The Mechanism of Action of Serine Transhydroxymethylase

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1. The preparation of stereospecifically tritiated glycines and the determination of their absolute configurations by the use of D-amino acid oxidase are described. 2. The reaction catalysed by serine transhydroxymethylase, which results in the conversion of glycine into serine, has been separated into at least four partial reactions. It is suggested that the first event in this conversion is the formation of a Schiff base intermediate of glycine and pyridoxal phosphate. The next important step involves the removal of the 2*S*-hydrogen atom of glycine to give a carbanion intermediate. Experiments pertinent to the mechanism of conversion of this carbanion intermediate into serine are described. 3. The enzyme preparation catalysing the conversion of glycine into serine also participates in the conversion of glycine into threonine and allothreonine. In both these conversions, glycine $\rightarrow$ serine and glycine $\rightarrow$ threonine, the 2*S*-hydrogen atom of glycine is eliminated and the 2*R*-hydrogen atom of glycine is retained. 4. In the light of these experiments the mechanism of action of serine transhydroxymethylase is discussed. It is suggested that methylenetetrahydrofolate is the carrier of formaldehyde, from which formaldehyde may be liberated at the active site of the enzyme, thus allowing the overall reaction to take place.

The enzyme serine transhydroxymethylase (serine-tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2.1.2.1) catalyses the interconversion of glycine and serine (eqn. 1), and has been studied by a number of investigators (for reviews see Sakami, 1955; Huennekens & Osborn, 1959; Rabinowitz, 1960):

Pyridoxal phosphate (Lascelles & Woods, 1950; Arnstein & Neuberger, 1953; Blakley, 1957) and tetrahydrofolate (Kisliuk & Sakami, 1955; Blakley, 1957) are required for the reaction.

By analogy with other pyridoxal-dependent reactions it has been suggested that the first major event in the conversion of glycine into serine (Scheme 1) is the activation of glycine (I) through the formation of a Schiff base intermediate (structure III) (Snell, 1953; Braunstein & Shemyakin, 1953). In the present work we have studied the subsequent conversion of the intermediate (III) into serine (VII) and have provided experimental evidence for the involvement of a carbanion or its equivalent species (IV) in the conversion of glycine into serine. The absolute stereochemistry of hydrogen elimination in the conversion of glycine into serine has also been examined. A part of this work has been described in preliminary communications (Akhtar & Jordan, 1968, 1969).

MATERIALS AND METHODS

Materials. Radioactive compounds were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Pyridoxal phosphate and bovine serum albumin were obtained from L. Light and Co. Ltd., Colnbrook, Bucks., U.K. L-Glutamate-pyruvate transaminase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Silica gel for chromatography was obtained from E. Merck A.-G., Darmstadt, Germany. All other chemicals were purchased from British Drug Houses Ltd., Poole, Dorset, U.K. Scintillation fluid NE 220 was supplied by Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K. DL-Tetrahydrofolate was prepared by the catalytic hydrogenation of folic acid over platinum oxide (Hatefi, Talbert, Osborn & Huennekens, 1960).

Radioactive compounds. [2*RS*-³H₂]Glycine and [2-¹⁴C]glycine were diluted with carrier glycine and each was spotted on a silica-gel plate (20 cm $\times$ 9 cm $\times$ 1.5 mm). The plates were developed in butanol-acetic acid-water (4:1:1, by vol.). After drying, the glycines (*R_F* 0.18) were eluted from the silica with dilute ammonia solution and were recrystallized twice before use.

[³H, ¹⁴C]Formaldehyde was prepared from glucose as follows. [6-³H]Glucose and [1-¹⁴C]glucose in 1 ml of water were diluted with 5 mg of non-radioactive glucose. Then 20 mg of NaBH₄ was added at 0°C and the solution was allowed to stand for 1 h. The remaining borohydride was decomposed by the addition of 1 or 2 drops of acetic acid. Then 35 mg. of NaIO₄ was added and the solution was kept in the dark for 6 h. After this time the solution was made alkaline by the addition of a few drops of

Scheme 1.

m-NaOH; the [³H,¹⁴C]formaldehyde was recovered by freeze-drying.

L-[2-³H]Alanine was prepared by exchange between L-alanine and tritiated water in the presence of L-glutamate-pyruvate transaminase (EC2.6.1.2). The reaction mixture contained 220 μmol of L-alanine, 50 μmol of sodium phosphate buffer, pH 7.5, 11 units of L-glutamate-pyruvate transaminase and [³H]water (10¹¹ c.p.m.) in a total volume of 2 ml. Incubation was carried out for 2 h at 37°C. After this time the tritiated water was removed by freeze-drying. The last traces of tritiated water were removed by adding 5 ml of benzene-ethanol (1:1, v/v) and evaporating to dryness several times. The residue was dissolved in a small amount of water and spotted on a thick-layer plate. The plate was developed in propanol-aq. ammonia (sp.gr. 0.88) (2:1, v/v). L-Alanine (*R_F* 0.3) was eluted with water. The solution was evaporated to a small volume and the tritiated L-alanine was crystallized by the addition of acetone. Then 20 mg of carrier L-alanine was added and the compound was recrystallized. The yield of L-[2-³H]alanine was 33 mg (7 × 10⁶ c.p.m./mg).

Measurement of radioactivity. All radioactive samples were counted in a Beckman Liquid Scintillation System CPM 200 at a counting efficiency of 23% for ³H and 79% for ¹⁴C as compared with the Beckman standard samples provided with the machine. Corrections for quenching were carried out by means of external standardization by using ³H and ¹⁴C quench curves.

Purification of enzymes. (a) Serine transhydroxymethylase was partially purified from rabbit liver by the method of Scrimgeour & Huennekens (1962) except that mm-EDTA was used in all stages. The enzyme was assayed by the acetylacetone method (Scrimgeour & Huennekens, 1962) and had a specific activity of 0.35 μmol of formaldehyde consumed/min per mg of protein.

(b) D-Amino acid oxidase was partially purified from

sheep kidney and was assayed manometrically (Kubo *et al.* 1960). The specific activity was 46.8 μmol of O₂/h per mg of protein with D-alanine as substrate (with glycine as substrate the rate of oxygen uptake was 11% of this value). Details of incubation conditions are shown in Table 3.

Determination of protein. This was carried out by the biuret method, with bovine serum albumin as standard (Layne, 1957).

Recovery of glycine from incubation mixtures. Incubations were terminated by heating to 100°C on a water bath for 2 min. Then 100 mg of glycine was added, and the solution was evaporated to a small volume and spotted on a thick-layer plate. Plates were developed in methanol-acetone-aq. ammonia (sp.gr. 0.88) (20:10:3, by vol.). After elution with water the glycine (*R_F* 0.25) was converted into its benzyloxycarbonyl derivative (Greenstein & Winitz, 1961).

Separation of glycine and serine. After incubation 100 μg of serine was added to each tube and the solutions were immediately treated with 1-fluoro-2,4-dinitrobenzene (Stahl, 1965). The DNP derivatives of glycine and serine were extracted into ether and spotted on thick-layer plates, which were developed in methylene chloride-acetone-acetic acid (18:3:2, by vol.). The DNP derivatives of glycine and serine (*R_F* 0.47 and 0.10) were eluted from the silica with methanol.

Separation of glycine and threonine (plus allothreonine). After incubation 100 μg of threonine was added and the solutions were treated with 1-fluoro-2,4-dinitrobenzene. After chromatography the DNP derivatives of glycine and threonine (plus allothreonine) (*R_F* 0.47 and 0.19 respectively) were eluted from the silica with methanol.

In incubations where serine and threonine (plus allothreonine) were synthesized 50 μg of carrier serine and 50 μg of carrier threonine were added before processing.

After development of the plates, they were gently dried in a current of cool air and were redeveloped in the same solvent system to obtain maximum separation of the DNP derivatives of serine and threonine (R_{Gly} 0.21 and 0.34 respectively).

Separation of glycine and glyoxylate. After incubation 7 mg of glyoxylic acid was added to each tube. The protein and salts were precipitated by the addition of 5 ml of acetone. After centrifugation the supernatant was carefully evaporated below 35°C to a small volume and spotted on a thick-layer plate. The plate was developed in chloroform-methanol-acetic acid (5:5:1, by vol.). Glyoxylate (R_F 0.62) was detected by spraying a small strip of the plate with iodine solution, and was eluted from the silica with acetone. Glycine (R_F 0.25) was recovered by elution with water.

Preparation of [2R-³H]glycine. A mixture (total volume 2.5 ml) containing 18.6 μ mol of [2RS-³H₂]glycine (10⁹ c.p.m.), 45 nmol of pyridoxal phosphate, 100 μ mol of potassium phosphate buffer, pH 7.5, 3.0 μ mol of tetrahydrofolate and 1.1 mg of serine transhydroxymethylase was incubated at 37°C under nitrogen for 1 h, when half the tritium radioactivity had been lost from the glycine. After incubation the enzyme was destroyed by heating at 100°C for 2 min. The solution was concentrated and spotted on a thick-layer plate together with 30 mg of carrier glycine. The plate was developed in methanol-acetone-aq. ammonia (sp.gr. 0.88) (20:10:3, by vol.). The glycine band (R_F 0.35) was eluted with a cold solution of dilute ammonia. The solution was concentrated and the glycine was crystallized by the addition of acetone. [2-¹⁴C]Glycine was added as required.

Preparation of [2S-³H]glycine. This was carried out by a method similar to that described above except that 267 μ mol of non-radioactive glycine was used in a final volume of 2 ml containing 4×10^{11} c.p.m. of [³H]water. The incubation was carried out for 30 min, after which time the tritiated water was removed by freeze-drying and evaporation with benzene-ethanol (1:1, v/v). Then 10 mg of non-radioactive glycine was added and the glycine was separated as described above for [2R-³H]glycine. [2-¹⁴C]Glycine was added as required.

Measurement of radioactive formaldehyde. [³H,¹⁴C]-Formaldehyde was diluted with 4 mg of non-radioactive formaldehyde and 10 ml of dimedone reagent (0.5% dimedone, pH 6.2, buffered with 0.1 M-sodium phosphate) was added slowly. The solution was cooled and filtered, and the dimedone derivative (m.p. 183°C) was crystallized from methanol.

RESULTS

Equilibration of one of the α -hydrogen atoms of glycine and the synthesis of stereospecifically labelled glycines. Schirch & Jenkins (1964) noted that serine transhydroxymethylase catalyses an exchange between the α -hydrogen atom of glycine and the solvent, the rate of which is greatly increased in the presence of tetrahydrofolate. We have now extended this observation to the synthesis of stereospecifically labelled glycines, which have been used in the study of the mechanism of the glycine $\rightarrow$ serine reaction.

Table 1. *Conditions for the labilization of one of the α -hydrogen atoms of glycine*

Incubations were carried out for 1 h in an atmosphere of nitrogen at 37°C. Each tube contained 18.6 μ mol of [2RS-³H₂,2-¹⁴C]glycine (³H/¹⁴C ratio 2.18, 1.2×10^8 c.p.m. of ¹⁴C), 0.03 μ mol of pyridoxal phosphate, 75 μ mol of potassium phosphate buffer, pH 7.5, 2.0 μ mol of DL-tetrahydrofolate, 2.5 μ mol of formaldehyde and 0.5 mg of serine transhydroxymethylase (specific activity 0.35 μ mol of formaldehyde consumed/min per mg of protein) in a total volume of 1.05 ml. Omissions are as indicated below. After incubation glycine was recovered by chromatography and was converted into its benzyloxycarbonyl derivative for measurement of radioactivity as described in the Materials and Methods section.

Omission(s)	³ H/ ¹⁴ C ratio of recovered glycine	% loss of tritium
None (boiled enzyme)	2.18	—
None (complete system)	1.03	52.8
Formaldehyde	0.97	55.6
Tetrahydrofolate	2.14	2.0
Formaldehyde and tetrahydrofolate	2.08	4.1
Pyridoxal phosphate	1.34	38.8

Table 2. *Time-course of labilization of one of the α -hydrogen atoms of glycine*

Incubations were carried out for various times under the conditions stated in Table 1. Formaldehyde was omitted.

Incubation time (min)	³ H/ ¹⁴ C ratio of recovered glycine	% loss of tritium
0	2.19	—
15	1.87	14.2
30	1.51	30.7
45	1.39	36.2
60	1.02	53.2
120	0.98	55.0

When randomly tritiated [2RS-³H₂]glycine was incubated with serine transhydroxymethylase in the absence of both tetrahydrofolate and formaldehyde, a slow exchange of tritium with the hydrogen atoms of the solvent occurred. In the presence of tetrahydrofolate, but without formaldehyde, the exchange was dramatically accelerated (Table 1) and continued until the radioactivity associated with one of the α -hydrogen atoms of glycine had been lost (Table 2). On taking advantage of this stereospecific equilibration, [2RS-³H₂]glycine when incubated with the enzyme gave [2R-³H]glycine, and the incubation of non-radioactive glycine with the enzyme in the presence of tritiated water yielded [2S-³H]glycine containing tritium with the opposite orientation.

In order to obtain a quantitative measure of the stereospecificity of tritium, [2R-³H]glycine and [2S-³H]glycine (for structures see Scheme 2) were

reincubated with serine transhydroxymethylase under the conditions described in Table 1. There was a rapid loss of 88% of the radioactivity from [2*S*-³H]glycine, showing that the tritium was stereospecifically located. [2*R*-³H]glycine, on the other hand, lost only 12% of the tritium label at a rate comparable with that observed with [2*S*-³H]glycine. A further loss of label from [2*R*-³H]glycine occurred on prolonged incubation; however, the rate of this loss was about one-tenth of the initial fast rate. This showed that the tritium radioactivity in the [2*R*-³H]glycine was at least 88% stereospecifically located. It is most likely that the very slow loss of tritium in the latter case, which is independent of tetrahydrofolate, is due to contaminating enzymes.

The assignment of absolute configuration to these specifically tritiated glycines was made by using D-amino acid oxidase (EC 1.4.3.3). It has been shown that D-amino acid oxidase, which normally converts D-alanine into pyruvate, may also accept glycine as a substrate, the product in the latter case being glyoxylate (Neims & Hellerman, 1962). However, to confirm that the α -hydrogen atom of an L-amino acid was undisturbed in the presence of D-amino acid oxidase the behaviour of α -tritiated L-alanine with the enzyme was studied. L-Alanine was labelled with tritium in the α -position by an exchange reaction in tritiated water by using L-glutamate-pyruvate transaminase, as described in the Materials and Methods section. Accordingly L-[2-³H]alanine (27 μ mol; 2×10^6 c.p.m.) was incubated with D-amino acid oxidase under the conditions described in Table 3, except that catalase was omitted. After the incubation the solution was freeze-dried and the radioactivity of the water was measured for possible tritium radioactivity. No

radioactivity was found in the water, showing that the α -hydrogen atom of L-alanine was stable in the presence of D-amino acid oxidase.

Since in the conversion of D-alanine into pyruvate by D-amino acid oxidase the α -hydrogen atom is removed, we have assumed that the α -hydrogen atom of glycine with the *S*-configuration will be removed in the formation of glyoxylate by the enzyme. This is more clearly shown in Scheme 2. When stereospecifically tritiated glycine, obtained from the incubation of non-radioactive glycine with serine transhydroxymethylase in the presence of tritiated water, was oxidized with D-amino acid oxidase 85% loss of tritium activity occurred, thus showing that the labelled hydrogen atom of glycine had the *S*-configuration. In another experiment glycine, obtained by the equilibration of [2*RS*-³H₂]glycine with the medium in the presence of serine transhydroxymethylase, when incubated with D-amino acid oxidase gave glyoxylate retaining 83% of the tritium radioactivity, thus indicating that the precursor glycine contained tritium associated with the *R*-configuration. As expected, the oxidation of randomly tritiated glycine with D-amino acid oxidase yielded glyoxylate containing half the original tritium radioactivity. These results are displayed in Table 3.

Synthesis of serine from tritiated glycines. When serine was synthesized from [2*RS*-³H₂, 2-¹⁴C]glycine (Table 4) the biosynthetic serine had a ³H/¹⁴C ratio half that of the precursor glycine, showing that one of the two hydrogen atoms of glycine was lost in the

Scheme 2.

Table 3. *Synthesis of glyoxylate from stereospecifically tritiated glycine by D-amino acid oxidase*

Each tube contained 27 μ mol of radioactive glycine as indicated, 100 μ mol of sodium pyrophosphate buffer, pH 8.3, 0.2 μ mol of FAD, 1 mg of catalase and 2.2 mg of D-amino acid oxidase in a total volume of 1.65 ml. Incubations were carried out at 38°C for 3 h.

Radioactive glycine	³ H/ ¹⁴ C ratio of glycine	³ H/ ¹⁴ C ratio of glyoxylate	% loss of tritium
Glycine obtained from the incubation of [2 <i>RS</i> - ³ H ₂]glycine with serine transhydroxymethylase (plus [2- ¹⁴ C]glycine)	1.71	1.41	17.5
[2 <i>RS</i> - ³ H ₂ , 2- ¹⁴ C]-Glycine	2.91	1.43	50.9
Glycine obtained from the incubation of non-radioactive glycine with serine transhydroxymethylase in the presence of [³ H]water (plus [2- ¹⁴ C]glycine)	2.60	0.42	83.9

Table 4. *Synthesis of L-serine and L-threonine from stereospecifically tritiated glycines*

Serine was synthesized as follows. Each tube contained 27 μmol of radioactive glycine as indicated (8×10^5 c.p.m. of ^{14}C), 75 μmol of potassium phosphate buffer, pH 7.5, 0.03 μmol of pyridoxal phosphate, 2.0 μmoles of DL-tetrahydrofolate, 2.5 μmol of formaldehyde and 0.8 mg of serine transhydroxymethylase in a total volume of 1.25 ml. Incubations were carried out for 30 min at 37°C under nitrogen. Serine was isolated as its DNP derivative. Threonine was synthesized as follows. Each tube contained 18.6 μmol of radioactive glycine as indicated (8×10^5 c.p.m. of ^{14}C), 75 μmol of potassium phosphate buffer, pH 7.5, 0.03 μmol of pyridoxal phosphate, 8.0 μmol of acetaldehyde and 0.5 mg of serine transhydroxymethylase in a total volume of 1.0 ml. Incubations were carried out at 37°C for 1.5 h. Threonine was isolated as its DNP derivative.

Radioactive glycine	$^3\text{H}/^{14}\text{C}$ ratio of glycine	$^3\text{H}/^{14}\text{C}$ ratio of serine	% loss of tritium
[2R- ^3H ,2- ^{14}C]- Glycine	1.71	1.41	13.5
[2RS- $^3\text{H}_2$,2- ^{14}C]- Glycine	2.91	1.44	49.5
[2S- ^3H ,2- ^{14}C]Glycine	2.60	0.34	86.9
	$^3\text{H}/^{14}\text{C}$ ratio of glycine	$^3\text{H}/^{14}\text{C}$ ratio of threonine	% loss of tritium
[2R- ^3H ,2- ^{14}C]Glycine	1.63	1.33	18.4
[2S- ^3H ,2- ^{14}C]Glycine	1.65	0.21	87.2

reaction. Studies carried out with stereospecifically tritiated glycines substantiated this conclusion and demonstrated that in the conversion of glycine into serine it is the S-hydrogen atom of glycine that is removed and that the R-hydrogen atom of glycine becomes the α -hydrogen atom of serine. These results provide the additional information that in the overall reaction glycine $\rightarrow$ L-serine the cleavage of a C-H bond of glycine and the formation of a new C-C bond of serine occurs with retention of configuration (Scheme 2).

Effect of increasing formaldehyde concentrations on the exchange process and on the synthesis of serine. To gain further insight into the mechanism of this stereospecific proton exchange ($\text{III} \rightleftharpoons \text{IV}$ in Scheme 1), we studied the relative extent of the exchange reaction and the net formation of serine from glycine in the presence of increasing concentrations of formaldehyde. Table 5 shows that the exchange process is most efficient in the total absence of formaldehyde. Increasing concentrations of formaldehyde inhibit the exchange reaction, but as expected promote the occurrence of the overall reaction, resulting in the formation of serine.

Synthesis of serine from [^3H , ^{14}C]formaldehyde. To investigate the fate of the hydrogen atoms attached to the carbon atom of formaldehyde, the synthesis

Table 5. *Effect of increasing concentrations of formaldehyde on the labilization of one of the α -hydrogen atoms of glycine and on the net synthesis of serine*

Each tube contained 18.6 μmol of [2RS- $^3\text{H}_2$,2- ^{14}C]-glycine ($^3\text{H}/^{14}\text{C}$ ratio 2.94, 8×10^5 c.p.m. of ^{14}C), 75 μmol of potassium phosphate buffer, pH 7.5, 0.03 μmol of pyridoxal phosphate, 2.0 μmol of DL-tetrahydrofolate, 0.8 mg of serine transhydroxymethylase and formaldehyde as indicated in a total volume of 1.25 ml. Incubations were carried out for 10 min. at 37°C under nitrogen. After incubation glycine and serine were separated as their DNP derivatives.

Formaldehyde (μmol)	$^3\text{H}/^{14}\text{C}$ ratio of recovered glycine	% loss of tritium	Serine formed (μmol)	$^3\text{H}/^{14}\text{C}$ ratio of serine
0	2.22	26.00	—	—
3.3	2.50	16.67	1.68	1.45
6.6	2.84	5.33	2.30	1.44
13.2	3.16*	0	2.76	1.39

* This slight increase in $^3\text{H}/^{14}\text{C}$ ratio may be attributed to an isotopic discrimination against [2RS- $^3\text{H}_2$]glycine.

of serine from glycine and [^3H , ^{14}C]formaldehyde with serine transhydroxymethylase was studied. A mixture containing 60 μmol of glycine, 2.5 μmol of [^3H , ^{14}C]formaldehyde ($^3\text{H}/^{14}\text{C}$ ratio 1.11, 250 000 c.p.m. of ^{14}C) and 0.8 mg of serine transhydroxymethylase was incubated for 30 min with the other components shown in Table 4. Serine was recovered as its DNP derivative after the removal of any residual formaldehyde by freeze-drying. The radioactive formaldehyde was measured as its dimedone derivative and had a $^3\text{H}/^{14}\text{C}$ ratio of 1.11. The $^3\text{H}/^{14}\text{C}$ ratio of the serine synthesized was also 1.11.

Biosynthesis of threonine and allothreonine. An analogous reaction resulting in the formation of threonine and allothreonine from glycine and acetaldehyde has been known for some time:

Unlike the condensation of glycine and formaldehyde, these related reactions do not require the

participation of tetrahydrofolate. Recently evidence has been put forward suggesting that serine transhydroxymethylase, in addition to catalysing the interconversion of glycine and serine (eqn. 1), is also responsible for the condensation of glycine and acetaldehyde to form threonine and allothreonine (eqn. 2). The evidence in support of a single enzyme catalysing all three reactions includes a constant ratio of specific activities during the purification of the enzyme and similarity in affinity constants for substrates and inhibitors (Schirch & Gross, 1968).

Relative rates of conversion of glycine into serine and threonine (plus allothreonine). We have shown that the incubation of glycine and acetaldehyde with our preparation of serine transhydroxymethylase resulted in the formation of threonine (plus allothreonine), which was isolated and determined as its DNP derivative. In a parallel incubation with similar concentrations of glycine and formaldehyde in the presence of tetrahydrofolate, it was shown that the rate of serine synthesis was about 20 times that of threonine (plus allothreonine) synthesis. Details of this experiment are as follows. Incubation mixtures contained 18.6 μmol of $[2\text{-}^{14}\text{C}]$ glycine (10⁶ c.p.m.), 0.03 μmol of pyridoxal phosphate, 75 μmol of potassium phosphate buffer, pH 7.5, 0.5 mg of serine transhydroxymethylase, 2.5 μmol of formaldehyde and 2.0 μmol of tetrahydrofolate

Table 6. *Effect of increasing concentrations of acetaldehyde on the labilization of the S-hydrogen atom of $[2\text{S-}^3\text{H}]$ glycine and on the net synthesis of threonine*

Each tube contained 9.5 μmol of $[2\text{S-}^3\text{H}, 2\text{-}^{14}\text{C}]$ glycine ($^3\text{H}/^{14}\text{C}$ ratio 2.07, 3×10^5 c.p.m. of ^{14}C), 75 μmol of potassium phosphate buffer, pH 7.5, 0.03 μmol of pyridoxal phosphate, 0.5 mg of serine transhydroxymethylase and acetaldehyde as indicated in a total volume of 1.0 ml. Incubations were carried out at 37°C for 2 h. After incubation glycine and threonine were separated as their DNP derivatives.

Acetaldehyde (μmol)	$^3\text{H}/^{14}\text{C}$ ratio of recovered glycine	% loss of tritium	Threonine formed (μmol)	$^3\text{H}/^{14}\text{C}$ ratio of threonine
0	1.85	11.12	—	—
0.8	1.86	10.14	0.22	0.21
2.4	1.94	6.28	0.61	0.20
8.0	2.03	1.93	1.43	0.23

in a total volume of 1.2 ml. The reaction was terminated after 15 min and serine was recovered as its DNP derivative. Threonine (plus allothreonine) was synthesized under identical conditions except that formaldehyde and tetrahydrofolate were replaced by 2.5 μmol of acetaldehyde.

Scheme 3.

In 15 min the total radioactivity incorporated into serine was 16068 c.p.m. The radioactivity incorporated into threonine (plus allothreonine) was 1072 c.p.m. There was no incorporation of radioactivity into serine in the absence of tetrahydrofolate.

Stereochemistry of threonine biosynthesis. When threonine (plus allothreonine) was synthesized from $[2R\text{-}^3\text{H}]\text{glycine}$ 81.6% of the tritium label was retained, whereas the use of $[2S\text{-}^3\text{H}]\text{glycine}$ resulted in a loss of 87.2% of the tritium radioactivity from the precursor glycine in the biosynthetic threonine (plus allothreonine). These results are summarized in Table 4 and establish that the stereochemical courses of threonine (plus allothreonine) and serine formation are identical, the *S*-hydrogen atom of glycine being lost and the *R*-hydrogen atom remaining undisturbed in both cases.

A slow exchange of one of the two α -hydrogen

atoms of glycine in the absence of tetrahydrofolate has been mentioned above. The extents of the exchange of $[2S\text{-}^3\text{H}]\text{glycine}$ and of the formation of threonine (plus allothreonine) were studied at various concentrations of acetaldehyde. Since the exchange reaction is slow in the absence of tetrahydrofolate, $[2S\text{-}^3\text{H}]\text{glycine}$ was used instead of $[2RS\text{-}^3\text{H}_2]\text{glycine}$ in order to amplify the loss of tritium.

Table 6 shows that increasing amounts of acetaldehyde resulted in an increased synthesis of threonine (plus allothreonine) whereas the exchange reaction was progressively depressed. This observation is comparable with the relationship between serine synthesis and the exchange reaction in the presence of increasing concentrations of formaldehyde (Table 5). In view of the relatively crude enzyme preparation employed in the present work, the stereochemical and other similarities observed

Mechanism 1

Mechanism 2

Mechanism 3

Scheme 3 (continued)

between the glycine $\rightarrow$ serine and glycine $\rightarrow$ threonine (plus allothreonine) reactions may be regarded as coincidental. However, the stereochemical features emerging from the present work are consistent with the conclusion arrived at by Schirch & Gross (1968), through the use of kinetic techniques, that the glycine $\rightarrow$ serine and glycine $\rightarrow$ threonine reactions may be catalysed by the same enzyme.

DISCUSSION

Involvement of a carbanion in the serine transhydroxymethylase reaction. We interpret these results in terms of the mechanism displayed in Scheme 1. It is suggested that the first event in the manipulation of the intermediate (III) by the enzyme is the cleavage of a C-H bond to give a resonance-stabilized carbanion or its equivalent species (IV). This carbanion, when methylenetetrahydrofolate is present, is available for the physiological reaction resulting in the formation of a new C-C bond. However, in the absence of formaldehyde (or its equivalent), the carbanion cannot be quenched in this way and is therefore neutralized by reprotonation, resulting in the exchange of one of the two α -hydrogen atoms of glycine with the protons of the solvent (reaction 3 in Scheme 1). The demonstration in the present work that a carbanion type intermediate is involved in the glycine-serine reaction is in agreement with the original hypothesis of Metzler, Longenecker & Snell (1954).

The validity of the above conclusions rests on the assumption that the partial reaction resulting in the exchange of one of the α -hydrogen atoms of glycine and the overall reaction for the synthesis of serine are manifestations of the activity of the same enzyme. Although the enzyme preparation employed in this present work was relatively crude, the following considerations make it highly improbable that the partial reaction reported in this work was catalysed by a contaminating enzyme. (1) The *S*-hydrogen atom of glycine is exchanged in the partial reaction and it is also this hydrogen atom that is eliminated in the overall reaction to form serine. This point is particularly relevant since under optimum conditions of serine biosynthesis (Table 5) the exchange reaction was completely absent, thus showing that the removal of the *S*-hydrogen atom of glycine occurs during the process of serine formation and not as a consequence of the pre-equilibration of the glycine pool. (2) The *S*-hydrogen atom of glycine will correspond to the α -hydrogen atom of D-amino acids. The contaminating enzymes in our preparation are more likely to be those involved in the metabolism of L-amino acids and these enzymes should, in principle,

labilize the hydrogen atom of glycine with the *R*-configuration. The fact that only 87% stereospecificity was observed in the synthesis of the labelled glycines may be due to a contaminating enzyme that labilized the *R*-hydrogen atom of glycine. However, the rate of the latter exchange was only about 10% of the rate of loss of the *S*-hydrogen atom, and so does not affect the main conclusions of the present paper. In fact, Besmer & Arigoni (1968) have utilized the enzyme L-alanine aminotransferase, which catalyses the exchange of the *R*-hydrogen atom of glycine, to synthesize a stereospecifically deuterated glycine. (3) The dependence on both tetrahydrofolate and pyridoxal phosphate for the exchange reaction adds to the burden of evidence that the partial reaction under consideration and the glycine-serine interconversion are the properties of the same enzyme. We are unaware of another reaction of amino acids that involves the manipulation of a C-H bond and has also a requirement for both tetrahydrofolate and pyridoxal phosphate.

The requirement for tetrahydrofolate in the exchange reaction highlights another interesting aspect of the serine transhydroxymethylase reaction. It may be assumed either that tetrahydrofolate is essential in the reaction responsible for the formation of the glycine-pyridoxal phosphate-enzyme complex (reaction 1 in Scheme 1) or that the binding of tetrahydrofolate to the glycine-pyridoxal phosphate-enzyme complex exposes or activates a group on the enzyme that is responsible for the deprotonation reaction (reaction 2 in Scheme 1). The spectroscopic studies of Schirch & Ropp (1967) indicate that glycine binds about four times more favourably to enzyme-tetrahydrofolate than to enzyme alone, thus suggesting that reaction 1 in Scheme 1 may be facilitated about fourfold by the presence of tetrahydrofolate. The results in Table 1 show that in the absence of tetrahydrofolate little exchange takes place. We therefore infer that the main site of activation by tetrahydrofolate is at the deprotonation step. A maximum of fourfold difference in reaction 1 by the presence of tetrahydrofolate should not so dramatically affect the overall exchange process unless it was also necessary for reaction 2 (Schirch & Jenkins, 1964).

Consideration of the reaction of the carbanion (IV) with formaldehyde or its equivalent to form serine. In the overall reaction, resulting in the formation of serine from glycine, the carbanion species (IV) proposed above must react with an electron-deficient carbon atom. It has been suggested that methylenetetrahydrofolate (X') can give rise to structure (X), which contains such an electron-deficient centre (C* in structure X; Scheme 3) (Jaenicke, 1961). The reaction of compound (IV) with compound (X) would result in the formation of

the intermediate (XI) containing methylene-tetrahydrofolate, glycine and pyridoxal phosphate all linked together by covalent bonds. The further conversion of this intermediate (XI) into serine must involve the cleavage of a C-N bond (bond 'a' in structure XI; Scheme 3).

At least three mechanisms can be envisaged for this process (Scheme 3). Mechanism 1 involves the cleavage of the C-N bond of compound (XI) by β -elimination to give the intermediate (XII). The latter on hydration would yield a pyridoxal phosphate-serine Schiff base (VI), which would finally give serine. Mechanism 2 involves a reaction sequence in which the C-N bond of compound (XI) is first oxidized to compound (XIII). Further conversion of this new Schiff base into serine involves its hydrolysis to compound (XIV) and then reduction to compound (VI). Finally, mechanism 3 would entail a direct displacement of tetrahydrofolate by OH^- to give the pyridoxal phosphate-serine Schiff base (VI).

Mechanism 1 would require that both the original α -hydrogen atoms of glycine be lost during the conversion of glycine into serine. The results shown in Table 4 indicate, however, that in the serine transhydroxymethylase reaction only one of the two α -hydrogen atoms of glycine is removed. These results are consistent with the observations made by Schirch & Mason (1963), who showed that serine transhydroxymethylase was able to catalyse the conversion of α -methylserine into D-alanine (although the reverse reaction was not observed), indicating that only one hydrogen atom was necessary for the reaction. The above evidence therefore eliminates mechanism 1.

Mechanism 2 would require that, in the formation of serine from glycine and formaldehyde, one of the two hydrogen atoms of the latter would be disturbed. The complete retention of both the hydrogen atoms of formaldehyde in serine precludes the participation of mechanism 2 in the cleavage of the C-N bond in the intermediate (XI). It is possible that the retention of both the hydrogen atoms of formaldehyde might be due to the fact that the hydrogen atom removed in the oxidation reaction (XI) $\rightarrow$ (XIII) is reintroduced in the reduction reaction (XIV) $\rightarrow$ (VI). It is, however, difficult at present to envisage a mechanism by which a hydride ion in the reaction (XI) $\rightarrow$ (XIII) is transferred to such a group on the enzyme complex that it can be returned to the intermediate (XIV) without exchanging with the protons of the solvent.

We now come to the consideration of mechanism 3. This mechanism would require the cleavage of the C-N bond of intermediate (XI) by the nucleophilic attack of OH^- . As far as we are aware, the aspects of the problem concerned with the transfer of the formaldehyde moiety between tetrahydrofolate and

glycine have not been critically discussed in the literature. However, a mechanistic illustration of the glycine-serine reaction by Brode & Jaenicke (1960) suggests that these authors favour mechanism 3. The key reaction (XI) $\rightarrow$ (VI) of this mechanism may be illustrated in the following generalized form:

Such displacement reactions are unfavourable and occur in organic chemistry only under very drastic conditions. [We are not opposed to the general principle of cleavage of the C-N bond by a nucleophilic displacement of the type shown in the conversion (XI) $\rightarrow$ (VI). In fact such a displacement must be involved in the transfer of a methyl group from methyltetrahydrofolate to vitamin B₁₂ in the biosynthesis of methionine. In this case, however, the negatively charged species is a highly reduced cobalt atom, which is endowed with a unique nucleophilic character. This aspect is discussed in detail elsewhere (Akhtar, 1969).] One may argue, of course, that some unrecognized feature of the pteridine moiety of tetrahydrofolate may permit the cleavage of the C-N bond (bond 'a' in Scheme 3) through a direct attack by a nucleophilic species (mechanism 3 in Scheme 3). There now remains the difficulty of explaining the mechanism of the reverse reaction. In the conversion of serine into glycine, the Schiff base of serine will be required to react by the process shown, (VI) $\rightarrow$ (XI), in Scheme 3. This involves the displacement of a hydroxyl group located at a non-activated carbon atom by an -NHR group. A critical examination of the literature suggests that biological reactions involving such displacements generally occur through the prior conversion of a hydroxyl group into a better leaving group such as phosphate or pyrophosphate. An appropriate example is the conversion of 5-phosphoribosyl 1-pyrophosphate and orotic acid into orotidine 5'-phosphate, which occurs through the elimination of pyrophosphate.

In view of the points raised in the discussion above, we suggest that methylenetetrahydrofolate (or its equivalent) may not be involved in the neutralization of the carbanion species (IV), but that tetrahydrofolate may act solely as the formaldehyde carrier that serves to liberate formaldehyde at the active site where it can react directly with the carbanion to give the intermediate (VI) in Scheme 1. In the reverse reaction, serine $\rightarrow$ glycine, tetrahydrofolate may be necessary for the transport of formaldehyde away from the active site. These latter points are discussed in detail elsewhere (Jordan, 1969).

In the enzymic formation of threonine (plus

allothreonine) from glycine and acetaldehyde, irrespective of which particular enzyme catalyses the reaction, the carbanion species of the type (IV) must attack the carbonyl group of acetaldehyde directly to form the new C-C bond as shown, (IV) $\rightarrow$ (VIII), in Scheme 1. It is to be noted that the carbonyl group of formaldehyde is more reactive than the carbonyl group of acetaldehyde. It follows that, if the carbanion species above is sufficiently nucleophilic to react with acetaldehyde, this species must be adequate to carry out a similar reaction with formaldehyde. Hence it seems unnecessary to involve tetrahydrofolate in any activation of formaldehyde. We are of the opinion that biological systems have evolved the use of tetrahydrofolate as a carrier of formaldehyde so that enzymes in general can be protected from the harmful effects of this highly reactive compound.

Overall stereochemistry of serine and threonine (plus allothreonine) biosynthesis. The conversion of [2R-³H]glycine into either serine or threonine (plus allothreonine) resulted in the retention of tritium radioactivity in each case (Table 4) thus showing that the *R*-hydrogen atom of glycine remained undisturbed during the reactions. We may therefore assume that the steric courses of these conversions are identical and that the *R*-hydrogen atom of glycine becomes the α -hydrogen atom of either serine or threonine (plus allothreonine). Since the *R*-hydrogen atom of glycine has the same absolute configuration as the α -hydrogen atom of L-amino acids (Scheme 2), we conclude that the overall conversion of glycine into serine or threonine (plus allothreonine) occurs with retention of configuration.

Note added in proof. Besmer & Arigoni (1968, and references cited therein) have synthesized a stereospecifically deuterated glycine by the overall conversion serine $\rightarrow$ glycine. These workers have also shown that in the serine $\rightarrow$ glycine reaction the α -hydrogen atom of serine remains undisturbed (personal communication from Professor Arigoni). These results of Besmer & Arigoni are therefore in general agreement with those obtained in the present work by studying the glycine $\rightarrow$ serine conversion.

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