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PURIFICATION AND PROPERTIES OF SERINE HYDROXYMETHYLASE FROM SOLUBLE AND MITOCHONDRIAL FRACTIONS OF RABBIT LIVER*

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SUMMARY

Serine hydroxymethylase (L-serine tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2 1 2 1) isoenzymes were purified separately from the soluble and mitochondrial fractions of rabbit liver The purified preparations appeared to contain a single component in each case as judged from ultracentrifugation and electrophoresis on polyacrylamide or starch gels The isoenzymes had similar properties with respect to pH optimum, electrophoretic mobility as well as the reactions they catalyzed The isoenzymes, however, were different immunochemically, the antiglobulin prepared against one of the isoenzymes did not inhibit the enzymic activity of the other The molecular weights of the soluble fraction and the mitochondrial fraction enzymes, determined by the sedimentation equilibrium method, were approx 185 000 and 170 000, respectively Both isoenzymes contained 4 moles of pyridoxal phosphate per mole of enzyme

INTRODUCTION

The preceding paper[1] has shown that two types of serine hydroxymethylase (L-serine tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2 1 2 1) occur in the soluble and mitochondrial fractions of rat liver Partially purified preparations of serine hydroxymethylase from the two fractions differ in pH optimum, electrophoretic mobility and stability

Previously, SCHIRCH AND MASON[2] obtained an homogeneous preparation of serine hydroxymethylase from acetone powder or frozen preparations of rabbit liver With this enzyme preparation, the spectral properties and various aspects of enzyme action were investigated[2-5] The occurrence of serine hydroxymethylase isoenzymes in rat liver has prompted us to reinvestigate the rabbit liver enzyme and it was found that the latter also contains isoenzymes with similar properties

The purpose of this communication is to describe the purification and properties of serine hydroxymethylase isoenzymes from the soluble and mitochondrial fractions

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of rabbit liver and to compare the results with those previously obtained with rabbit liver enzyme

EXPERIMENTAL PROCEDURE

(±)-L-Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid as described by HATEFI *et al.*[6] $NADP^+$ was obtained from the Sigma Chemical Co. Other chemicals were purchased from commercial sources and were used without further purification. Brushite was prepared by slow admixing of Na_2HPO_4 and $CaCl_2$ solutions according to the method of TISELIUS *et al.*[7]

5,10-Methylenetetrahydrofolate dehydrogenase was purified from *Achromobacter eurydice* grown on L-glutamate as the sole carbon source. After protamine sulfate treatment of the extract of sonicated cells, the supernatant solution obtained by centrifugation was fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 40 and 52% saturation was dissolved in a small amount of 0.05 M potassium phosphate buffer (pH 6.5) and was dialyzed against the same buffer. The dialyzed enzyme was diluted with 0.05 M potassium phosphate buffer (pH 6.5) to give a protein concentration of about 1% and was heated at 56° for 3 min. After removing the denatured protein by centrifugation, the supernatant solution was concentrated by precipitation with $(NH_4)_2SO_4$. After dialysis of the concentrated solution against 0.02 M potassium phosphate buffer (pH 7.1), a volume containing approx. 320 mg of protein was poured onto a column (2 cm × 10 cm) of DEAE-cellulose which had been equilibrated with 0.02 M potassium phosphate buffer (pH 7.1). The column was then washed with 150 ml of the same buffer, and elution was carried out using a linear gradient with 350 ml of 0.02 M potassium phosphate buffer (pH 7.1) in the mixing vessel and 350 ml of 0.2 M potassium phosphate buffer in the reservoir. 5,10-Methylenetetrahydrofolate dehydrogenase usually elutes soon after establishment of the gradient. Chromatography on DEAE-cellulose effects the separation of the dehydrogenase from serine hydroxymethylase. The fractions containing the dehydrogenase were combined and concentrated by precipitation with $(NH_4)_2SO_4$. The purified preparation was stable and could be stored at 5° for at least 6 months without appreciable loss of activity.

Antisera to the serine hydroxymethylases from the soluble and mitochondrial fractions were prepared separately in guinea pigs by intramuscular injections of the purified soluble fraction enzyme and the crystalline mitochondrial enzyme as antigens in Freund's complete adjuvant. 3 weeks after the first injection of 0.5 mg of each antigen (0.5 mg per guinea pig), the animals received second injections of the same amounts of antigens. Blood was collected by cardiac puncture 2 weeks after the second injection. The globulin fraction was prepared by precipitation with Na_2SO_4 and was dialyzed against 0.3% NaCl solution before use[8].

Protein was estimated by the method of LOWRY *et al.*[9]. The dry weight of protein was determined on a salt-free sample dried over P_2O_5 under reduced pressure.

Serine hydroxymethylase activity was determined by the rate of utilization of formaldehyde for serine synthesis.

$$\text{Serine} + \text{tetrahydrofolate} \underset{b}{\overset{a}{\rightleftharpoons}} \text{glycine} + \text{5,10-methylenetetrahydrofolate} + H_2O \qquad (1)$$

$$\text{5,10-Methylenetetrahydrofolate} + NADP^+ \underset{b}{\overset{a}{\rightleftharpoons}} \text{5,10-methenyltetrahydrofolate} + NADPH + H^+ \qquad (2)$$

from glycine (Reaction 1b, Assay 1), or by the rate of NADPH formation in a coupled system of serine hydroxymethylase and 5,10-methylenetetrahydrofolate dehydrogenase (Reactions 1a and 2a, Assay 2) Details of assay conditions have been previously given[1]

Sedimentation velocity and equilibrium measurements were made in a Hitachi UCA-1A analytical ultracentrifuge equipped with a phase plate schlieren diaphragm and a Rayleigh interference optical system Fringe displacement measurements were made with a Nikon optical microcomparator Molecular weights were determined by the high-speed sedimentation equilibrium method of YPHANTIS[10]

Spectra were made in a Cary Model-14 spectrometer, and absorbance measurements were made in a Shimadzu QB-50 spectrophotometer

RESULTS

Purification of serine hydroxymethylases

Freshly excised rabbit liver was homogenized with 5 vol of 0 25 M sucrose in a Waring blender After removing connective tissues and cellular debris by passing the homogenate through a layer of gauze, the nuclear fraction was separated by centrifugation (700 × *g*, 10 min) in the usual manner The precipitate thus obtained was again homogenized in one-half the original volume of 0 25 M sucrose in a glass homogenizer fitted with a plastic pestle, and the supernatant obtained by centrifugation was combined with the first supernatant The combined supernatant solution was centrifuged at 10 000 × *g* for 10 min, and the mitochondrial pellet obtained was washed once with the sucrose solution and stored at −20° until use Since no serine hydroxymethylase activity was found to be associated with the microsomal fraction, the 10 000 × *g* supernatant fluid was used as the starting material for the purification of serine hydroxymethylase from the soluble fraction

Purification of serine hydroxymethylase from the soluble fraction

Step 1. To the 10 000 × *g* supernatant solution (1680 ml), 420 g of solid $(NH_4)_2SO_4$ were gradually added, the pH was held at 7 by addition of 2 M KOH After 30 min, the mixture was transferred to a disc of filter paper which had previously been coated with Super-Cel The filtrate was then treated with 168 g of solid $(NH_4)_2SO_4$, and the resulting precipitate was collected by filtration in the same manner and dissolved in 0 05 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA

Step 2 The solution from Step 1 was diluted with distilled water to give a protein concentration of about 10 mg/ml To the diluted solution were added 0 2 vol of 0 5 M potassium phosphate buffer (pH 6 5), and L-serine to a final concentration of 20 mM The mixture was then heated in a water bath at 63° for 3 min and after rapid cooling, the denatured protein was collected by centrifugation and discarded

Step 3 The supernatant solution from Step 2 was treated with $(NH_4)_2SO_4$ in the same manner as in Step 1, and the precipitate obtained by centrifugation was dissolved in a small volume of 0 05 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA The solution was then dialyzed against 0 01 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA and 1 4 mM 2-mercaptoethanol

Step 4 The dialyzed solution from Step 3, containing approx 300 mg of protein, was poured onto a column of brushite (3 cm × 17 cm) which had been equilibrated

with 0 01 M potassium phosphate buffer (pH 7 1), containing 1 4 mM 2-mercaptoethanol and 8 μM pyridoxal phosphate* The column was washed with 260 ml of the same buffer, and serine hydroxymethylase activity was eluted with 0 04 M potassium phosphate buffer (pH 7 1) containing 1 4 mM 2-mercaptoethanol and 8 μM pyridoxal phosphate The fractions having a high specific activity were combined, and 40 g of solid $(NH_4)_2SO_4$ were added per 100 ml solution The resulting precipitate was taken up in a small volume of 0 05 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA

Step 5 The concentrated enzyme solution from Step 4 was freed from insoluble material by brief centrifugation, and solid $(NH_4)_2SO_4$ was added to turbidity At this stage, a weak schlieren sheen could be observed Microscopic examination revealed the formation of small particles, but attempts to increase their size were unsuccessful Standing the mixture for several days at 5° resulted in the formation of a rather heavy precipitate

A summary of the typical purification procedure of serine hydroxymethylase from the soluble fraction is presented in Table I

TABLE I

PURIFICATION OF SERINE HYDROXYMETHYLASE FROM THE SOLUBLE FRACTION

Serine hydroxymethylase activity was determined by Assay 1 A unit of enzyme activity was defined as the amount of enzyme causing the disappearance of 1 μmole of formaldehyde per min at 37°

Fraction	*Volume (ml)*	*Protein content (mg/ml)*	*Total units*	*Specific activity (units/mg protein)*	*Yield (%)*
10 000 × *g* supernatant	1680	19 7	1880	0 057	(100)
1st $(NH_4)_2SO_4$	97	55 0	1460	0 27	77 9
Heat treatment	1030	1 3	1420	1 10	75 6
2nd $(NH_4)_2SO_4$	12 5	24 2	1010	3 34	53 8
Brushite	3 0	23 6	525	7 4	27 9
3rd $(NH_4)_2SO_4$	2 0	15 5	310	10 0	16 5

Purification of mitochondrial serine hydroxymethylase

Step 1 Packed mitochondria were suspended in distilled water to give a protein concentration of approx 20 mg/ml The suspension (700 ml) was made 1 mM and 20 mM with respect to EDTA and L-serine, respectively, and heated at 63° for 3 min The bulky precipitate obtained by centrifugation was washed once with 350 ml of 0 02 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA, and both supernatant solutions were combined

Step 2 To 840 ml of the heated extract, 184 g of solid $(NH_4)_2SO_4$ were added, the pH being maintained at 7 by addition of 2 M KOH After 30 min, the precipitate

* In view of the report of SCHIRCH AND MASON[2] that the purified preparation of serine hydroxymethylase was activated to a considerable degree by added pyridoxal phosphate, it was initially included in some buffer solutions used in steps where dilution of the enzyme solution might be encountered in order to avoid possible inactivation of the apoprotein Subsequent experiments have shown that a similar purification can be achieved in the absence of added pyridoxal phosphate (see DISCUSSION)

was collected by centrifugation and discarded, and 84 g of $(NH_4)_2SO_4$ were added to the supernatant fluid The resulting precipitate was collected by centrifugation and dissolved in a minimal volume of 0 05 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA

Step 3 The enzyme solution from Step 2 was dialyzed overnight against 0 005 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA, and the dialyzed enzyme was put on a column of brushite (3 cm × 14 cm) which had been equilibrated with 0 01 M potassium phosphate buffer (pH 7 1) containing 8 μM pyridoxal phosphate After washing the column with 300 ml of the same buffer, elution was carried out with 0 03 M potassium phosphate buffer (pH 7 1) containing 8 μM pyridoxal phosphate To the combined active fractions $(NH_4)_2SO_4$ (40 g per 100 ml) was added, and the resulting precipitate, collected by centrifugation, was dissolved in a small volume of 0 05 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA The mitochondrial serine hydroxymethylase could be crystallized from this concentrated solution by allowing it to stand for several days at 5° Fig 1 shows a photomicrograph of crystalline mitochondrial serine hydroxymethylase

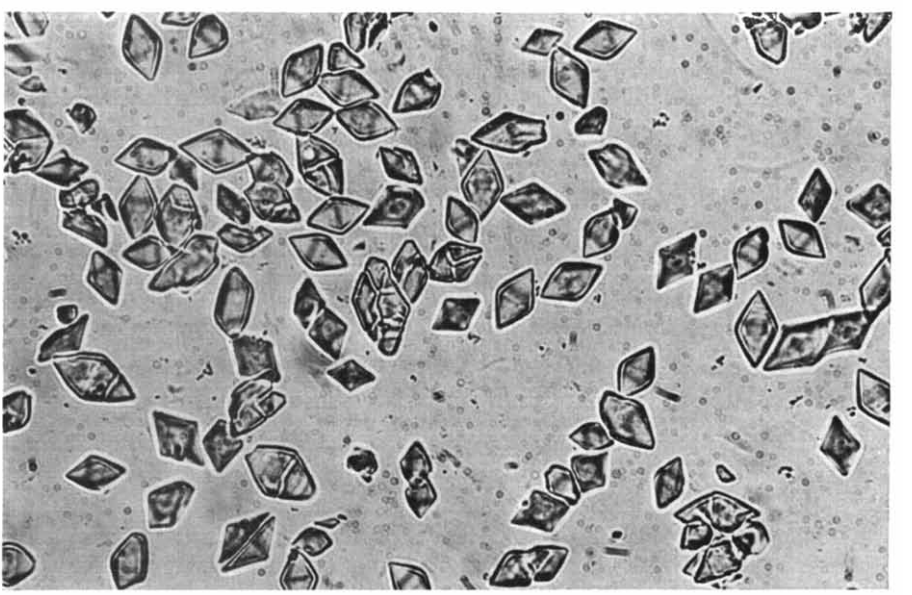

Fig 1 Photomicrograph of crystalline serine hydroxymethylase from the mitochondrial fraction Magnification, × 100

Table II summarizes the purification procedure of serine hydroxymethylase from the mitochondrial fraction

Properties of the soluble fraction and mitochondrial serine hydroxymethylases

Homogeneity Sedimentation patterns of the purified serine hydroxymethylases from the soluble and mitochondrial fractions obtained using an analytical ultracentrifuge, are shown in Fig 2 In both cases, a single symmetrical boundary was

TABLE II

PURIFICATION OF SERINE HYDROXYMETHYLASE FROM THE MITOCHONDRIAL FRACTION
Serine hydroxymethylase activity was measured by Assay 1

Fraction	*Volume (ml)*	*Protein content (mg/ml)*	*Total units*	*Specific activity (units/mg protein)*	*Yield (%)*
Mitochondrial suspension	750	13 1	640	0 066	(100)
Heat treatment	840	1 8	540	0 36	84 5
$(NH_4)_2SO_4$	9 4	26 2	425	1 73	66 5
Brushite	1 5	20 1	250	8 28	39 1
Crystallization	1 0	11 6	175	15 5	27 4

observed The sedimentation coefficients ($s_{20,w}$) of the soluble fraction and mitochondrial fraction enzymes calculated from the data presented in Fig 2, were 8 8 S and 8 5 S, respectively

Electrophoresis of the enzymes was performed on thin-layer polyacrylamide gel prepared in 0 01 M potassium phosphate buffer (pH 7 4), at 10° with a constant current of 2 mA/cm Protein was stained with 0 2% Amidoblack in methanol–water–acetic acid (5 5 1, by vol) Both the soluble fraction and mitochondrial fraction enzymes migrated toward the anode as one major band in each case A commercial crystalline bovine serum albumin, under the same experimental conditions, cleaved into one major band migrating well towards the anode, and 2 faint bands The electrophoretic mobilities of the serine hydroxymethylases purified from the two fractions were almost identical and migrated about one-fifth as fast as the major band of bovine serum albumin Starch-gel electrophoresis performed under the same conditions as above also revealed in each case a single band which had identical mobilities

Molecular weights The molecular weights of the soluble fraction and mitochondrial fraction serine hydroxymethylases were determined by the high-speed sedimentation equilibrium procedure of YPHANTIS[10] The plot of log of concentration *vs* square of distance from the center of rotation, r^2, gave a straight line in each case From the data presented in Fig 3, the apparent molecular weights of the serine

Fig 2 Sedimentation patterns of serine hydroxymethylases A The soluble fraction enzyme (Step 5) was dialyzed against 0 04 M potassium phosphate buffer (pH 7 1), containing 1 mM EDTA, and was centrifuged at 20° at 43 700 rev /min The protein concentration was 5 6 mg/ml Each picture was taken at about 18 and 48 min after maximum speed was reached B The crystalline mitochondrial enzyme was dialyzed against 0 04 M potassium pyrophosphate buffer (pH 8 5), containing 1 mM EDTA, and was sedimented at 15° at 43 700 rev /min The protein concentration was 11 2 mg/ml Pictures were taken at about 37 and 82 min after reaching maximum speed

Fig 3 Sedimentation equilibrium runs of the serine hydroxymethylases Plots are log c against r^2 A The soluble fraction enzyme (Step 5) was dialyzed against 0 02 M potassium phosphate buffer (pH 7 1) containing 1 mM EDTA The protein concentration was 0 43 mg/ml A 3 5-mm column of the enzyme solution was analyzed in a double-sector cell, the dialysate being placed in the reference cell The rotor speed was 25 090 rev /min, time, 21 h, temp, 20° B The crystalline mitochondrial enzyme was dialyzed against 0 05 M potassium pyrophosphate buffer (pH 8 5) containing 1 mM EDTA The protein concentration was 0 39 mg/ml The sedimentation equilibrium run was made under the same conditions as in (A)

hydroxymetylases were calculated as 185 000 and 170 000, respectively, assuming a partial specific volume of 0 73 ml/g

Absorption spectra The purified preparation of the soluble fraction serine hydroxymethylase showed absorption maxima at 278 and 428 mμ, characteristic of pyridoxal phosphate-containing enzymes (Fig 4A) The mitochondrial enzyme showed a similar spectrum with absorption maxima at 278 and 430 mμ (Fig 4B) The $A_{278 m\mu}$ $A_{430\ m\mu}$ ratio of the soluble fraction serine hydroxymethylase was 8 9, whereas that of the mitochondrial enzyme was 6 3 The spectra of the serine hydroxymethylases did not shift when the pH of the solution was varied between 5 4 and 9 2, in accordance with the finding of SCHIRCH AND MASON[3] The addition of D-alanine to serine hydroxy-

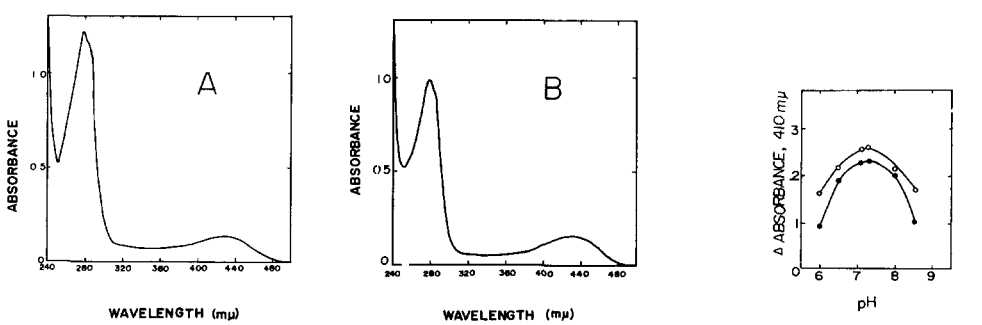

Fig 4 Absorption spectra of the serine hydroxymethylases A Spectrum of purified soluble fraction serine hydroxymethylase (Step 5) in 0 05 M potassium phosphate buffer (pH 7 1) The protein concentration was 1 56 mg/ml B Spectrum of mitochondrial serine hydroxymethylase in 0 05 M potassium phosphate buffer (pH 7 1) The protein concentration was 1 42 mg/ml

Fig 5 Serine hydroxymethylase activity as a function of pH The purified preparations of the soluble fraction (●—●) and mitochondrial (○—○) serine hydroxymethylases were used Activity was measured by Assay 1

methylase was previously shown to cause the appearance of a new peak at 495 mμ, the height of which was increased in the presence of tetrahydrofolate[2,4] Similar experiments carried out with the soluble fraction and mitochondrial fraction enzymes also confirmed these findings Addition of D-serine had no effect on the spectra of either enzyme

pH optima The pH optima of the soluble fraction and mitochondrial enzymes were found to lie at 7 3 when assayed with glycine and 5,10-methylenetetrahydrofolate as substrates The pH–activity curves were similar for both enzymes, but a somewhat steeper curve was obtained for the soluble fraction enzyme (Fig 5)

Reaction velocities in forward and reverse directions The serine hydroxymethylase reaction is readily reversible The initial velocities in the forward (Reaction 1a) and reverse (Reaction 1b) directions were determined under standard assay conditions, and the ratio of these values (forward/reverse) was calculated for both the soluble fraction and mitochondrial enzymes, a value of 1 7 was found for the former and 1 8 for the latter

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON SERINE HYDROXYMETHYLASE ACTIVITY

The enzyme was preincubated with each compound tested in 0 3 ml of 0 1 M potassium phosphate buffer (pH 7 3) for 10 min at 19° The activity measurements were made by Assay 1

Compound	*Concn (mM)*	*Original enzyme activity (%)*	
		Soluble fraction serine hydroxy-methylase	*Mitochondrial serine hydroxy-methylase*
o-Phenanthroline	1	71 2	59 6
α,α′-Dipyridyl	1	100	100
EDTA	10	87 0	83 0
p-Chloromercuribenzoate	0 004	7 4	71 5
	0 02	0	50 0
	0 1	0	33 2
Iodoacetate	10	100	100
N-Ethylmaleimide	0 1	72 6	93 7
	1	21 8	45 0
	10	0	35 5
Sodium arsenite	10	100	100
NH_2OH	1	84 5	100
Hydrazine	1	36 4	66 6

Effect of various compounds on serine hydroxymethylase activity To test the difference in susceptibility of the two enzymes to inhibition, their activities were measured in the presence of various inhibitors As shown in Table III, the soluble fraction enzyme was more susceptible to sulfhydryl reagents and also to carbonyl reagents The soluble fraction enzyme was very strongly inhibited by *p*-chloromercuribenzoate and *N*-ethylmaleimide The enzyme was not protected from inhibition by these compounds by the presence of either glycine or 5,10-methylenetetrahydrofolate Iodoacetate and $NaAsO_2$ had no effect on the reaction rate

Monovalent (K^+, Na^+, NH^+_4) and divalent (Mg^{2+}, Mn^{2+}, Ca^{2+}) cations, tested at

concentrations of 0 1 M and 0 01 M, respectively, did not influence the reaction rate Addition of thiol compounds such as 2-mercaptoethanol (10 mM) and dithiothreitol (1 mM) had also no effect Incubation of the enzymes with purine nucleoside mono-, di-, and triphosphates (each at 5 mM) resulted in no significant change in activity

Amounts of pyridoxal phosphate bound to enzyme The amounts of pyridoxal phosphate bound to the enzymes were determined by the phenylhydrazine method[11] The purified preparations of the soluble fraction and mitochondrial serine hydroxymethylases were dialyzed against 0 01 M potassium phosphate buffer (pH 7 1) and mixed with 2% phenylhydrazine in 1 M H_2SO_4 After removing the denatured protein by centrifugation, the absorbancies of the supernatant solutions were read at 410 mμ For the soluble fraction serine hydroxymethylase, 1 mole of pyridoxal phosphate was found to bind with 48 000 g of protein; the corresponding value for the mitochondrial enzyme was 42 000 g of protein These values and the apparent molecular weights of the enzymes (Fig 3), suggest that 1 mole of the enzyme binds 4 moles of pyridoxal phosphate

Reaction with α-methylserine SCHIRCH AND MASON[2] demonstrated that α-methylserine was cleaved to D-alanine and 5,10-methylenetetrahydrofolate by serine hydroxymethylase Tested at high concentrations of the soluble fraction and mitochondrial serine hydroxymethylases, cleavage of α-methylserine could be demonstrated by substituting the compound for L-serine in Assay 2 With a comparatively small amount of enzyme that readily catalyzed the breakdown of L-serine, no appreciable cleavage of α-methylserine occurred, and when the same compound was added to the reaction mixtures containing L-serine (Assay 2), it inhibited the reaction With L-serine as the variable substrate, α-methylserine inhibited the cleavage of serine competitively as judged from LINEWEAVER–BURK plots[12] Table IV compares the relative rates of transformation of α-methylserine by the two enzymes, and the K_i values found when this compound was added as the inhibitor of the serine hydroxymethylase reaction An approx 3-fold higher K_i value was obtained for the mitochondrial enzyme The

TABLE IV

REACTION WITH α-METHYLSERINE

The incubation mixture contained, in 1 4 ml, 150 μmoles of potassium phosphate buffer (pH 7 3), 0 2 μmole of (±)-L-tetrahydrofolate, 7 μmoles of 2-mercaptoethanol, 0 25 μmole of $NADP^+$, a sufficient amount of 5,10-methylenetetrahydrofolate dehydrogenase and serine hydroxymethylase from either the soluble fraction or the mitochondrial fraction The reaction was started by addition of 0 1 ml of 0 1 M L-serine or 0 1 M α-methyl-DL-serine, and the increase in absorbance at 340 mμ was read in a spectrophotometer at 19° The amounts of enzyme used from the soluble and mitochondrial fractions were 2 5 and 2 μg, respectively, where L-serine was the substrate With α-methyl-DL-serine as the substrate, the amounts of enzymes were increased 20-fold For determination of K_i values, L-serine was incubated under the same conditions but in the presence of 3 34, 6 67 and 13 4 mM α-methyl-DL-serine Calculation of K_i values are based on the assumption that only one isomer of α-methyl-DL-serine is active

	Relative activity with		*K_i for α-methyl-serine (mM)*
	L-Serine	*α-Methyl-DL-serine*	
Soluble fraction serine hydroxymethylase	100	1 24	3 9
Mitochondrial serine hydroxymethylase	100	1 12	11 2

K_m values for serine determined under these conditions were 1 3 mM for the soluble fraction enzyme and 1 0 mM for the mitochondrial enzyme

Immunochemical studies To examine the immunochemical distinction between the soluble fraction and the mitochondrial serine hydroxymethylases, antisera were prepared separately in guinea pigs with either the purified preparation of the soluble fraction or the mitochondrial serine hydroxymethylase as antigen Antiglobulins were prepared from these antisera by precipitation with Na_2SO_4 As shown in Fig 6, both the enzymes were inhibited by preincubation with the corresponding antiglobulins

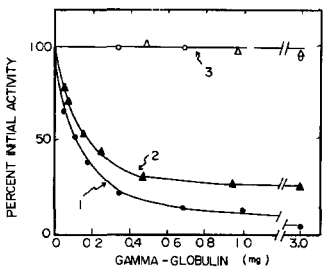

Fig 6 Inhibition of serine hydroxymethylase activity by antiglobulins The antiglobulins against the soluble fraction and mitochondrial serine hydroxymethylases were prepared as described in EXPERIMENTAL PROCEDURE The purified soluble fraction enzyme equivalent to 8 μg of protein and the crystalline mitochondrial enzyme equivalent to 6 μg of protein, were preincubated with antiglobulins in 0 3 ml of 0 1 M potassium phosphate buffer (pH 7 3) for 20 min at 32° The enzyme activity was measured under standard assay conditions by Assay 1 Curve 1, soluble fraction enzyme *plus* the antisoluble fraction serine hydroxymethylase, Curve 2, mitochondrial enzyme *plus* antimitochondrial enzyme, Curve 3, soluble fraction enzyme *plus* antimitochondrial enzyme or mitochondrial enzyme *plus* antisoluble fraction enzyme

Almost complete inhibition of the soluble fraction serine hydroxymethylase occurred on incubation with its antiglobulin, whereas the inhibition maximum was about 70% with the mitochondrial serine hydroxymethylase and its antiglobulin The activity of the soluble fraction serine hydroxymethylase was not inhibited by the antiglobulin to the mitochondrial serine hydroxymethylase, and conversely, the activity of the mitochondrial enzyme was not affected by the antiglobulin to the soluble fraction enzyme Normal serum was also without effect on the activities of both enzymes

DISCUSSION

Previous investigation in this laboratory has shown that serine hydroxymethylase isoenzymes occur in the soluble and mitochondrial fractions of rat liver[1] The isoenzymes purified from this source differ in their electrophoretic mobilities, pH optima and stabilities Preliminary experiments with livers of pig, ox, chicken, mouse and rabbit have indicated that serine hydroxymethylase activity resides both in the soluble and mitochondrial fractions, and also that both crude soluble and mitochondrial fractions of rabbit liver have about 8–10 times higher activity than those of other mammalian livers Although SCHIRCH AND MASON[2] obtained an apparently homogeneous preparation of serine hydroxymethylase from rabbit liver, no study was made as to the intracellular localization of the enzyme Demonstration of the occurrence of serine hydroxymethylase isoenzymes in rat liver and presumably in other mammalian

livers prompted us to investigate the isoenzymes in rabbit liver Serine hydroxymethylase was purified from the soluble and mitochondrial fractions of rabbit liver to an apparent homogeneity In contrast to the rat liver enzymes, the serine hydroxymethylases purified from the two fractions of rabbit liver have similar pH optima, electrophoretic mobilities and stabilities The reaction with α-methylserine and D-alanine previously reported by SCHIRCH AND MASON[2] and by SCHIRCH AND JENKINS[4,5] is common to both isoenzymes, although minor differences exist The property which clearly distinguishes these isoenzymes is their immunochemical reactivity The antiglobulin prepared against one of the isoenzymes does not inhibit the activity of the other, although it inhibits the corresponding enzyme (Fig 6) A similar immunochemical difference between isoenzymes which differ in their intracellular localization is reported for aspartate aminotransferase[13]

Previously, SCHIRCH AND MASON[2] obtained rabbit liver serine hydroxymethylase with a molecular weight of 331 000 that contained 4 moles of pyridoxal phosphate per mole of enzyme This preparation could be activated about 35% by added pyridoxal phosphate When a purified enzyme preparation was first incubated with excess pyridoxal phosphate and then dialyzed against 0 01 M potassium phosphate buffer (pH 7 3) containing 1 mM EDTA for 48 h, the dialyzed enzyme still contained 4 moles of pyridoxal phosphate per mole of enzyme From these observations, the authors suggested that the enzyme contained 6 moles of pyridoxal phosphate when fully active, and that 2 of these were easily removed by dialysis while the rest were firmly bound The serine hydroxymethylase isoenzymes purified by the present procedures seem to have rather firmly bound pyridoxal phosphate Neither prolonged dialysis against potassium phosphate–EDTA buffer, under the same conditions as above, nor gel filtration on Sephadex caused a reduction of specific enzyme activities nor were the isoenzymes activated by added pyridoxal phosphate The apparent molecular weights of the soluble fraction and mitochondrial serine hydroxymethylases, as determined by sedimentation equilibrium analysis, were about 185 000 and 170 000, respectively These values and the minimum molecular weight calculated on the basis of the coenzyme content suggest that both isoenzymes contain 4 moles of pyridoxal phosphate per mole If the fully active preparation of SCHIRCH AND MASON contains 6 moles of pyridoxal phosphate, the weight of protein bound by 1 mole of the coenzyme compares rather well with those of the isoenzyme reported here The reason for discrepancies with the results of SCHIRCH AND MASON regarding the molecular weight and activation by added pyridoxal phosphate is not clear However, it may be inferred that the portion of their preparation which is not bound with the coenzyme might be destroyed and removed during purification, possibly at the heat step involved in the purification methods employed here

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