Biochem. J. (1975) **145**, 159–168
Printed in Great Britain

Mechanistic, Inhibitory and Stereochemical Studies on Cytoplasmic and Mitochondrial Serine Transhydroxymethylases

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(*Received* 18 *July* 1974)

By using cytoplasmic and mitochondrial serine transhydroxymethylase isoenzymes from rabbit liver, it was shown that both enzymes exhibited similar ratios of serine transhydroxymethylase/threonine aldolase activities. Both enzymes catalysed the removal of the pro-*S* hydrogen atom of glycine, which was greatly enhanced by the presence of tetrahydrofolate. The cytoplasmic as well as the mitochondrial enzyme catalysed the synthesis of serine from glycine and [$^3H_2$]formaldehyde in the absence of tetrahydrofolate. The results are consistent with our previous suggestion that a role of tetrahydrofolate in the serine transhydroxymethylase reaction is to transport formaldehyde in and out of the active site (Jordan & Akhtar, 1970). The isoenzymes, however, showed remarkable differences in their inactivation by inhibitors. The serine transhydroxymethylase as well as the threonine aldolase activities of the cytoplasmic enzyme were inactivated in a similar fashion by chloroacetaldehyde, iodoacetamide, bromopyruvate and glycidaldehyde (2,3-epoxypropionaldehyde). These inhibitors had no effect on the two activities of the mitochondrial enzyme. The rate of inactivation of the cytoplasmic enzyme by glycidaldehyde was enhanced by the presence of glycine but decreased by the presence of serine. The implications of these results to the mechanism of catalysis and the nature of the active site of the enzymes are discussed.

Serine transhydroxymethylase (5,10-methylenetetrahydrofolate–glycine hydroxymethyltransferase, EC 2.1.2.1) catalyses the reversible reaction of eqn. (1) and also possesses the additional activity for the cleavage of allothreonine and/or threonine (eqn. 2).

$$\text{Glycine} + \text{methylenetetrahydrofolate} \rightleftharpoons \text{L-serine} + \text{tetrahydrofolate} \quad (1)$$

$$\text{Glycine} + \text{acetaldehyde} \rightleftharpoons \text{allothreonine (or threonine)} \quad (2)$$

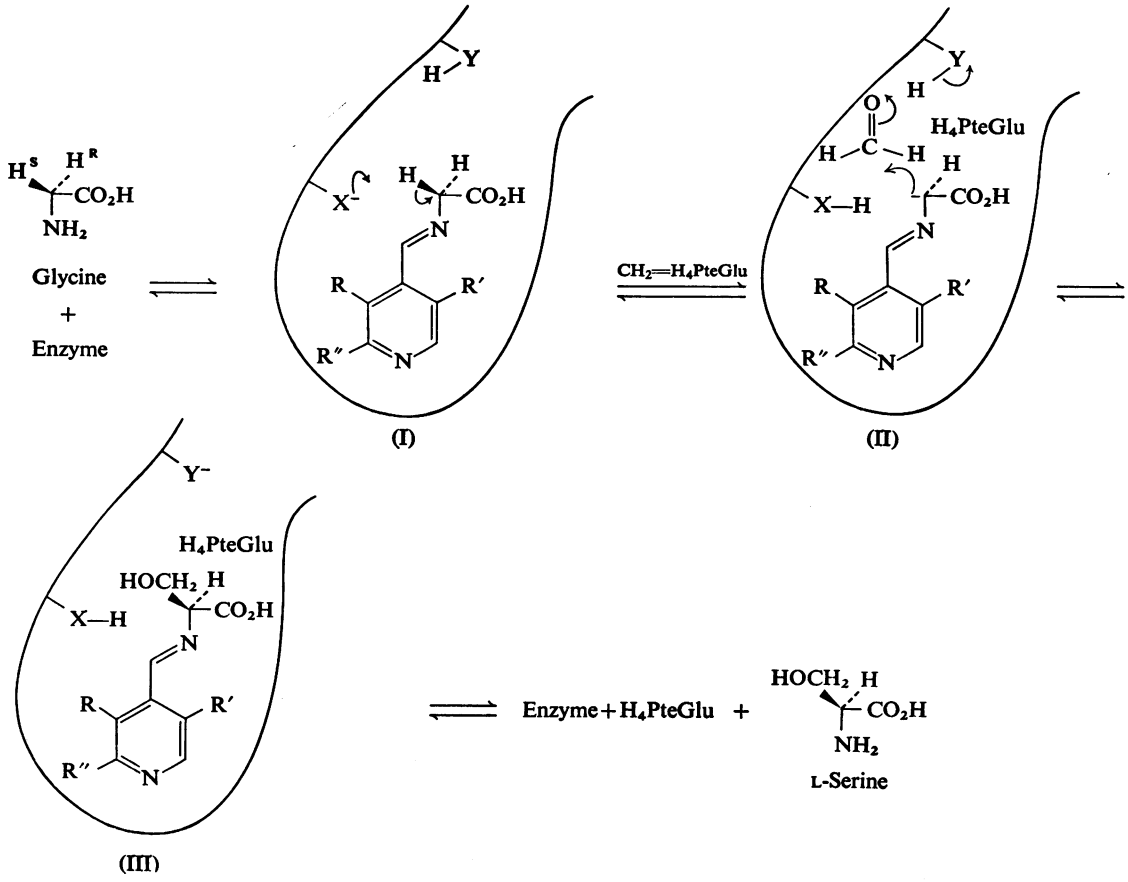
On the basis of mechanistic studies using rabbit liver cytoplasmic serine transhydroxymethylase (Schirch & Jenkins, 1964; Jordan & Akhtar, 1970; Akhtar & El-Obeid, 1972) we have suggested that the overall conversion occurs through the sequence shown in Scheme 1. This involves in the initial step the formation of a glycine–pyridoxal phosphate–enzyme complex (I), from which the pro-2*S* hydrogen atom of the glycine moiety is next removed to give the carbanion intermediate (II). The latter species (II) then reacts with formaldehyde, released at the active site from methylenetetrahydrofolate, to give the Schiff base of L-serine–pyridoxal phosphate–enzyme (III), which finally decomposes to the products (Jordan & Akhtar, 1970). In the threonine aldolase reaction the carbanion (II) reacts with acetaldehyde in an analogous fashion to give allothreonine (plus threonine).

The work of Fujioka (1969) has shown the existence of two types of serine transhydroxymethylase, one occurring in the cytoplasm and the other in the mitochondria. We have now explored facets of the active sites of both isoenzymes and have also compared their mechanism and stereochemistry.

Experimental

Materials

[2*RS*-$^3H_2$]Glycine, [2-^{14}C]glycine, [2-^{14}C]serine and NaB^3H_4 were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Silica gel for chromatography was obtained from E. Merck A.G., Darmstadt, Germany. Pyridoxal phosphate was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. 2-Chloroacetaldehyde was purchased from Fluka A.G., Basle, Switzerland. All other chemicals were purchased from British Drug Houses Ltd., Poole, Dorset, U.K. Brushite was prepared by slow admixing of Na_2HPO_4 and $CaCl_2$ solutions by the method of Tiselius *et al.* (1956). DL-Tetrahydrofolate was prepared by the catalytic hydrogenation of folic acid over platinum oxide (Hatefi *et al.*, 1960). [$^3H_2$]Formaldehyde was prepared from glucose and NaB^3H_4 by the method of Jordan & Akhtar (1970). [^{3}H]Acetaldehyde was prepared from diacetyl and NaB^3H_4 by adopting the



Scheme 1. *Proposed mechanism for the action of serine transhydroxymethylase* (*Jordan & Akhtar*, 1970)

H_4PteGlu, Tetrahydrofolate; R=OH; R′=$CH_2 \cdot O \cdot PO_3H_2$; R″=$CH_3$; X and Y, catalytic groups on the enzyme.

same principle. Butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] was obtained from CIBA (A.R.L.) Ltd., Duxford, Cambridge, U.K.

Methods

Preparation of serine transhydroxymethylase from rabbit liver. The enzyme was prepared as described in the Results section.

Assay of enzyme activity. The serine transhydroxymethylase activity was determined by the acetylacetone method (Scrimgeour & Huennekens, 1962) and the (allo)threonine aldolase activity as described previously (Akhtar & El-Obeid, 1972) with allothreonine as the substrate. A unit of enzyme activity is defined as the amount of enzyme causing the disappearance of 1 μmol of formaldehyde/min at 37°C. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preincubation of serine transhydroxymethylase with inhibitors. The enzyme (2 units) in 2ml of 1mM-potassium phosphate buffer (pH7.2) was treated with either of the inhibitors (see the Figures) at 37°C. Samples (0.1ml) were removed at various time-intervals and assayed for serine transhydroxymethylase and threonine aldolase activities.

2-Chloroacetaldehyde was shown to act as an efficient substrate for alcohol dehydrogenase used in the coupled-enzyme assay system for the determination of threonine aldolase activity. This compound was therefore removed from the enzyme solution as follows. Serine transhydroxymethylase (4 units) in a total volume of 4ml was treated with 10mM-chloroacetaldehyde at 37°C. Samples (1ml) of the incubation mixture were removed at various time-intervals and dialysed against 1mM-potassium phosphate buffer (pH7.2) for 2h at 0–5°C. The non-diffusible material was then separately assayed for serine transhydroxymethylase and threonine aldolase activities.

Preparation of t-butyloxycarbonylserine. This was prepared by a modification of the method described by Broadbent *et al.* (1967) as follows. L-Serine (1.05g, 10mmol), t-butyl-2,4,5-trichlorophenyl carbonate (3.42g, 11.5mmol), triethylamine (3.5ml, 25mmol), water (8ml) and 2-methylpropan-2-ol (12ml) were stirred overnight at 55–58°C. After removal of most of the 2-methylpropan-2-ol in vacuum, water (15ml) was added and the mixture acidified to pH3 with saturated aq. citric acid and then extracted with ethyl acetate (3×25ml). The combined extracts were washed with 1M-$NaHCO_3$ (1×10ml; 3×5ml) and the washings were acidified to pH3 at 0°C with saturated aq. citric acid. The product was then collected by extraction with ethyl acetate. After evaporation of the ethyl acetate under vacuum an oil was obtained. The yield was 70–75%.

Preparation of O-chloroacetylserine hydrochloride. Triethylamine (2.1ml, 15mmol) was added to the t-butyloxycarbonylserine (3.1g, 15mmol) in 75ml of dry ethyl acetate. The mixture was cooled at 0°C in an ice–salt bath. To this was added, with stirring over a period of 30min, a solution of chloroacetyl chloride (3.55g, 30mmol) in dry ethyl acetate (10ml) cooled to the same temperature. The mixture was stirred at 0°C for another 30min and then at 25°C for 3h, after which time it was washed with water several times. The ethyl acetate layer was dried over Na_2SO_4 and the solvent was evaporated under vacuum to give *N*-t-butyloxycarbonyl-*O*-chloroacetylserine.

This was dissolved in dry ether and HCl gas was passed into the solution at 0°C for 30min. The resulting precipitate was filtered off and purified by crystallization from methanol–ether. This was repeated several times. The *O*-chloroacetylserine hydrochloride had m.p. 138–140°C. (Found: C, 27.36; H, 4.4; N, 6.64; Cl, 32.14%. Calc. for $C_5H_9Cl_2NO_4$: C, 27.06; H, 4.16; N, 6.42; Cl, 32.52%).

Preparation of O-iodoacetylserine hydrochloride. *N*-t-Butyloxycarbonyl-*O*-chloroacetylserine (1.5g, 5mmol) was dissolved in 5ml of acetone. To this was added a solution of NaI (750mg, 5mmol) dissolved in a minimum amount of acetone. The mixture was left for 4h at 20°C and then overnight at −10°C. The NaCl precipitate was removed by filtration and the acetone evaporated off under vacuum to give an oil. The yield of *N*-t-butyloxycarbonyl-*O*-iodoacetylserine as calculated from the NaCl precipitate was 88%. The product was converted into *O*-iodoacetylserine hydrochloride by passing HCl gas into the ethereal solution of the derivative. (Found: C, 20.52; H, 3.25; N, 4.83; Cl, 12.44; I, 39.65%. Calc. for $C_5H_9ClINO_4$: C, 19.41; H, 2.93; N, 4.52; Cl, 11.46; I, 41.05%.)

Synthesis of N-haloacetyl-amino acids. The general procedure for the preparation of the *N*-haloacetyl-amino acids was a modification of the method of Fischer & Roesner (1910) as follows. Amino acid (0.1mol) was dissolved in water (50ml) containing NaOH (4g, 0.1mol). The mixture was cooled in an ice bath and stirred vigorously. Then 0.125mol of haloacetyl halide dissolved in dry ether (55ml) and an ice-cold solution of NaOH (4.9g, 0.125mol in 62ml of water) were added dropwise simultaneously over a period of 15–20min. The reaction mixture was kept at between pH8 and 9 by regulating the amounts added of both the haloacetyl halide and NaOH solutions. The mixture was then cooled and stirred for 1h, after which it was acidified to pH2–3 with 5M-HCl. The mixture was evaporated to dryness under reduced pressure. The residue was extracted several times with warm ethyl acetate. The solvent was removed by evaporation and the residue was crystallized from cold ethyl acetate–light petroleum (b.p. 60–80°C). It was then recrystallized from ethyl acetate and light petroleum.

Preparation of N-iodoacetyl-amino acids. The iodoacetyl derivatives were prepared by an exchange reaction between NaI and the chloroacetyl derivative, taking advantage of the fact that NaI is soluble in acetone whereas NaCl is not.

Equimolar amounts of *N*-chloroacetyl-amino acid and NaI were dissolved separately in the smallest amount possible of acetone (heating was necessary sometimes to dissolve the chloroacetyl derivative in acetone). The two solutions were mixed and the mixture was placed in a dark place at room temperature for about 1h. The precipitated NaCl was removed by filtration and the acetone evaporated. The residue was extracted with warm ethyl acetate and washed with a saturated solution of NaCl. The mixture was dried over anhydrous Na_2SO_4 and the ethyl acetate evaporated. The residue was brought to crystallization by rubbing with light petroleum (b.p. 60–80°C) and ethyl acetate. Recrystallization was effected by dissolving in ethyl acetate and precipitation with light petroleum.

Measurement of radioactivity. Radioactive samples were counted for radioactivity in 10ml of butyl-PBD in toluene (8g/litre). Samples containing ^3H or ^{14}C were counted in a Beckman CPM 200 liquid-scintillation system at a counting efficiency of 23% for ^3H and 79% for ^{14}C. Doubly labelled samples were counted in an Intertechnique SL40 Counter programmed to automatic quench correction.

Results

Serine transhydroxymethylase and threonine aldolase activities of the cytoplasmic and mitochondrial isoenzymes

The cytoplasmic enzyme from rabbit liver was prepared by the method described earlier (Akhtar & El-Obeid, 1972), except that a second heat-treatment step was added just before the calcium phosphate-gel

Table 1. *Labilization of one of the α-hydrogen atoms of glycine by the mitochondrial enzyme*

Incubations were carried out for 2h in an atmosphere of N_2 at 37°C. Each tube contained, in a final volume of 1ml: 18.6μmol of [2*RS*-$^3H_2$,2-^{14}C]glycine ($^3H/^{14}C$ ratio 4.67; 1.5×10^5c.p.m. of ^{14}C), 0.03μmol of pyridoxal phosphate, 75μmol of potassium phosphate buffer, pH7.2, 2.0μmol of DL-tetrahydrofolate and 0.1 unit of mitochondrial enzyme. Omissions are as indicated below. Parallel experiments were carried out by using the cytoplasmic enzyme for comparison. After incubation glycine was recovered as its benzyloxycarbonyl derivative for measurement of radioactivity.

	Omission	$^3H/^{14}C$ ratio of recovered glycine	% loss of ^3H
Mitochondrial enzyme			
	None (boiled enzyme)	4.67	—
	None (complete system)	2.26	51.60
	Tetrahydrofolate	4.52	3.30
Cytoplasmic enzyme			
	None (boiled enzyme)	4.67	—
	None (complete system)	2.32	50.32
	Tetrahydrofolate	4.54	2.57

treatment, which in this case was replaced by a column (2.5cm×10cm) of brushite. The column was equilibrated with 0.05M-potassium phosphate buffer (pH7.1) containing 8μM-pyridoxal phosphate and 1.4mM-2-mercaptoethanol. The enzyme was eluted with the same buffer. The mitochondrial enzyme was prepared by the method of Fujioka (1969). By using these procedures a 70–130-fold purification was obtained for the cytoplasmic enzyme (specific activity 2–4 units/mg of protein) and a 100–170-fold purification for the mitochondrial enzyme (specific activity 3–5 units/mg of protein). Throughout the purification of the cytoplasmic enzyme the ratios of serine transhydroxymethylase/allothreonine cleavage activities were identical (the ratios were 4.2–4.5:1). The same ratio was observed for the purified mitochondrial enzyme (4.4:1). These results for the cytoplasmic enzyme agree with previous studies (Schirch & Gross, 1968; Akhtar & El-Obeid, 1972). The threonine aldolase activity was also studied in the direction of threonine (and/or allothreonine) synthesis by an isotopic-exchange reaction involving unlabelled threonine and [^{3}H]acetaldehyde. The two enzymes were separately incubated with DL-threonine (25μmol) and [^{3}H]acetaldehyde (8μmol, 1.0×10^6c.p.m.) at 37°C for 2h under the conditions outlined in Table 1, except that DL-tetrahydrofolate was omitted. The amino acid was then recovered as its *N*-dinitrophenyl derivative (Jordan & Akhtar, 1970). With the cytoplasmic and mitochondrial enzymes 2.3×10^4 and 2.5×10^4c.p.m. respectively were incorporated into dinitrophenyl-threonine thus showing that both enzymes catalysed the exchange reaction to the same extent. These results therefore indicate that the mitochondrial serine transhydroxymethylase is similar to the cytoplasmic enzyme in that it possesses the additional activity responsible for the reversible cleavage of allothreonine and threonine.

Effect of chloroacetaldehyde, iodoacetamide and bromopyruvate on cytoplasmic and mitochondrial enzymes

The demonstration that the two isoenzymes catalyse not only the serine transhydroxymethylase but also the threonine aldolase reaction suggested that methylenetetrahydrofolate and acetaldehyde may interchangeably participate in the reaction with the carbanion species of type II (Scheme 1) at the active site of these enzymes. This pointed to the possibility that alkylating agents resembling acetaldehyde may interfere with their activity.

Results in Fig. 1 show that serine transhydroxymethylase as well as threonine aldolase activities of the cytoplasmic enzyme were inactivated in a coordinated fashion by chloroacetaldehyde, iodoacetamide and bromopyruvate, thus confirming the previous work (Akhtar & El-Obeid, 1972). None of the three compounds had any significant effect on the two activities of the mitochondrial enzyme.

Effects of analogues of glycine and L-serine on the activity of serine transhydroxymethylase

Next the reactive haloacyl group of the above reagents was incorporated within the skeleton of two amino acid substrates. For glycine the $-CO-CH_2-$halogen moiety was attached to the amino nitrogen atom (IV). For serine, however, the haloacyl moiety was attached to either the hydroxyl (V) or the amino (VI) group. The preparation of the former type of compounds, which were synthesized for the first time, is described in detail in the Experimental section.

The compounds tested as possible inhibitors included *N*-chloroacetyl, *N*-bromoacetyl and *N*-iodoacetyl derivatives of DL-serine and glycine, as

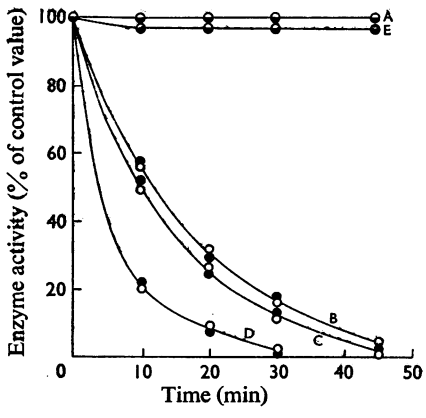
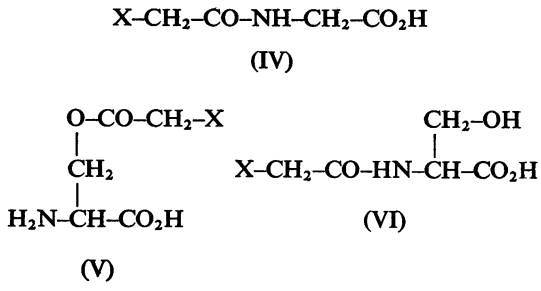


Fig. 1. *Effect of alkylating agents on cytoplasmic and mitochondrial enzymes*

Enzymes were preincubated in the presence or absence of inhibitor and samples were removed at times indicated for determination of serine transhydroxymethylase (●) and threonine aldolase (○) activities as described in the Experimental section. Curve A, no addition, cytoplasmic or mitochondrial enzymes; curve B, preincubation of cytoplasmic enzyme with 7.5mM-chloroacetaldehyde; curve C, preincubation of cytoplasmic enzyme with 7.5mM-iodoacetamide; curve D, preincubation of cytoplasmic enzyme with 1mM-bromopyruvate; curve E, preincubation of mitochondrial enzyme with chloroacetaldehyde or iodoacetamide or bromopyruvate; the determinations, which were identical, are represented by a composite curve.



well as *O*-iodoacetyl-, *O*-chloroacetyl- and *O*-bromoacetyl-L-serine. The inhibitors (15mM), neutralized to pH7.0, were separately incubated with serine transhydroxymethylase and samples were removed at different time-intervals and assayed for the serine transhydroxymethylase and threonine aldolase activities. The *N*-iodoacetyl derivatives of L-serine and glycine and *O*-iodoacetylserine slightly inhibited both of the activities of the cytoplasmic enzyme (Fig. 2). The extent of the inactivation was only 20% after 45min preincubation. None of the other compounds had any noticeable inhibitory effect on either of the enzyme activities. Two explanations could account for the rather weak inhibitory effect of these compounds on the enzyme activities. First, the chemical modification of the substrate impaired the structural requirement for binding to the enzyme, secondly, the structural analogues do bind to the enzyme but in such a manner that the alkylating part of the molecule is directed away from the sensitive group(s) at the enzyme active site. These alternatives were examined by investigating the ability of the derivatives to protect the enzyme against inactivation by iodoacetamide.

The cytoplasmic serine transhydroxymethylase was preincubated at 37°C with iodoacetamide in the presence or the absence of 25mM of either of the *N*-haloacetyl derivatives of serine or glycine (neutralized to pH7.0) and at different time-intervals samples were removed and assayed for the enzyme activity towards serine synthesis. The results (Figs. 3 and 4) show that all the analogues tested protected the enzyme against inactivation by iodoacetamide. The protection obtained with these analogues is comparable with that of the corresponding amino acid substrates, i.e. L-serine and glycine. These results suggest that each of the analogues still retains the ability to bind to the enzyme and that the enzyme–analogue complex is stable against inactivation by

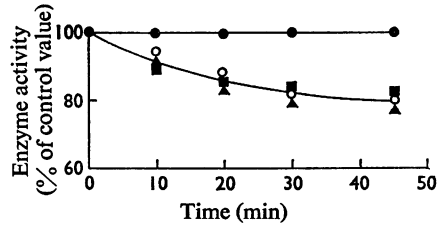


Fig. 2. *Effect of amino acid analogues on serine transhydroxymethylase and threonine aldolase activities*

Enzyme was preincubated in the presence or absence of inhibitor and samples were removed at the times indicated for the determination of serine transhydroxymethylase and threonine aldolase activities as described in the Experimental section. ●, No addition, or enzyme preincubated with *N*-chloroacetylglycine, *N*-bromoacetylglycine, *N*-chloroacetyl-L-serine or *N*-bromoacetyl-DL-serine; ○, enzyme preincubated with 15mM-*N*-iodoacetyl-DL-serine; ▲, enzyme preincubated with 15mM-*N*-iodoacetylglycine; ■, enzyme preincubated with 15mM-*O*-iodoacetyl-L-serine. (Serine transhydroxymethylase and threonine aldolase inactivations were identical and only one series of symbols has been used for clarity.)

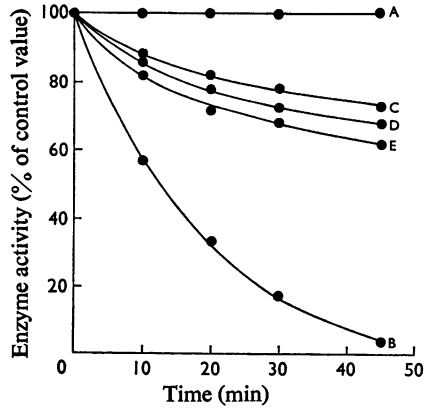


Fig. 3. *Protection by glycine and glycine analogues against the inactivation of cytoplasmic serine transhydroxymethylase by iodoacetamide*

Enzyme was preincubated as indicated and samples were removed at times indicated for the determination of serine transhydroxymethylase as described in the Experimental section. Curve A, no addition; curve B, enzyme preincubated with 7.5mM-iodoacetamide; curve C, enzyme preincubated with 7.5mM-iodoacetamide and 25mM-glycine; curve D, enzyme preincubated with 7.5mM-iodoacetamide and 25mM-*N*-chloroacetylglycine; curve E, enzyme preincubated with 7.5mM-iodoacetamide and 25mM-*N*-iodoacetylglycine.

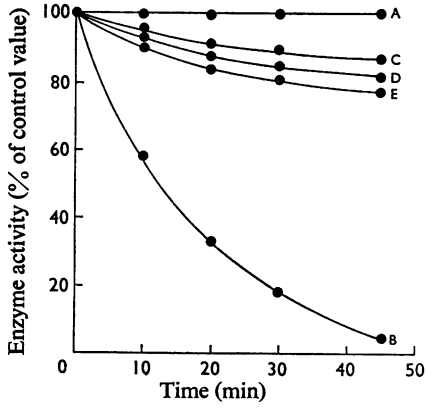


Fig. 4. *Protection by serine and serine analogues against the inactivation of cytoplasmic serine transhydroxymethylase by iodoacetamide*

Enzyme was preincubated as indicated and samples were removed for determination of serine transhydroxymethylase activity as described in the Experimental section. Curve A, no addition; curve B, enzyme preincubated with 7.5mM-iodoacetamide; curve C, enzyme preincubated with 7.5mM-iodoacetamide and 25mM-L-serine; curve D, enzyme preincubated with 7.5 mM-iodoacetamide and 25mM-*N*-chloroacetyl-DL-serine or *N*-bromoacetyl-DL-serine; curve E, enzyme preincubated with 7.5mM-iodoacetamide and 25mM-*N*-iodoacetyl-DL-serine.

iodoacetamide. The fact that these compounds did not act as efficient alkylators for the enzyme may be due to their binding in such a manner that the haloacetyl moiety is inaccessible for reaction with the nucleophilic group at the active site. If we assume that iodoacetamide and chloroacetaldehyde react with a nucleophilic group located in the close vicinity of the aldehyde-binding site, it is clearer why the C–halogen bond of *N*-haloacyl derivatives of serine and glycine is then inaccessible to the same group.

Effect of glycidaldehyde on the activity of serine transhydroxymethylase

The effect of an epoxy aldehyde on the serine transhydroxymethylase and threonine aldolase activities of the two isoenzymes was studied. Fig. 5 shows that glycidaldehyde (2,3-epoxypropionaldehyde) inactivated the two activities of the cytoplasmic enzyme in a time-dependent fashion. At a similar concentration glycidol, the alcohol analogue of glycidaldehyde, showed no inhibitory effect on either of the enzyme activities, suggesting the requirement of an aldehyde

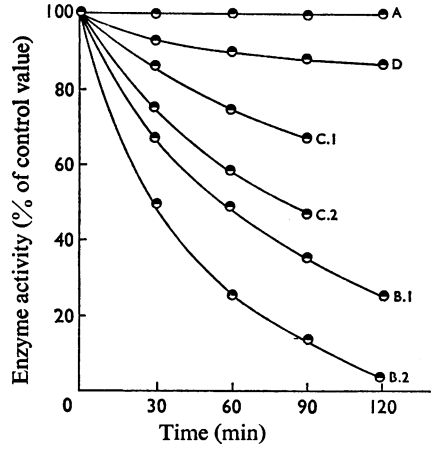


Fig. 5. *Contrasting effects of glycine and L-serine on the inactivation of cytoplasmic serine transhydroxymethylase and threonine aldolase activities by glycidaldehyde*

Enzyme was preincubated as indicated and samples were removed for determination of serine transhydroxymethylase (●) and threonine aldolase (○) activities as described in the Experimental section. Curve A, no addition; curve B.1, enzyme preincubated with 10mM-glycidaldehyde; curve B.2, enzyme preincubated with 10mM-glycidaldehyde and 25mM-glycine; curve C.1, enzyme preincubated with 5mM-glycidaldehyde; curve C.2, enzyme preincubated with 5mM-glycidaldehyde and 25mM-glycine; curve D, enzyme preincubated with 10mM-glycidaldehyde and 25mM-L-serine.

group probably for binding of the compound to the enzyme. The presence of L-serine in the incubation mixture of the enzyme and glycidaldehyde prevented the enzyme inactivation markedly, whereas glycine greatly enhanced the inactivation reaction (Fig. 5). The time required for 10mM-glycidaldehyde to cause a 50% loss of enzyme activity (the inactivation half-time) was about 60min. In the presence of glycine the inactivation half-time was decreased to 30min. In view of the potential mechanistic importance of the stimulatory effect of glycine on the inactivation, the time-course of this process was also studied by using 5mM-glycidaldehyde. From the data, the inactivation half-time was calculated as 80 and 160min in the presence and absence of glycine respectively. It should be borne in mind that since glycidaldehyde is only sparingly soluble in water the concentration of the compound referred to throughout this study corresponds to the amount of compound suspended in water rather than the 'effective concentration'.

In the presence of tetrahydrofolate the cytoplasmic serine transhydroxymethylase activity was partially protected against inactivation by glycidaldehyde (Fig. 6). This observed protection by tetrahydrofolate was greatly improved when glycine was also included in the incubation mixture, a result that agrees with earlier results (Schirch & Ropp, 1967) that tetrahydrofolate has a greater affinity for the enzyme–glycine complex than for the free enzyme.

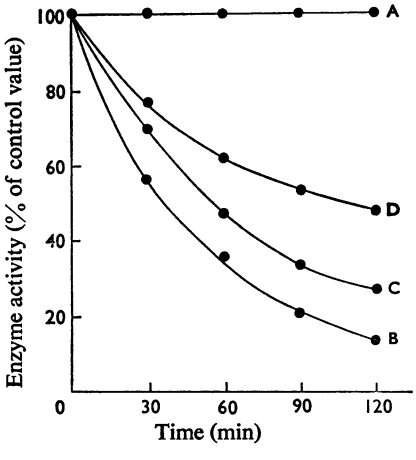


Fig. 6. *Protection by glycine and/or tetrahydrofolate against the inactivation of cytoplasmic serine transhydroxymethylase by glycidaldehyde*

Enzyme was preincubated as indicated and samples were removed for determination of serine transhydroxymethylase activity as described in the Experimental section. Curve A, no addition; curve B, enzyme preincubated with 8mM-glycidaldehyde; curve C, enzyme preincubated with 8mM-glycidaldehyde and 4mM-tetrahydrofolate; curve D, enzyme preincubated with 8mM-glycidaldehyde, 4mM-tetrahydrofolate and 25mM-glycine.

Effect of inhibitors on the mitochondrial serine transhydroxymethylase

The fact that the mitochondrial serine transhydroxymethylase was not significantly inhibited by chloroacetaldehyde, iodoacetamide, bromopyruvate or glycidaldehyde under conditions that resulted in the complete loss of the activity of the cytoplasmic enzyme suggested some structural differences between the two isoenzymes. The group(s) on the enzyme being modified appear to be exposed in the cytoplasmic isoenzyme but are inaccessible in the mitochondrial isoenzyme, probably owing to steric factors. In view of these striking differences between the active-site reactivities of the two isoenzymes with respect to inactivation, their mechanistic and steric courses were compared.

Exchange of the α-hydrogen atom of glycine with the proton of the medium

The previous observation that the cytoplasmic serine transhydroxymethylase catalysed the exchange of the α-hydrogen atom of glycine with the protons of the medium (Jordan & Akhtar, 1970; Schirch & Jenkins, 1964) was extended to the mitochondrial enzyme. [2-$^3H_2$, 2-^{14}C]Glycine was incubated at 37°C with the mitochondrial enzyme for 2h, and for comparison another incubation was run with the cytoplasmic enzyme. Glycine was recovered from both runs as the crystalline benzyloxycarbonyl derivative (Jordan & Akhtar, 1970) and showed the loss of about 3% of the original ^3H radioactivity when tetrahydrofolate was absent. In the presence of tetrahydrofolate the extent of the loss was dramatically increased (Table 1). When the rate of ^3H exchange in the presence of tetrahydrofolate was studied, with either the mitochondrial enzyme or the cytoplasmic enzyme, in both cases there was an initial rapid loss of ^3H which ceased after half the original ^3H radioactivity had been removed (Table 2).

The absolute stereochemistry of the hydrogen of glycine being labilized by the mitochondrial enzyme was next studied. [2*S*-^3H]Glycine was prepared by the exchange reaction involving unlabelled glycine in the presence of ^3H_2O by using the cytoplasmic enzyme. The stereochemistry of the [^3H]glycine formed by this procedure had been established before (Jordan & Akhtar, 1970). It was found that when [2*S*-^3H,2-^{14}C]glycine was incubated with the mitochondrial enzyme in the presence of tetrahydrofolate, 94.3% of the ^3H associated with the α-hydrogen atom of glycine was lost. This experiment

Table 2. *Time-course of the labilization of one of the α-hydrogen atoms of glycine by the mitochondrial and cytoplasmic enzymes*

Incubations were carried out for various time-intervals under the conditions stated in Table 1 by using 0.1 unit of either enzyme.

	Incubation time (min)	$^3H/^{14}C$ ratio of recovered glycine	% loss of ^3H
Mitochondrial enzyme	0	0.62	—
	30	0.43	31
	60	0.37	40
	90	0.30	52
	120	0.29	53.0
Cytoplasmic enzyme	0	0.62	—
	30	0.41	34
	60	0.31	50.0
	90	0.31	50.0
	120	0.30	51.0

shows that, like the cytoplasmic enzyme, the mitochondrial enzyme also catalyses the removal of the α-hydrogen atom of glycine with pro-*S* configuration.

Tetrahydrofolate-independent reaction catalysed by the two isoenzymes

To provide further support for the crucial prediction that formaldehyde rather than methylenetetrahydrofolate reacts with the carbanion (II) of Scheme 1, experiments were undertaken to demonstrate the serine–glycine interconversion in the absence of tetrahydrofolate. Cytoplasmic serine transhydroxymethylase was incubated with [2-^{14}C]serine at 37°C for 2h and after the addition of carrier glycine the incubation mixture was heated to 100°C and the amino acids were isolated as their *N*-dinitrophenyl derivatives and purified by t.l.c. (Jordan & Akhtar, 1970). The *N*-dinitrophenyl derivative of glycine contained 4700 c.p.m.; this incorporation was about 2% of that observed in the presence of tetrahydrofolate (212400c.p.m.). In a control experiment with the boiled enzyme, the incorporation of radioactivity into glycine from [2-^{14}C]serine was 650c.p.m. Next, with both cytoplasmic and mitochondrial enzymes, the reaction was studied in the direction of serine biosynthesis by using glycine and [$^3H_2$]formaldehyde. Both enzymes (0.2 unit) were separately incubated with [$^3H_2$]formaldehyde (15μmol; 1.0×10^6c.p.m.), pyridoxal phosphate (0.03μmol), phosphate buffer (75μmol, pH7.2) and DL-tetrahydrofolate (2.0μmol, when present) in a final volume of 1ml for 2h at 37°C in an atmosphere of N_2. Serine was then isolated

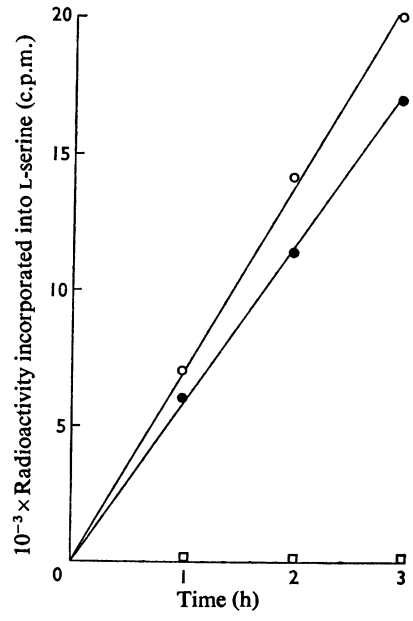


Fig. 7. *Time-course of serine biosynthesis in absence of tetrahydrofolate by both cytoplasmic and mitochondrial enzymes*

Incubations were carried out for different time-intervals at 37°C. Each tube contained in a final volume of 1ml: 15μmol of glycine, 10μmol of [^{3}H]formaldehyde (5.0×10^6c.p.m.), 0.03μmol of pyridoxal phosphate, 75μmol of potassium phosphate buffer, pH7.2, and 1 unit of enzyme activity. A similar set of experiments in which the enzyme was boiled was conducted as a control. ○, Cytoplasmic enzyme; ●, mitochondrial enzyme; □, boiled cytoplasmic or mitochondrial enzyme.

as its *N*-dinitrophenyl derivative. It was found that with both the enzymes the incorporation into serine in the presence and the absence of DL-tetrahydrofolate was 1.5×10^5–1.6×10^5c.p.m. and 2.8×10^3–3.1×10^3c.p.m. respectively. That the tetrahydrofolate-independent incorporation of formaldehyde into serine was in fact due to an enzymic reaction was further shown by the time-course study in Fig. 7 in which larger amounts of enzyme and formaldehyde of higher specific radioactivity were used. Fig. 7 shows that an approximately linear incorporation of radioactivity from [$^3H_2$]formaldehyde into serine occurs during the 3h incubation period with both the enzymes. It is particularly gratifying that in view of the relatively large amount of radioactivity incorporated into serine in the presence of the enzyme, the non-enzymic incorporation with the boiled enzyme was practically zero.

Discussion

In this paper we have compared the mechanistic and stereochemical courses of the cytoplasmic and mitochondrial serine transhydroxymethylases from rabbit liver and have also studied the effect of a number of potentially active-site-directed inhibitors on their activities.

The two isoenzymes possess in addition to serine transhydroxymethylase activity the additional property for the reversible cleavage of allothreonine. The ratio of serine transhydroxymethylases/allothreonine aldolase activities was found to be about 4:1 for both isoenzymes. Related studies on the rat liver enzymes have shown that the cytoplasmic serine transhydroxymethylase also catalyses the aldolase cleavage of allothreonine. The mitochondrial enzyme from rat liver, however, does not possess the allothreonine aldolase activity (Palekar *et al.*, 1973).

The stereochemical studies have revealed that both the isoenzymes from rabbit liver catalysed the exchange of the α-hydrogen atom of glycine with pro-*S* configuration. This suggests that in both cases the cleavage of the α-C–H bond of glycine and the formation of the new C–C bond of serine occurs with the retention of configuration (for previous work on the cytoplasmic enzyme see Jordan & Akhtar, 1970). Until a few years ago, the occurrence of biological condensation reactions with retention of configuration appeared to be a general rule. However, examples of enzymic reactions with inversion mechanisms are being discovered with increasing frequency (for reviews see Akhtar & Wilton, 1972; Abboud *et al.*, 1974).

The two isoenzymes catalysed the reversible interconversion of glycine and serine in the absence of tetrahydrofolate. The possibility that the serine transhydroxymethylase activity exhibited by the mitochondrial and cytoplasmic isoenzymes in the absence of tetrahydrofolate may be due to a small percentage of enzyme molecules containing tightly bound tetrahydrofolate is most unlikely in view of the instability of this compound and its physiological role as a carrier of C_1 units between different enzymes.

Although the rate of interconversion of glycine and serine by both enzymes in the absence of tetrahydrofolate was 2% of that in the presence of tetrahydrofolate, that it occurs at all provides further support for the enzymic mechanism discussed in detail elsewhere (Jordan & Akhtar, 1970) and outlined in Scheme 1. A key feature of this mechanism (Scheme 1) is the proposal that in the serine transhydroxymethylase reaction, tetrahydrofolate acts as a carrier of formaldehyde. In the direction of serine biosynthesis methylenetetrahydrofolate serves to liberate formaldehyde at the active site, where it can react directly with the carbanion (II) to give the intermediate (III) in Scheme 1. In the reverse reaction, serine to glycine, tetrahydrofolate may be necessary for the transport of formaldehyde away from the active site.

The formation of serine from labelled glycine in the absence of tetrahydrofolate by the cytoplasmic enzyme has been reported by Chen & Schirch (1973). These workers have interpreted these results in terms of the role of tetrahydrofolate suggested above and previously advocated by us on several occasions (Jordan & Akhtar, 1970; Akhtar & El-Obeid, 1972; El-Obeid & Akhtar, 1973).

The demonstration that the threonine aldolase reaction is the property of serine transhydroxymethylase suggested that methylenetetrahydrofolate and acetaldehyde may interchangeably participate at the active site of serine transhydroxymethylase. This realization prompted the study of the effect of several analogues of acetaldehyde containing suitable alkylating groups on the activities of the two isoenzymes.

Iodoacetamide, bromopyruvate, chloroacetaldehyde and glycidaldehyde inactivated both the activities of the cytoplasmic enzyme in an identical fashion. None of these compounds, however, had any noticeable inhibitory effect on either of the mitochondrial enzyme activities. These results highlight the fact that in spite of close similarities between both the isoenzymes with respect to mechanistic and stereochemical properties the two isoenzymes have marked structural differences. Similar differences have been reported by Fujioka (1969), who noted that the activity of the cytoplasmic serine transhydroxymethylase was not inhibited by the antibody to the mitochondrial enzyme, and conversely that the activity of the mitochondrial enzyme was not affected by the antibody to the cytoplasmic enzyme.

Extensive data on the protection offered by substrates against inactivation by the inhibition have been obtained. However, of particular mechanistic interest is the observation that whereas serine protected both the activities of the cytoplasmic enzyme against inactivation by glycidaldehyde, glycine enhanced the inactivation reaction. These experiments allow two main conclusions to be drawn. First, the group on the enzyme that is modified by glycidaldehyde is protected by the $-CH_2OH$ moiety of L-serine. The enzymic group participating in the inactivation process must be therefore involved in the manipulation or the binding of the $-CH_2OH$ moiety of L-serine and hence by implication of the C_1 unit in the forward reaction. Secondly, the activity of the group under discussion is enhanced by the binding of glycine to the enzyme. The latter observation, when taken in conjunction with previous studies (Schirch & Jenkins, 1964; Jordan & Akhtar, 1970) showing that the binding of tetrahydrofolate to the enzyme activates the group participating in the deprotonation reaction (I $\rightarrow$ II, Scheme 1), emphasizes that the binding of each substrate to the enzyme promotes a conformational change resulting in

enhanced activity of the groups participating in the subsequent stage of the reaction.

We thank the Science Research Council for a grant and H. A. E.-O. thanks the University of Khartoum (Sudan) for a Research Scholarship.

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