_4$ at room temperature for 2h] or for enzyme activity (by equilibration at 37°C for 10min in 0.1M-DL-*threo*-β-phenylserine in 0.1M-potassium phosphate buffer, pH7.4, followed by incubation for 10min at 37°C in 0.1% 2,4-dinitrophenylhydrazine in 2.4M-HCl). Destaining of Coomassie Brilliant Blue G-stained gels was not necessary (Reisner *et al.*, 1975).

Tube-gel isoelectrofocusing

The isoelectric point (pI) of the enzyme was determined by isoelectrofocusing on polyacrylamide gels as described by Chua *et al.* (1978) with some modifications. Electrophoretic runs were carried out with LKB Ampholine carrier ampholytes, pH3.5–10.0. The polyacrylamide gel was prepared as follows. A solution of acrylamide (1.42g) and *NN'*-methylenebisacrylamide (700mg) in 18.5ml of deionized water was filtered through Whatman no. 1 filter paper. Sucrose (1g), *NNN'N'*-tetramethylethylenediamine (13.4μl), 0.004% riboflavin (1ml), ammonium persulphate (6.67mg) and carrier Ampholyte, pH3.5–10.0 (1ml), were mixed with the filtrate. The gel solution was deaerated and cast in tubes (4mm × 120mm), and these were exposed to u.v. light for 30min. Anodic and cathodic chambers contained 10mM-H_3PO_4 and 20mM-NaOH respectively. The gels were prerun at a constant voltage of 400V for 3h at 4°C. Samples containing 0.1% (v/v) Ampholyte were loaded on the gels and electrophoresed. The power source was adjusted to a constant voltage of 400V for a further period of 4h. After completion of the isoelectrofocusing, the gel was stained with the stain fixative

prepared by mixing 100ml each of 2% (w/v) Coomassie Brilliant Blue R-250 and 2M-H_2SO_4. The precipitate formed was discarded and the green supernatant was titrated with 10M-KOH, until the solution turned pale blue. Trichloroacetic acid was added to the solution (12g/100ml) and used as stain fixative. An equivalent gel isoelectrophoresed under identical conditions was sliced into 1cm pieces and the pH measured in water after 15min equilibration.

Ultracentrifugal measurements

Sedimentation-velocity studies were performed in a single-sector cell with a Spinco model E analytical ultracentrifuge, equipped with schlieren optics. The ultracentrifuge was operated at 50740rev./min for the determination of sedimentation coefficient, which was corrected to $s_{20,w}$ (Schachman, 1957).

Molecular-weight determination

The molecular weight of monkey liver serine hydroxymethyltransferase was estimated by gel filtration (Andrews, 1965) on Sephacryl S-200 (superfine grade gel equilibrated with 50mM-potassium phosphate buffer, pH7.4, containing 0.2M-KCl. The following proteins of known molecular weight served as markers: horse heart ferritin (480000), bovine liver catalase (232000), human IgG (156000) and horse heart cytochrome *c* (12384). The molecular weight was calculated from a plot of log(molecular weight) against elution volume. The partition coefficient (K_D) of standard proteins and the enzyme were determined in separate runs. The Stokes radius of the enzyme was obtained by interpolation on a calibration curve prepared by plotting Stokes radius of standard proteins versus $K_D^{\frac{1}{2}}$.

The subunit molecular weight of serine hydroxymethyltransferase was determined by SDS/polyacrylamide-gel disc electrophoresis as described by Weber & Osborn (1969).

Immunological techniques

Antibodies to the purified monkey liver serine hydroxymethyltransferase were raised in male albino rabbits (about 2.5kg). The enzyme from either step 4 or step 5 in 50mM-potassium phosphate buffer, pH7.4 (1mg/ml), was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple points at weekly intervals. After 4 weeks a booster dose of 1mg of enzyme was administered. After 7 days the rabbits were bled through the marginal ear vein. Blood was allowed to clot initially for 2h at room temperature and later for 12h at 4°C. Control sera (pre-immune sera) were obtained from the rabbits before the first injection of the antigen. The IgG fraction was prepared by repeated $(NH_4)_2SO_4$ fractionation (0–52% saturation) and negative adsorption on DE-52 DEAE-cellulose. All sera and IgG fractions were stored at −20°C. Ouchterlony double-immunodiffusion analysis on agar was performed as described by Ouchterlony (1958). Immunoelectrophoresis on agar was performed as described by Clausen (1970).

Results

Distribution of serine hydroxymethyltransferase in the various organs of adult male monkey

It is clear from Table 1 that the enzyme is present in large amounts in the liver. In skeletal muscle, brain and pancreas the specific activity of serine hydroxymethyltransferase was about 10% and in the kidney 25% of that in the liver. For this reason liver was chosen for further studies.

Subcellular distribution of serine hydroxymethyltransferase in monkey liver

The cytosol fraction contained 75% of the enzyme activity, whereas the mitochondria had 5% and the microsomal fraction 3% of the total activity. The cytosolic fraction had the highest specific activity. The recovery was 83% (Table 2). The relatively low recovery was due to loss of the enzyme during the washing procedure.

Table 1. *Distribution of serine hydroxymethyltransferase in the organs of the monkey (Macaca radiata)*
For details of the procedure used to obtain the enzyme from different organs and assay of the enzyme see the Experimental section.

Organ	Activity (Units/g of tissue)	Specific activity (unit/mg of protein)
Liver	4.42	0.027
Kidney	0.74	0.007
Spleen	0.27	0.003
Testes	0.21	0.003
Lung	0.20	0.003
Pancreas	0.19	0.002
Small intestine	0.17	0.003
Heart	0.09	0.003
Brain	0.05	0.002
Skeletal muscle	0.07	0.001

Table 2. *Subcellular distribution of monkey liver serine hydroxymethyltransferase*
For details see the Experimental section.

Fraction	Total activity (units)	Specific activity (unit/mg of protein	Recovery (%)
Homogenate	15.20	0.020	100
Nuclei	0.03	0.003	0.2
Mitochondria	0.70	0.009	4.6
Microsomal fraction	0.50	0.007	3.3
Cytosol	11.40	0.029	75.0

Enzyme purification

The major protein peak eluted from the Blue Sepharose CL-6B affinity column with 1M-KCl coincided with the enzyme activity (Fig. 1). L-Serine (10mM), NADH (10mM) or NADPH (50mM) failed to elute the enzyme from the Blue Sepharose column. Polyacrylamide-gel disc electrophoresis of fractions eluted from Blue Sepharose column revealed a single protein band [Fig. 1, inset (*a*)], indicating the absence of contaminating proteins. The specific activity of the pooled fractions from this affinity column was 3.3 units/mg. The specific activities of several preparations of the enzyme were in the range 3.2–3.5 units/mg.

The enzyme from the Ultrogel AcA-34 step was adsorbed on a folate–AH-Sepharose column. Elution with buffer containing 1M-KCl gave a single symmetrical protein peak that coincided with the

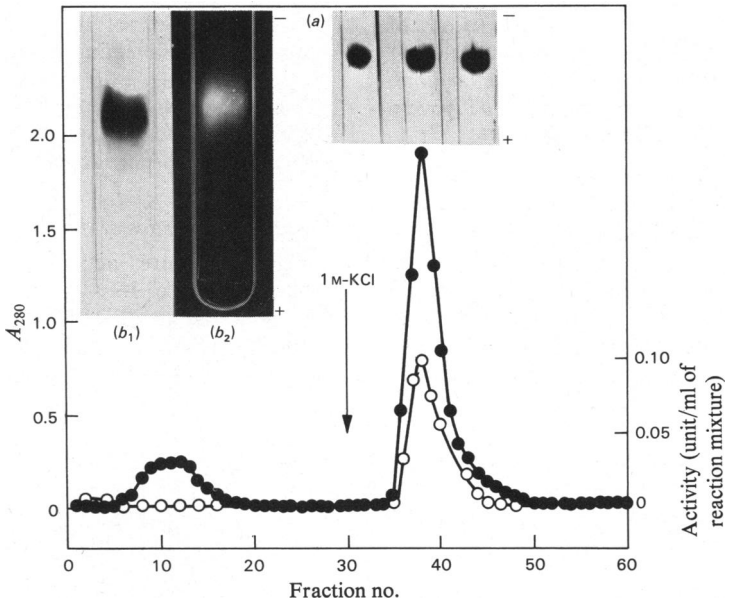

Fig. 1. *Elution profile of monkey liver serine hydroxymethyltransferase from a Blue Sepharose CL-6B affinity column*
The enzyme (step 4, Table 3; 111 units; specific activity 3.04 units/mg) was loaded on a Blue Sepharose column (1cm × 20cm). The column was washed free of unadsorbed protein with 250ml of 50mM-potassium phosphate buffer, pH 7.4, containing 0.05mM-pyridoxal 5'-phosphate, 1mM-2-mercaptoethanol and 20mM-EDTA. The enzyme was eluted with the buffer containing 1M-KCl, and fractions (2.0ml) were assayed for protein (A_{280}) (●) and enzyme activity (○) as described in the Experimental section. The arrow (↓) indicates the point at which 1M-KCl was included in the eluting buffer. The inset (*a*) shows polyacrylamide-gel disc electrophoresis of fractions 37, 38 and 39. Inset (*b*) shows the electrophoretic pattern of the pooled fractions. Gels were stained for protein (b_1) and for activity (b_2).

Table 3. *Summary of purification of serine hydroxymethyltransferase from monkey liver*
For details see the Experimental section.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)
1. Crude extract (from 80g of liver)	8326	150	0.018	100
2. $(NH_4)_2SO_4$ precipitate (25–50% saturation)	3320	140	0.042	93
3. CM-Sephadex ion-exchange chromatography (pooled fractions)	68	131	1.93	87
4. Ultrogel AcA-34 gel chromatography	36.5	111	3.04	74
5. (*a*) Blue Sepharose CL-6B affinity chromatography	23	76	3.3	51
(*b*) Folate–AH-Sepharose 4B	21	71	3.4	47

enzyme activity. Electrophoresis of the enzyme eluted from the folate–AH-Sepharose affinity column also gave a single protein band and was identical in all respects with the enzyme prepared by using Blue Sepharose (results not given).

All the studies reported in the present paper were carried out with the enzyme purified by using the Blue Sepharose affinity column rather than the folate affinity matrix. The five-step purification procedure described in the present paper resulted in an overall purification of 180-fold, with 50% recovery. Table 3 summarizes the results of the purification. Enzyme (2mg/ml) could be stored at 0°C without loss of activity for at least 2 months, and it was stable at −20°C for 6–8 months. Pyridoxal 5′-phosphate, 2-mercaptoethanol or dithiothreitol and EDTA were necessary for the stability of the enzyme.

Criteria of purity

On polyacrylamide-gel disc electrophoresis a single protein band was obtained that coincided with the enzyme activity band [Fig. 1, inset (*b*)]. Tube-gel isoelectrofocusing on polyacrylamide gels gave a single protein band, located by staining with Coomassie Brilliant Blue R-250 stain fixative. Enzyme activity could not be detected in a similar gel run below pH 5.0 owing to inactivation of the enzyme (results not given). The pI of the protein was determined to be 4.2 ± 0.1. Immunoelectrophoresis of the purified enzyme gave a single precipitin line (Fig. 2*a*) on either side of the antigen track. Ouchterlony double-immunodiffusion assay with rabbit IgG rasied against the partially purified enzyme gave a single precipitin line with the purified enzyme (well 4) and two precipitin bands with the enzyme at the Ultrogel AcA-34 step (well 3 in Fig. 2*b*). Normal rabbit IgG (well 2), however, did not give any precipitin line with the anti-enzyme IgG. On ultracentrifugation of the purified enzyme a single symmetrical sedimenting peak was obtained with an $s_{20,w}$ value of 8.9 ± 0.1 S (Fig. 3). The enzyme, on passage through Sephacryl S-200 (superfine grade, gel column, emerged as a single symmetrical peak with constant specific activity (results not given).

Catalytic properties of the enzyme

Catalytic-centre activity of the enzyme was calculated to be $1.7 \times 10^{-3} s^{-1}$, on the basis of a molecular weight of 2.08×10^5 (4mol of pyridoxal 5′-phosphate/mol of the enzyme) and the $V_{max.}$ calculated from the double-reciprocal plot. The

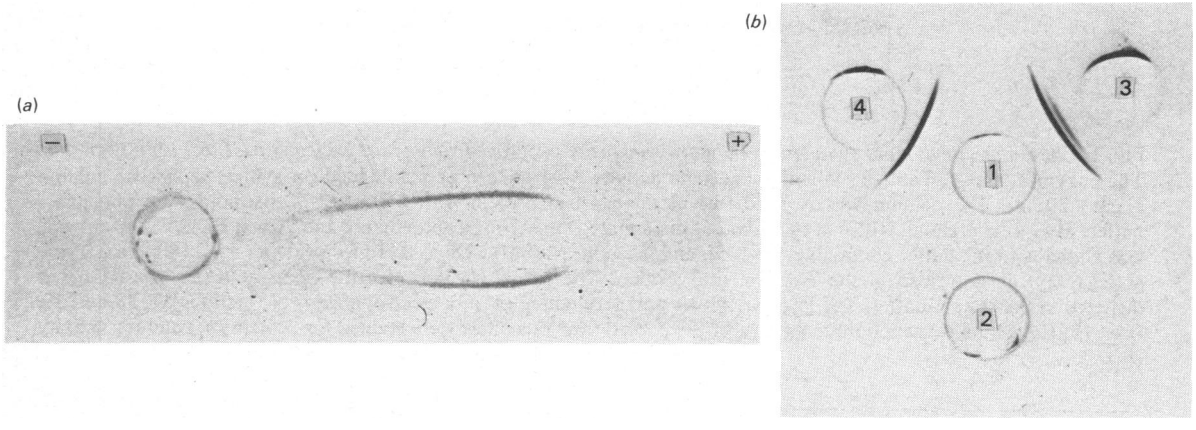

Fig. 2. (*a*) *Immunoelectrophoresis of monkey liver serine hydroxymethyltransferase and* (*b*) *Ouchterlony double-diffusion analysis*

(*a*) Agar (1.5%, w/v) in 60mM-sodium veronal buffer, pH 8.6, was layered on a glass plate (3.5cm × 9.0cm) and allowed to form an uniform layer of gel. The enzyme (40μg) was placed in the well punched at the centre of the plate, and electrophoresed at 4°C for 10h at 250V. After electrophoresis, troughs running through the entire length of the gel were cut on either side of the antigen well. Rabbit anti-enzyme IgG (antibody raised against the enzyme at step 4, 5mg/0.5ml) poured in each trough and allowed to diffuse overnight in a humidity chamber at 37°C and for 1 week at room temperature (Clausen, 1970). The gel was deproteinized with 0.15M-NaCl, and stained with Amido Black 10B and destained with 7% (v/v) acetic acid. For details see the text. (*b*) Agar (1.25%, w/v) in 25mM-potassium phosphate buffer, pH 7.4, was poured into Petri dishes and allowed to gel. Four wells were punched in the plate. Well 1 contained 500μg of rabbit anti-enzyme IgG; well 2, 500μg of control IgG (pre-immune sera); well 3, 600μg of enzyme from step 4 (Table 3); well 4, 300μg of the purified enzyme (step 5). The plates were kept in humidity chambers for 24h at 37°C and for 1 week at room temperature. Deproteinization of the gel, staining and destaining were performed as described for (*a*). For details see the text.

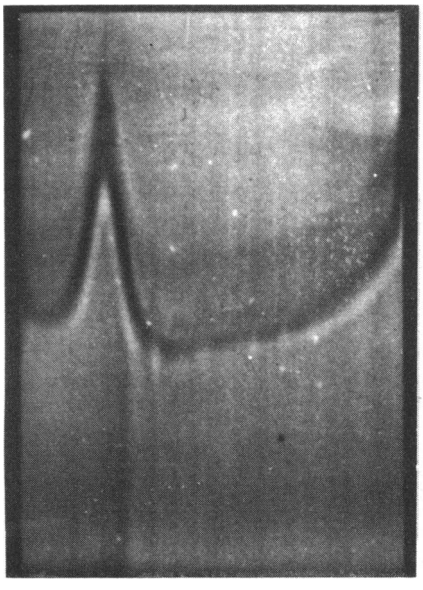

Fig. 3. *Sedimentation pattern of purified monkey liver serine hydroxymethyltransferase*
Ultracentrifugation analysis of the monkey liver enzyme (5mg/ml) in 50mM-potassium phosphate buffer, pH 7.4, was performed at 18°C in a Spinco model E analytical ultracentrifuge with schlieren optics. The pattern reproduced here was taken 7min after a speed of 50740rev./min had been reached, with the phase-plate angle at 40°. The enzyme moved as a single symmetrical peak during centrifugation for 75min. Sedimentation is from left to right.

purified enzyme showed (i) a linear relationship between initial reaction rate and enzyme concentration up to 28μg/ml of reaction mixture, (ii) a linear rate of formaldehyde formation for 30min when serine (3.6mM), H_4folate (1.8mM) and protein (up to 28μg) were used, and (iii) optimum enzyme activity at pH 7.4 and 60°C at 3.6mM-serine and 2mM-H_4folate concentrations.

Regulatory properties of the enzyme

The purified enzyme exhibited a sigmoid saturation pattern when H_4folate was the varied substrate (Fig. 4). The $S_{0.5}$ (H_4folate) and h value of 0.90mM and 2.5 were calculated by using the Hill equation (Segel, 1975). The L-serine saturation curve was hyperbolic with a K_m (L-serine) value of 0.70mM (inset to Fig. 4). NADH (10mM) and NADPH (50mM) abolished the sigmoidicity of the H_4folate saturation curve, and an h value of 1.1 was obtained. On the other hand, NAD^+ (50mM) increased the sigmoidicity and gave a h value of 3.3. These results are in good agreement with our previous findings with the partially purified monkey liver enzyme (Ramesh & Appaji Rao, 1978).

Reconstitution and reactivation of the enzyme

Enzyme purified in the absence of pyridoxal 5'-phosphate, EDTA and a thiol compound (dithiothreitol or 2-mercaptoethanol) had a specific activity of 0.12 unit/mg, as compared with 3.3 units/mg, when the enzyme was purified in the presence of these compounds. Addition of any one of these three compounds separately resulted in a 10-fold enhancement of the specific activity. However, dithiothreitol (1mM), pyridoxal 5'-phosphate (1mM) and EDTA (20mM) added together led to a 29-fold increase in the specific activity. The reactivation of the apoenzyme by EDTA could be due to the removal of contaminating metal ions from the apoenzyme or the protection of H_4folate against air oxidation, or it could be acting as electron donor, similar to its function in the photoreduction of flavin (Strickland & Massey, 1973). These results are summarized in Table 4.

Visible-absorption spectrum of monkey liver serine hydroxymethyltransferase

The spectrum of the purified enzyme had an absorption maximum centred at 420nm (Fig. 5), characteristic of pyridoxal 5'-phosphate-dependent enzymes, due to the internal protonated Schiff base between pyridoxal 5'-phosphate and the ε-amino group of a lysine residue of the enzyme (Fasella, 1967).

Molecular weight of monkey liver serine hydroxymethyltransferase

The molecular weight of the native enzyme was estimated to be 208000 ± 5000, by using a calibrated Sepachryl S-200 (superfine grade) gel column. The Stokes radius of the enzyme ($K_D = 0.18$), determined from the regression line of standard proteins, was 4.0nm.

SDS/polyacrylamide-gel disc electrophoresis of the purified enzyme revealed a single protein band (inset to Fig. 6). The molecular weight of this polypeptide chain was estimated to be 52000 ± 1400. The validation experiment for the SDS/polyacrylamide-gel disc electrophoresis determination of molecular weight indicated that the enzyme does not have anomalous properties, since the position in a plot of relative electrophoretic mobility versus log(molecular weight) falls on a straight line generated by four standard marker proteins (Fig. 6).

Thermal stability of the enzyme

The enzyme was stable at 50°C for 20min. At 65°C there was a gradual loss of enzyme activity (80% loss in 20min). However, at 70°C the enzyme

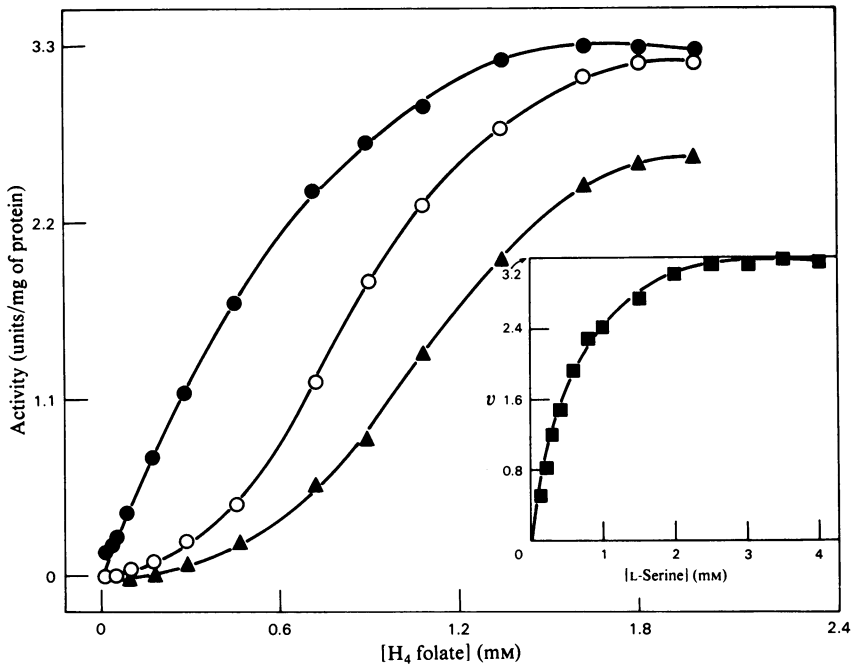

Fig. 4. *Co-operative interactions of H_4folate and the effects of nicotinamide nucleotides on these interactions with monkey liver serine hydroxymethyltransferase*

The enzyme (1.0μg/100μl of reaction mixture) was preincubated with the concentrations of H_4folate (○) indicated and the reaction was started by the addition of 3.6mM-L-[3-^{14}C]serine. After incubation for 15min at 37°C, the amount of [^{14}C]formaldehyde formed was determined (Taylor & Weissbach, 1965). The enzyme after preincubation with H_4folate was incubated for 5min with 10mM-NADH (●) or 50mM-NAD^+ (▲) and assayed for enzyme activity. The inset shows the saturation pattern of the enzyme with L-serine, at saturating concentration of H_4folate (1.8mM).

Table 4. *Reactivation of monkey liver serine hydroxymethyltransferase*

Enzyme preparation A was purified in the absence of pyridoxal 5′-phosphate (pyridoxal-*P*), 2-mercaptoethanol and EDTA. Each of these compounds were added to the enzyme and incubated for 30min at 37°C, and samples were assayed for enzyme activity. Enzyme preparation B was purified in the presence of EDTA (20mM), 2-mercaptoethanol (1mM) and pyridoxal 5′-phosphate (0.05mM). For details see the Experimental section.

Enzyme preparation	Specific activity (units/mg of protein)
Enzyme A	0.12
Enzyme A + 20mM-EDTA	0.91
Enzyme A + 1mM-pyridoxal-*P*	1.11
Enzyme A + 1mM-dithiothreitol	1.07
Enzyme A + 1mM-pyridoxal-*P* + 20mM-EDTA	1.25
Enzyme A + 1mM-pyridoxal-*P* + 1mM-dithiothreitol	3.49
Enzyme A + 1mM-pyridoxal-*P* + 20mM-EDTA + 1mM-dithiothreitol	3.50
Enzyme B	3.30
Enzyme B + 1mM-pyridoxal-*P* + 20mM-EDTA + 1mM-dithiothreitol	3.30

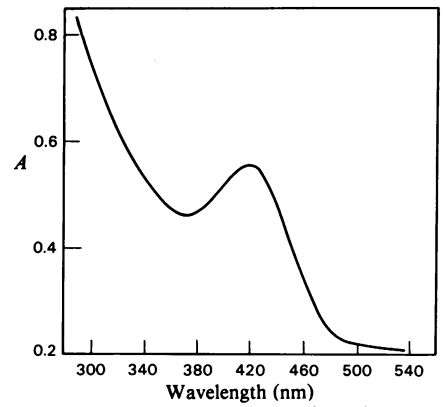

Fig. 5. *Visible-absorption spectrum of monkey liver serine hydroxymethyltransferase*

The spectrum of the enzyme (1.94mg/ml) in 50mM-potassium phosphate buffer, pH7.4, was recorded in a Cary model 14 spectrophotometer.

was rapidly inactivated, with complete loss of activity occurring in 6min. Considerable protection (80%) of the enzyme against thermal inactivation was observed when L-serine (18mM) was added. Protection of the enzyme by H_4folate against ther-

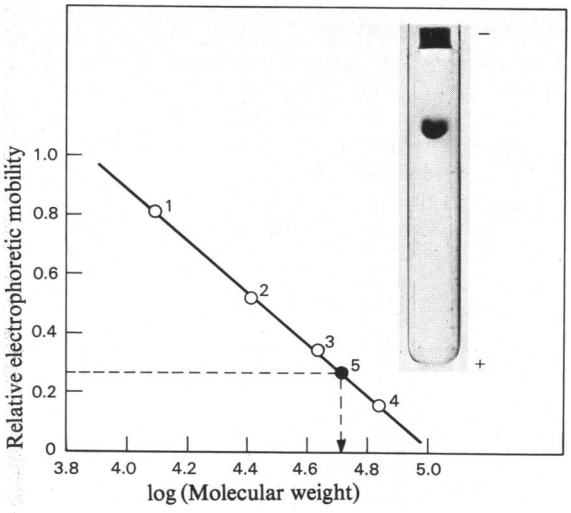

Fig. 6. *Subunit composition and determination of the molecular weight of monkey liver serine hydroxymethyltransferase*

The enzyme (50 μg) was denatured in the presence of 2-mercaptoethanol and SDS, each at 1% (v/v) concentration in 0.43 M-glycine/Tris buffer (7:1), pH 8.4, by heating at 90°C for 5 min. The denatured sample was mixed with 10 μl each of 0.05% Bromophenol Blue and 8 M-sucrose. The marker proteins 1 (cytochrome *c*, mol.wt. 12384), 2 (α-chymotrypsinogen, mol.wt. 25600), 3 (ovalbumin, mol.wt. 43000) and 4 (conalbumin, mol.wt. 68000) were similarly denatured and subjected to electrophoresis along with the denatured monkey liver enzyme (5), at 8 mA/tube at 30°C, until the marker dye reached the end of the tube (about 6 h). The proteins were stained with Coomassie Brilliant Blue R and the mobility of the proteins relative to the migration of the dye were plotted against log (molecular weight). The subunit molecular weight (52000) of the enzyme was determined by extrapolation. The inset shows the electrophoretic pattern of monkey liver serine hydroxymethyltransferase on SDS/polyacrylamide gel, demonstrating the presence of identical subunits.

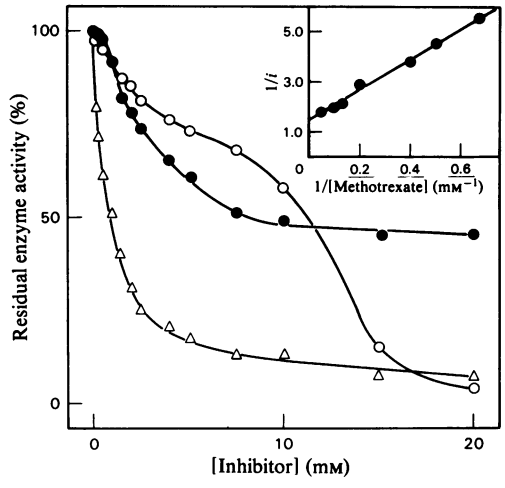

Fig. 7. *Inhibition of monkey liver serine hydroxymethyltransferase by antifolate drugs*

The enzyme was preincubated successively for 5 min at 37°C with H_4folate (1.8 mM) and methotrexate (●) or aminopterin (○) or dichloromethotrexate (△). The enzyme activity was assayed as described in the Experimental section. The enzyme activity in the absence of inhibitor was normalized to 100. The inset shows the plot of reciprocal of fractional inhibition (*i*) versus the reciprocal of methotrexate concentration. Aminopterin also gave similar results, whereas dichloromethotrexate completely inhibited the enzyme activity.

mal inactivation could not be attempted because of the instability of H_4folate. Allosteric effectors (NADH, NAD^+ and NADPH) failed to protect the enzyme against thermal inactivation.

Effects of antifolate drugs on monkey liver serine hydroxymethyltransferase

Inhibition of the enzyme activity by methotrexate, aminopterin and dichloromethotrexate followed different patterns (Fig. 7). Inhibition by methotrexate was partial (67% at infinite concentration determined by extrapolation; inset to Fig. 7), whereas that by aminopterin was sigmoidal. Dichloromethotrexate inhibited the enzyme activity in a hyperbolic manner. The concentrations of the drugs required for 50% inhibition were 1.2 mM (dichloromethotrexate), 8.1 mM (methotrexate) and 14.7 mM (aminopterin).

Effects of folate analogues on the enzyme activity

Folate, H_2folate, 5-CH_3-H_4folate and 5-CHO-H_4folate inhibited the monkey liver serine hydroxymethyltransferase activity to different extents, and the inhibition was concentration-dependent (Fig. 8). Partial-inhibition analysis (Webb, 1963) showed that H_2folate was a partial inhibitor (inset to Fig. 8), whereas 5-CH_3-H_4folate and 5-CHO-H_4folate completely inhibited the enzyme activity. An intercept value greater than 1 (inset to Fig. 8) when $1/i$ was plotted against 1/[I] suggested that H_2folate was a partial inhibitor (Webb, 1963), whereas 5-CH_3-H_4folate and 5-CHO-H_4folate completely inhibited the enzyme activity, indicated by the intercept value of 1 (not shown in the inset). The theoretical basis for the partial inhibition analysis is as follows.

When [I] is a partial competitive inhibitor:

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

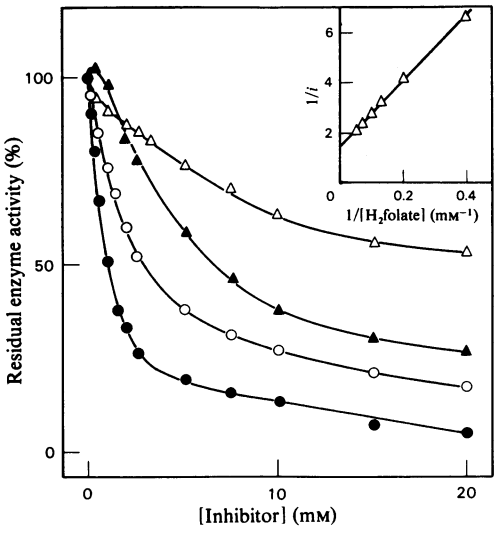

Fig. 8. *Effects of folate derivatives on monkey liver serine hydroxymethyltransferase*
Enzyme (1 μg/100 μl of reaction mixture) was incubated with folate (▲), H_2folate (△), 5-CH_3-H_4folate (○) or 5-CHO-H_4folate (●) after preincubation with 1.8 mM-H_4folate for 5 min at 37°C. The enzyme activity in the absence of any inhibitor was normalized to 100. The inset shows the reciprocal of fractional inhibition (i) versus reciprocal of H_2folate concentrations. A similar analysis with folate, 5-CH_3-H_4folate and 5-CHO-H_4-folate, however, gave an intercept value of 1.0, indicating that these inhibited the activity completely at infinite concentration.

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 completely competitive inhibitor is given by:

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

The ordinate intercept value is always equal to 1. α in eqn. (1) and expression (2) represents the factor by which K_i and K_s are altered when EI and ES react with the substrate or inhibitor.

Table 5. *Inhibition data for the effects of glycine and* D-*cycloserine on monkey liver serine hydroxymethyltransferase*
For details see the text.

Varied substrate	Inhibitor	Inhibition pattern	Slope replot	K_i (mM)
L-Serine	Glycine	Competitive	Linear	2.0
H_4folate	Glycine	Non-competitive	Linear	10.5
L-Serine	D-Cycloserine	Competitive	Linear	0.27

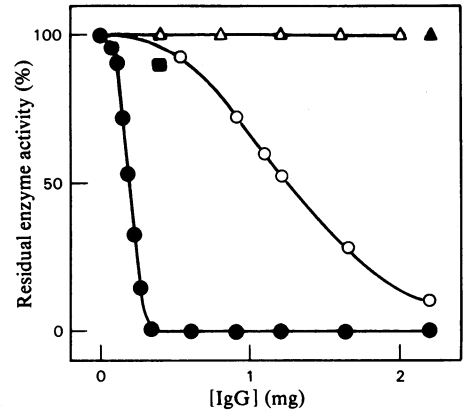

Fig. 9. *Interaction of anti-(monkey liver serine hydroxymethyltransferase) IgG with the purified serine hydroxymethyltransferases from monkey, sheep and rabbit livers*
The antibodies raised in the rabbits to the homogeneous monkey liver enzyme (step 5) were incubated for 30 min at 37°C and then for 12 h at 4°C with 20 μg of monkey liver enzyme (●) or with 22 μg of sheep liver enzyme (○) or with 20 μg of rabbit liver enzyme (▲) or 20 μg of control IgG (pre-imune sera) with 20 μg of monkey liver enzyme (△). The precipitated enzyme–antibody complex was centrifuged at 3000 *g* for 10 min and the enzyme activity in the supernatant was measured. The immunoprecipitate obtained with 350 μg of IgG antibodies and 20 μg of monkey liver enzyme was dispersed in the buffer and enzyme activity was assayed. (■). The activity in the absence of added antibodies was normalized to 100.

Effects of glycine and D-*cycloserine on the enzyme activity*

The product of the reaction, glycine (2–30 mM), competitively inhibited the enzyme when L-serine was the varied substrate at saturating concentration of H_4folate (1.8 mM) and non-competitively when H_4folate was the varied substrate at 3.6 mM-L-serine.

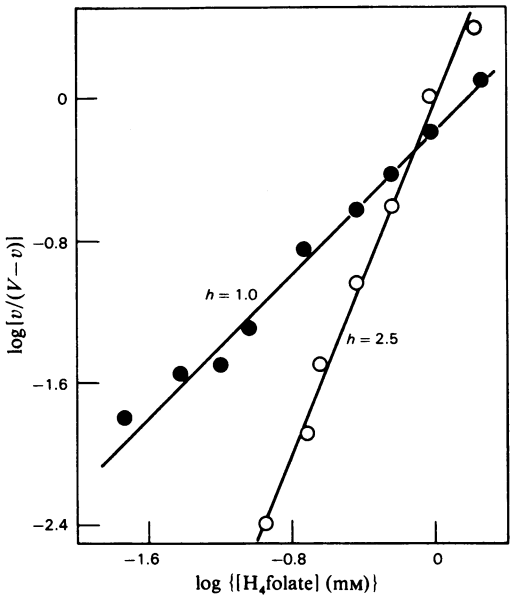

Fig. 10. *Hill plots showing the H_4folate saturation of monkey liver serine hydroxymethyltransferase–antibody complex*

The enzyme (40μg) was incubated for 30min at 37°C and then for 12h at 4°C with 700μg of anti-(monkey liver enzyme) IgG. The immune precipitate obtained on centrifugation at 3000*g* for 10min was dispersed in 50mM-potassium phosphate buffer, pH 7.4. This dispersion (corresponding to 1.1μg of the free enzyme/100μl of reaction mixture) was used for the H_4folate saturation (●). A mixture of 40μg of enzyme and 700μg of control IgG (pre-immune sera), treated similarly, served as the control for H_4folate saturation (○).

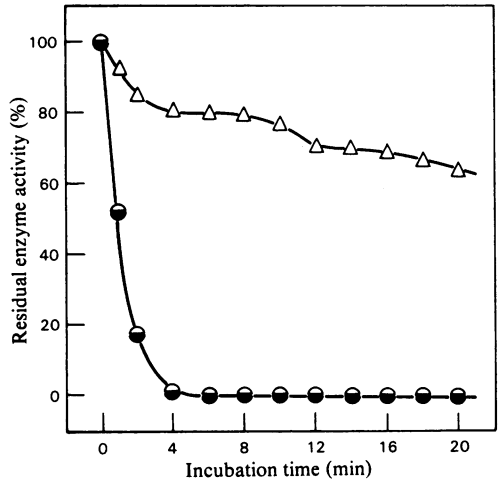

Fig. 11. *Heat-inactivation pattern of monkey liver serine hydroxymethyltransferase–antibody complex*

The enzyme–antibody complex obtained as described in the legend for Fig. 10 was dispersed uniformly in 50mM-potassium phosphate buffer, pH 7.4. This dispersion was heated at 70°C, and samples were withdrawn at regular time intervals, and rapidly cooled in ice and assayed for enzyme activity (△). A mixture of the enzyme and normal IgG (pre-immune sera) (○) or the enzyme alone (●), treated identically, served as controls. The activity of the unheated enzyme was normalized to 100.

Secondary slope and intercept replots were linear and K_I values were determined from the slope replots. D-Cycloserine, a rigid cyclic analogue of D-alanine, inhibited the activity of the enzyme competitively when L-serine was the varied substrate at 1.8mM-H_4folate. The secondary slope replot was linear. These results are summarized in Table 5.

Interaction of rabbit anti-enzyme IgG with monkey liver serine hydroxymethyltransferase

A progressive loss of enzyme activity in the supernatant solution occurred when increasing amounts of anti-enzyme antibodies were added (Fig. 9), and the inhibition pattern was not altered in the presence of NADH or NAD^+. It was noted that antibodies to the monkey liver enzyme cross-reacted and inhibited purified sheep liver serine hydroxymethyltransferase, although at fairly high concentrations of IgG. In contrast, the purified rabbit liver enzyme neither cross-reacted nor was inhibited by the antibodies to the monkey liver enzyme. Comparable quantities of control antibodies (pre-immune sera) had no effect on the enzymes from sheep, monkey and rabbit livers.

The insoluble enzyme–antibody complex, when dispersed in the buffer, had 90% of the original activity. The complex gave a hyperbolic saturation pattern with both H_4folate and L-serine. In contrast with the free enzyme, the enzyme–antibody complex gave an *h* value of 1.0 and $s_{0.5}$ (H_4folate) 0.7mM (Fig. 10). NADH and NAD^+ had no effect on the H_4folate saturation pattern obtained with the enzyme–antibody complex. The enzyme–antibody complex was heat-stable. In contrast with the free enzyme, the complex retained 70% of the activity when heated at 70°C for 20min (Fig. 11).

Discussion

Serine hydroxymethyltransferase has been isolated from several mammalian and microbial sources (Nakano *et al.*, 1968; Fujioka, 1969; Schirch, 1971; Jones & Priest, 1976; Ulevitch & Kallen, 1977; Mansouri *et al.*, 1972). None of these preparations showed any co-operative interactions with substrates or other metabolites, whereas the partially purified enzyme from pig kidney and monkey liver exhibited both homotropic and heterotropic interactions (Harish Kumar *et al.*, 1976; Ramesh & Appaji Rao, 1978). This apparent discrepancy seems to be resolved by the isolation of an homogeneous enzyme exhibiting allosteric properties (Table 3 and Figs. 2–4).

The special features of the present method for purification of the monkey liver enzyme were the use of a Blue Sepharose affinity matrix and the omission of thermal denaturation in the presence of L-serine, which is a major step used by earlier workers for the purification of this enzyme (Nakano *et al.*, 1968; Fujioka, 1969; Akhtar & El-Obeid, 1972; Cheng & Haslam, 1972; Rowe & Lewis, 1973; Palekar *et al.*, 1973; Jones & Priest, 1976; Ulevitch & Kallen, 1977). The allosteric effects of nicotinamide nucleotides on the monkey liver enzyme (Ramesh & Appaji Rao, 1978) suggested that the Blue Sepharose affinity matrix could be effectively used in the purification of the serine hydroxymethyltransferase. It is well known that Blue Sepharose binds proteins that contain a dinucleotide fold in their secondary structure (Thompson *et al.*, 1975). The enzyme could also be purified by using a column of folate–AH-Sepharose. However, the Blue Sepharose affinity procedure was preferred, as this matrix was stable.

The monkey liver enzyme was homogeneous as determined by polyacrylamide-gel disc electrophoresis [Fig. 1, inset (*a*)], isoelectrofocusing, immunoelectrophoresis (Fig. 2*a*), immunodiffusion (Fig. 2*b*), analytical ultracentrifugation (Fig. 3) and gel filtration on Sephacryl S-200 (superfine grade) columns. The enzyme had a molecular weight of 208000 ± 5000 and was composed of four identical subunits of molecular weight 52000 ± 1400 (Fig. 6). The monkey liver enzyme was similar in its molecular weight and $s_{20,w}$ value to the enzyme isolated from rabbit liver (Fujioka, 1969; Martinez-Carrion *et al.*, 1972), lamb liver (Ulevitch & Kallen, 1977) and sheep liver (R. Manohar, personal communication). The monkey liver enzyme, like the enzyme from other sources, contained bound pyridoxal 5′-phosphate (Fig. 5). The pH optimum of the monkey liver enzyme determined at 3.6mM-serine and 1.8mM-H_4folate was 7.4, and this value was similar to that reported for this enzyme from other sources. The pI of the monkey liver enzyme was 4.2 ± 0.1, although the enzyme was adsorbed strongly on CM-Sephadex, suggesting that enzyme had a net positive charge. The reason for this discrepancy can be ascertained only after the determination of the amino acid composition of the enzyme. The catalytic-centre activity of the monkey liver enzyme calculated by using the extrapolated value of $V_{max.}$ with L-serine as the substrate was $1.7 \times 10^{-3} s^{-1}$; values for this enzyme from other sources with L-serine as the substrate are not available. However, when L-threonine was used as the substrate (this reaction does not require H_4-folate), the values were $0.76 \times 10^{-3} s^{-1}$ [rabbit liver (Schirch & Diller, 1971)] and $0.09 s^{-1}$ [lamb liver (Ulevitch & Kallen, 1977)].

The next important property of the purified monkey liver serine hydromethyltransferase that distinguishes it from those of rabbit, lamb, rat and bovine livers is the sigmoid saturation with H_4folate (Fig. 4). The presence of positive homotropic co-operative interactions with H_4folate was indicated by the curvilinear double-reciprocal plot and h value of 2.5. As already mentioned, the presence of L-serine [a positive heterotropic effector (Ramesh & Appaji Rao, 1978)] and the use of a heat-denaturation step during purification of this enzyme from other sources may have resulted in the loss of an allosteric binding site (Harish Kumar *et al.*, 1976). Evidence in support of this argument is provided by the oservations that (i) the monkey liver enzyme purified by using a heat-denaturation step (70°C for 5min) in the presence of L-serine (15mM) failed to exhibit positive homotropic co-operative interactions with H_4folate or heterotropic allosteric effects with nicotinamide nucleotides. When the purified enzyme was heated in the presence of L-serine, the allosteric properties were abolished, (ii) purification of this enzyme from rabbit, sheep and rat livers by the procedure adopted for the monkey liver enzyme gave a homogeneous enzyme that retained positive homotropic co-operative interactions with H_4folate (K. S. Ramesh & N. Appaji Rao, unpublished work), (iii) all these enzyme preparations showed positive heterotropic interactions with nicotinamide nucleotides and (iv) the $s_{0.5}$ (H_4folate) values determined by us were 0.90mM (monkey liver), 0.50mM [mouse liver (Harish Kumar *et al.*, 1976)], 0.74mM (sheep liver) and 1.21mM (rabbit liver) compared with the K_m values (H_4folate) of 0.07mM [ox liver (Rowe & Lewis, 1973)], 0.0072mM [rat liver (Nakano *et al.*, 1968)] and 0.077mM [rabbit liver (Schirch, 1971)]. The decrease in K_m or $s_{0.5}$ values as a consequence of desensitization of allosteric enzymes has been extensively documented (Gerhart & Pardee, 1962; Mansour & Martensen, 1978). Thus it is clear from our observations that the predominant form of the enzyme and its kinetics depend on the method of isolation and assay of the enzyme.

In view of the high temperature optimum (60°C) of the monkey liver serine hydroxymethyltransferase and the extensive use of heat-denaturation steps in the purification of this enzyme from other sources, it was decided to study the thermal stability of the monkey liver enzyme. It is evident that L-serine protects the enzyme against thermal inactivation, whereas allosteric effectors such as NAD^+ and NADH failed to afford any protection. It is well known that substrates or effectors often influence the stability of native enzyme conformations during thermal denaturation. The thermal denaturation of a protein occurs co-operatively, starting from an open site. The ligand-induced conformational changes might either hinder or facilitate the accessibility of the open site for initiation of the denaturation process. In the presence of L-serine this open site may be blocked and hence denaturation prevented. This protection by the substrate is unusual, since denaturation of allosteric proteins is usually facilitated by substrate binding, owing to weakening of subunit interaction (Bernhard, 1968).

Antifolates have greater affinity for dihydrofolate reductase (5,6,7,8-tetrahydrofolate–$NADP^+$ oxidoreductase, EC 1.5.1.3) than for any other enzyme of folate metabolism (Bertino, 1963; Werkheiser, 1963). The beneficial effects of heavy-dose chemotherapy with methotrexate (Frei *et al.*, 1975) suggested the prevalence of secondary site(s) of interaction with the drug. The inhibition of monkey liver serine hydroxymethyltransferase by classical antifolate compounds (Fig. 7) suggests that this enzyme might be an alternative target, especially when the circulating concentration of this drug is very high. A noteworthy feature of the inhibition by methotrexate is the partial inhibition observed even at infinite concentration of the drug (determined by extrapolation), suggesting that this vital enzyme in the folate-metabolic pathway is not completely blocked in the methotrexate therapy. As a consequence the reactions requiring folate coenzyme, except thymidylate synthetase (EC 2.1.1.45) may continue to function even at high concentrations of methotrexate.

It is to be expected that folate coenzymes regulate their metabolism by affecting the first enzyme in the folate pathway, namely serine hydroxymethyltransferase. 5-CH_3-H_4folate and 5-CHO-H_4folate, the end products of the pathway, inhibited the enzyme, the concentrations required to cause 50% inhibition being 3.0mM and 1mM respectively. The predominant folate derivative in the liver is 5-CH_3-H_4folate (Noronha & Silverman, 1962; Shin *et al.*, 1972; Brown *et al.*, 1974). H_2folate was a partial inhibitor of the enzyme. Folate activated the enzyme at low concentrations (<1mM) and inhibited in a sigmoidal fashion with increasing concentrations, suggesting that folate interacted at multiple site(s) on the enzyme. The product of the reaction, glycine, and a substrate analogue, D-cycloserine, competitively inhibited the enzyme when L-serine was the varied substrate (Table 5).

Recovery of full catalytic activity in the insoluble enzyme–antibody complex (Fig. 9) suggests that either the substrate can diffuse through the complex to reach the active site or the antigenic determinant site may be located distal to the substrate-binding site. Antibody binding to the enzyme has resulted in the loss of the nicotinamide nucleotide-binding site and also the site involved in the positive co-operative interactions with H_4folate (Fig. 10). The loss of positive homotropic co-operative interaction of fructose 6-phosphate with the phosphofructokinase (EC 2.7.1.11)–antibody complex has been observed; however, the allosteric interaction with fructose 1,6-bisphosphate was not lost on binding of the antibody to the enzyme (Bartholomé-Dönnicke & Hofer, 1975). The loss of allosteric properties of serine hydroxymethyltransferase on its binding to antibody is reminiscent of the observation that this enzyme from L-1210 solid tumours in mice failed to exhibit homotropic interactions with H_4folate. The enzyme isolated from liver and kidney of tumour-bearing mice also failed to exhibit homotropic interactions (Harish Kumar *et al.*, 1976). The enzyme from tumour tissue regained its allosteric properties on $(NH_4)_2SO_4$ fractionation of the homogenate, suggesting that a dissociable factor was lost during purification (P. M. Harish Kumar, J. H. Mangum & N. Appaji Rao, unpublished work). These results were interpreted to suggest the possibility of the production of a chemical messenger by the tumour that is transported to, and produces biochemical changes in, other tissues of the animal (Harish Kumar *et al.*, 1976). The loss of allosteric properties of the monkey liver enzyme on complexing with its antibody suggests that the production of a proteinaceous factor by the tumour could be one of the mechanisms by which the enzyme activity is regulated in neoplastic tissues. The protection of the enzyme against thermal inactivation by enzyme antibodies (Fig. 11) suggests that the binding of antibody prevents the initiation of denaturation. These results demonstrate that the specific antibodies to the enzyme induce a conformational change that is manifested by altered catalytic, regulatory and physicochemical properties.

The results described in the present paper clearly indicate that serine hydroxymethyltransferase is a regulatory protein, and emphasize the importance of using gentle methods of purification of allosteric enzymes.

We thank Professor C. S. Vaidyanathan and Professor John H. Mangum for their valuable discussion and

suggestions. The technical assistance of Miss V. Sowmithri is acknowledged. This investigation was supported by a research grant from the Department of Science and Technology, Government of India, New Delhi, India. K. S. R. is the recipient of a Senior Research Fellowship of the Department of Atomic Energy, Government of India, India.

References

Akhtar, M. & El-Obeid, H. A. (1972) *Biochim. Biophys. Acta* **258**, 791–799

Andrews, P. (1965) *Biochem. J.* **96**, 595–606

Bartholomé-Dönnicke, M. & Hofer, H. W. (1975) *Biochim. Biophys. Acta* **397**, 347–354

Bernhard, S. (1968) in *The Structure and Function of Enzymes*, pp. 154–161, W. A. Benjamin, New York and Amsterdam

Bertino, J. R. (1963) *Cancer Res.* **23**, 1286–1306

Blobel, G. & Potter, V. R. (1966) *Science* **154**, 1662–1665

Brown, J. P., Davidson, G. E & Scott, J. M. (1974) *Biochim. Biophys. Acta* **343**, 78–88

Cheng, C. & Haslam, J. L. (1972) *Biochemistry* **11**, 3512–3518

Chua, K. E., Crossman, E. J. & Gilmour, C. A. (1978) *Sci. Tools* **25**, 9–11

Clausen, J. (1970) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E., eds.), vol. 1, part 3, pp. 454–465, North-Holland, Amsterdam and London

Conn, E. E. & Stumpf, P. K. (1972) *The Outlines of Biochemistry*. 3rd edn., pp. 127–133, John Wiley and Sons, New York

Fasella, P. (1967) *Annu. Rev. Biochem.* **36**, 185–210

Frei, E., Jaffe, N., Tattersall, M. H. N., Pitman, S. & Parker, L. (1975) *N. Engl. J. Med.* **292**, 846–851

Fujioka, M. (1969) *Biochim. Biophys. Acta* **185**, 338–349

Gerhart, J. C. & Pardee, A. B. (1962) *J. Biol. Chem.* **237**, 891–896.

Harish Kumar, P. M., North, J. A., Mangum, J. H. & Appaji Rao, N. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1950–1953

Hatefi, Y., Talbert, P. T., Osborn, M. J. & Huennekens, F. M. (1960) *Biochem. Prep.* **7**, 89–92

Jones, C. W. & Priest, D. G. (1976) *Arch. Biochem. Biophys.* **174**, 305–311

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mansour, T. E & Martensen, T. M. (1978) *J. Biol. Chem.* **253**, 3628–3634

Mansouri, A., Dector, J. B. & Silber, R. (1972) *J. Biol. Chem.* **247**, 348–352

Martinez-Carrion, M., Critz, W. & Quashnock, J. (1972) *Biochemistry* **11**, 1613–1615

Nakano, Y., Fujika, M. & Wada, H. (1968) *Biochim. Biophys. Acta* **159**, 19–26

Noronha, J. M. & Silverman, M. (1962) *J. Biol. Chem.* **237**, 3299–3302

Ouchterlony, Ö. (1958) *Prog. Allergy* **5**, 1–78

Palekar, A. G., Tate, S. S. & Meister, A. (1973) *J. Biol. Chem.* **248**, 1158–1167

Ramesh, K. S. & Appaji Rao, N. (1978) *Biochem. J.* **174**, 1055–1058

Reisner, A. H., Nemes, P. & Bucholtz, C. (1975) *Anal. Biochem.* **64**, 509–516

Rowe, P. B. & Lewis, G. P. (1973) *Biochemistry* **12**, 1962–1969

Schirch, L. (1971) *Methods Enzymol.* **17**, 335–340

Schirch, L. & Diller, A. (1971) *J. Biol. Chem.* **246**, 3961–3966

Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32–103

Segel, I. H. (1975) *Enzyme Kinetics*, pp. 371–375, John Wiley and Sons, New York

Shin, Y. S., Williams, M. A. & Stokstad, E. L. R. (1972) *Biochem. Biophys. Res. Commun.* **47**, 35–43

Strickland, S. & Massey, V. (1973) *Proc. Int. Symp. Oxidases and Related Redox Systems 2nd* 189

Taylor, R. T. & Weissbach, H. (1965) *Anal. Biochem.* **13**, 80–84

Thompson. S. T., Cass, K. H. & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 669–672

Ulevitch, R. J. & Kallen, R. G. (1977) *Biochemistry* **16**, 5342–5350

Webb, J. L. (1963) *Enzymes and Metabolic Inhibitors*, vol. 1, pp. 149–191, Academic Press, New York

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Werkheiser, W. C. (1963) *Cancer Res.* **23**, 1277–1285