The Interconversion of Serine and Glycine: Role of Pteroylglutamic Acid and other Cofactors

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(Received 12 March 1954)

Considerable evidence has now been accumulated for the interconversion of glycine and serine in the animal body and in micro-organisms. Following the discovery by Shemin (1946) of the conversion of isotopically labelled serine into glycine in rats and guinea pigs, the reverse process, that is, the biosynthesis of serine from labelled glycine was also found to occur in the intact animal (Sakami, 1948, 1949). Incorporation of labelled glycine into serine has been observed in rat-liver slices and homogenates (Siekevitz, Winnick & Greenberg, 1949), and serine synthesis from glycine and [^{14}C]formate or [^{14}C]formaldehyde has been studied in soluble liver extracts (Deodhar & Sakami, 1953; Berg, 1953). Serine degradation to glycine and formaldehyde has been demonstrated in liver slices, extracts and homogenates (Vilenkina, 1952).

Synthesis of serine from glycine and formate by washed cells of *Streptococcus faecalis* R has been shown to require pteroylglutamic acid (PGA) in catalytic amounts (Lascelles & Woods, 1950), and there is also some evidence for the participation of PGA or a derivative in serine-glycine interconversion in mammalian tissues. Thus in folic acid-deficient rats less isotope from administered H^{14}COOH is fixed into serine of body proteins than in normal animals, but the rate of incorporation of ^{14}C into C-3 of serine becomes normal when the animals are fed PGA (Plaut, Bethell & Lardy, 1950). Elwyn & Sprinson (1950) have shown that [^{15}N]-serine is utilized less efficiently for hippuric acid synthesis in folic acid-deficient animals, and it was calculated that in folic acid deficiency the conversion of serine into glycine was reduced to one-sixth the normal rate. The addition of PGA or related compounds to tissue preparations has been shown to cause an increase in serine synthesis from formate (Kelley, 1951) and in serine breakdown to glycine and formaldehyde (Vilenkina, 1952). Recently, Rauen & Jaenicke (1953) have briefly reported evidence for serine synthesis from N^{10} -formylPGA (II) and glycine in tissue homogenates and extracts. In the present investigation soluble enzyme preparations catalysing serine synthesis and break-

down (Blakley, 1954) have been used to study the role in these processes of PGA, its derivatives and some other cofactors.

Another aspect of serine synthesis which has received attention in this work is the chemical identity of the immediate precursor of C-3 of serine. Labelled formate is incorporated into this position of the serine molecule by intact animals (Sakami, 1948), liver slices (Siekevitz & Greenberg, 1949) and liver extracts (Deodhar & Sakami, 1953), but Kruhøffer (1951) has reported that in rat liver slices labelled glycine is incorporated into serine much more efficiently than labelled formate, and that in liver homogenates formate is not incorporated into serine even when incorporation of glycine into serine occurs. Furthermore, Siegel & Lafaye (1950) found that in rat-liver homogenates isotopic formaldehyde is much more rapidly incorporated into serine and into the methyl group of methionine than is formate or methanol. Evidence has been obtained in the present investigation for the view, consistent with these observations, that formaldehyde is an important precursor of serine C-3.

EXPERIMENTAL

Materials

The following commercial preparations were used: PGA (Roche Products, Ltd., Welwyn Garden City, Hertfordshire); cobalamin (Glaxo Laboratories, Greenford, Middlesex); pyridoxal phosphate (gift from Roche Products, Ltd.); glycine, L-ascorbic acid (British Drug Houses Ltd., London); aminotrihydroxymethylmethane (THAM), adenosine diphosphate, adenosine 5-phosphate (Light and Co., Colnbrook, Buckinghamshire); leucovorin (5-formyl-5:6:7:8-tetrahydroPGA), 4-amino-4-deoxyPGA (aminopterin), teropterin (sodium pteroyltriglutamate) (gifts from Lederle Laboratories Division, American Cyanamid Co., New York).

Adenosine triphosphate. Chromatographically pure adenosinetriphosphate (ATP) was prepared by fractionation of a commercial product (Boots Pure Drug Co. Ltd., Nottingham) on ion-exchange resin.

DL-Homocysteine. This was prepared by reduction of DL-homocystine (Riegel & du Vigneaud, 1935).

D- and L-serine. These were prepared by resolution of DL-serine (British Drug Houses, Ltd.) by the method of Fischer & Jacobs (1906).

N^{10} -FormylPGA (II). PGA was formylated by refluxing with 98% (w/v) formic acid and acetic anhydride (Gordon, Ravel, Eakin & Shive, 1948).

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TetrahydroPGA and N^{10} -formyltetrahydroPGA were prepared by catalytic hydrogenation of PGA and N^{10} -formylPGA respectively in aqueous solution (O'Dell, Vandebelt, Bloom & Piffner, 1947). When the theoretical amount of hydrogen had been consumed the identity of the products was checked by determination of their ultraviolet absorption spectra and extinction coefficients (Pohland, Flynn, Jones & Shive, 1951).

Labelled compounds. $[2-^{14}C]$ Glycine was obtained from the Radiochemical Centre, Amersham, and diluted with non-isotopic glycine. The diluted material contained $2.68 \times 10^{-2} \mu\text{C}/\text{mg}$. $[2-^{14}C]$ L-Serine was produced enzymically from L-serine and $[2-^{14}C]$ glycine (conditions as in Table 1 with the complete system) and isolated by chromatography on Zeokarb 225 and crystallization of the *p*-hydroxyazobenzene-*p'*-sulphonate. The bulk of the sulphonic acid was subsequently removed from the serine salt by crystallizing out the sodium salt. Final purification of the serine was achieved by treatment with charcoal and crystallization from water-ethanol. The pure product contained $7.23 \times 10^{-3} \mu\text{C}/\text{mg}$. $[^{14}C]$ Formaldehyde was prepared by catalytic oxidation of $[^{14}C]$ methanol (Arnstein, 1951). Another sample, free from methanol, was obtained by periodate oxidation of DL-[3- ^{14}C]serine (Sakami, 1950) in a small volume of water. The formaldehyde was collected by distillation. Sodium $[^{14}C]$ formate was obtained from the Radiochemical Centre, Amersham.

Methods

Yeast extract. A dried yeast preparation (Aluzyme no. 40 powder; Phillips Yeast Products Ltd., Park Royal, London, N.W. 10) was mixed with 5 times its weight of water, the mixture boiled for 1 min. and centrifuged; the supernatant was used without further treatment.

Enzyme preparation. Adult pigeons were decapitated, bled and the livers quickly removed, washed, blotted dry, and frozen. Subsequent steps were performed in a cold room (5°). After the frozen livers had been dispersed in pure chilled acetone in a Waring Blendor, the solid material was filtered off, washed with cold acetone, and the bulk of the acetone quickly removed on the filter and then in an evacuated desiccator. The last traces of acetone were removed at less than 1 mm. pressure and room temperature. The powder was extracted by homogenizing with 10 vol. THAM buffer, pH 7.7 (0.01 M) at 5°. After standing 30 min. at 5°, the homogenate was centrifuged to remove the bulk of the insoluble material and the supernatant dialysed 16–48 hr. at 5° against 20 vol. THAM buffer, pH 7.7 (0.01 M). In some cases the dialysed extract was prepared for use by centrifuging at 100 000 *g* for 30 min. to remove suspended particles. Otherwise the dialysed preparation was first held at 53° for 20 min. before chilling and centrifuging. Water-clear supernatants were obtained in all cases.

Isolation of serine. In experiments on net serine synthesis from $[2-^{14}C]$ glycine and formaldehyde a solution of 250 μ -moles of L-serine was added at the end of the incubation period, and mixed with the contents before deproteinizing with 1 ml. of 30% (w/v) trichloroacetic acid. After incubation of $[2-^{14}C]$ glycine with L-serine the reaction mixture (5 ml.) was directly deproteinized with trichloroacetic acid (1 ml. of 30%). In either case the ppt. was centrifuged off in the reaction tube. The supernatant was transferred to a column of Zeokarb 225 resin (200–400 mesh, H⁺ form; 35 cm. $\times$ 2.5 cm. diameter) and elution with 1.5 N-HCl was

begun. The eluate was collected in 10 ml. fractions, serine appearing in about tubes 12–20 and glycine in about tubes 24–30. The fractions of the eluate containing serine were free from glycine. They were combined, evaporated to dryness under reduced pressure, and the residue dissolved in 3 ml. of water. Solid *p*-hydroxyazobenzene-*p'*-sulphonic acid was added and dissolved by warming until a very slight excess was present as judged by the persistence of the deep red colour of the reagent and the greatly increased difficulty in dissolving more reagent. The serine *p*-hydroxyazobenzene-*p'*-sulphonate which crystallized out on cooling to 0° was filtered off with gentle suction on to a filter paper in a perforated Perspex disk (1.2 cm.²) clamped in a special holder (Popják, 1950). After washing with 2 ml. ice-cold water, the disk was removed from the holder and blotted dry. The crystals were dried in the disk over P₂O₅ *in vacuo* and their radioactivity was determined.

Isolation of glycine. Unlabelled carrier glycine was added to each tube at the end of the incubation period to bring the total amount of unlabelled glycine added to 14.5 mg./tube. The contents of the tubes were mixed and deproteinized with tungstic acid (0.28 ml. 0.6 N-H₂SO₄ followed by 0.20 ml. 10% (w/v) sodium tungstate). The supernatant (about 2.5 ml. containing about 15 mg. glycine) was transferred to a 100 ml. flask in which it was shaken with 10 ml. water, 10 ml. of a 10% (w/v) solution of 1-fluoro-2,4-dinitrobenzene in methanol and 0.3 g. NaHCO₃ for 4 hr. at room temp. Isolation of *N*-2,4-dinitrophenylglycine (DNP-glycine) was then achieved by the method of Campbell & Work (1952), and the isolated material crystallized from ethyl acetate-cyclohexane, dried and packed into a 1 cm.² Polythene disk for counting.

Degradation of serine. This was performed according to the method of Sakami (1950) with the exception that formaldehyde formed by periodate oxidation of serine was distilled off, converted into its dimedone derivative, and the latter filtered directly into a polythene disk, dried (100°, 30 min.) and its radioactivity determined. After crystallization from ethanol-water, samples were recounted.

Formaldehyde. This was determined by the method of Nash (1953) on portions of the supernatant after tungstic acid deproteinization.

Radioactivity measurements. These were all carried out with a helium-filled bell-shaped Geiger-Müller counter on infinite thickness samples mounted in Polythene or Perspex disks (Popják, 1950). When experiments were performed in duplicate the radioactivity of the duplicate samples usually agreed within $\pm 5\%$, and the activity of samples did not change by more than this amount on rechromatographing and recrystallizing.

RESULTS

Reaction between L-serine and $[2-^{14}C]$ glycine

In preliminary experiments, $[2-^{14}C]$ glycine was incubated with pigeon-liver homogenates and extracts and acetone-dried pigeon liver in the presence of various cofactors. Unlabelled L-serine was used as carrier for the isolation of radioactive serine formed enzymically, and for convenience was added with the $[^{14}C]$ glycine at the beginning of the experiment. At the end of the incubation period the serine was isolated and its radioactivity determined.

In some cases duplicate samples of serine were combined, degraded, and the radioactivity of the degradation products determined.

It was found that while ^{14}C is readily incorporated into position 2 of serine under these conditions, labelling of position 3 by homogenates is small and is negligible when extracts are used (Table 1).

The principal source of C-3 of the $[2\text{-}^{14}\text{C}]$ serine formed in these experiments was shown to be the L-serine added as carrier and present during the incubation. Thus, when carrier L-serine was added at the end of the incubation immediately before deproteinization, or when D-serine was added instead of L-serine to the incubation mixture, the serine subsequently isolated was only slightly radioactive (Table 2).

Table 1. *Radioactivities of degradation products from $[^{14}\text{C}]$ serine formed from $[2\text{-}^{14}\text{C}]$ glycine and L-serine*

Incubations were performed at 37° in evacuated Thunberg tubes each containing 0.05 M L-serine, 0.02 M $[2\text{-}^{14}\text{C}]$ glycine ($2.68 \times 10^{-2} \mu\text{C}/\text{mg.}$), 0.05 M THAM buffer, pH 7.7, and pigeon-liver extract (dialysed 16 hr.) in a total volume of 5.00 ml. The complete system contained, in addition, PGA ($4.5 \times 10^{-5} \text{M}$) and yeast extract (0.50 ml.). Time 6 hr.

	Specific activity of serine degradation products (counts/min./cm. ²)		
	BaCO ₃ from C-1	BaCO ₃ from C-2	Formaldehyde dimedone from C-3
Complete system	2	1211	7
Yeast extract omitted	0	170	5
PGA omitted	0	41	2

Table 2. *Formation of $[2\text{-}^{14}\text{C}]$ serine from $[2\text{-}^{14}\text{C}]$ glycine and L-serine by liver extract under various conditions*

Incubations were performed at 37° in evacuated Thunberg tubes each containing 0.05 M L-serine, 0.02 M $[2\text{-}^{14}\text{C}]$ glycine ($2.68 \times 10^{-2} \mu\text{C}/\text{mg.}$), 0.05 M THAM buffer, pH 7.7, enzyme and PGA at the concentrations indicated, in a total volume of 5.00 ml. Time 6 hr.

		Specific activity of serine <i>p</i> -hydroxy-azobenzene- <i>p'</i> -sulphonate (counts/min./cm. ²)
Expt. 1. Liver extract dialysed for 48 hr.		
Complete system contained $7.2 \times 10^{-5} \text{M}$ PGA and 0.50 ml. yeast extract		
Complete system		522
Complete system; incubation stopped at zero time		1
Complete system; boiled enzyme		1
PGA omitted		23
Yeast extract omitted		55
Expt. 2. Liver extract dialysed for 16 hr.		
Complete system contained $1.1 \times 10^{-4} \text{M}$ PGA and 0.30 ml. yeast extract		
Complete system		823
Complete system; serine added at end of incubation		52
Expt. 3. Liver extract dialysed for 16 hr.		
0.50 ml. yeast extract present in all cases		
PGA ($1.1 \times 10^{-4} \text{M}$)		1013
PGA ($2.2 \times 10^{-4} \text{M}$)		947
PGA ($2.2 \times 10^{-4} \text{M}$) (D-serine instead of L-serine)		1

Breakdown of serine to glycine and formaldehyde

Liver extracts supplemented with PGA or certain of its derivatives converted serine into formaldehyde and glycine, which was isolated by chromatographic separation of its DNP-derivative. The extent of the transformation was determined by using L- $[2\text{-}^{14}\text{C}]$ serine, adding unlabelled carrier glycine to the reaction mixture immediately before deproteinization, and measuring the radioactivity of the DNP-glycine subsequently isolated. Formaldehyde was determined in samples of the deproteinized reaction mixture. The results of such experiments, shown in Table 3, indicate that the breakdown of serine to glycine is rapid, up to $10 \mu\text{moles}$ of glycine being formed per ml. of reaction mixture during the incubation period of 6 hr. Although yeast extract and ascorbic acid were added to the reaction mixture in the experiments of Table 3, they were subsequently found unnecessary (Table 8). The inclusion of unlabelled glycine (0.01 M) in the solution during the incubation caused an approximately 50 % increase in serine breakdown. The results on the conversion of serine into glycine of Expt. 3, Table 10, were confirmed by paper chromatography of portions of the incubated reaction solution removed immediately before addition of carrier glycine.

As seen in Table 3, the yields of formaldehyde formed from serine are uniformly low when compared with the glycine simultaneously produced. Recovery of formaldehyde incubated with supplemented liver extract in the absence of other substrates was approximately 80 %, so that the low

Table 3. *Formation of [¹⁴C]glycine and formaldehyde from L-[2-¹⁴C]serine by liver extracts under various conditions*

Incubations performed at 37° in evacuated Thunberg tubes each containing 0.02M L-[2-¹⁴C]serine ($7.23 \times 10^{-3} \mu\text{C}/\text{mg.}$), 0.05M THAM buffer, pH 7.7, and liver extract (dialysed 40 hr. and kept at 53° for 20 min.) in a total volume of 2.50 ml. The complete system contained $1.5 \times 10^{-4}\text{M}$ PGA and 0.20 ml. yeast extract, and 0.01M L-ascorbic acid was also present in each case in Expt. 1. Yeast extract and ascorbic acid were, however, unnecessary for full activity (Table 8). Time 6 hr.

	Specific activity of DNP-glycine (counts/min./cm. ²)	Glycine formed (μmoles)	Formaldehyde formed (μmoles)
Expt. 1			
Complete system	352	19.3	7.5
Complete system; boiled enzyme	10	0.5	0.6
Complete system + 0.01M glycine	512	29.3	3.2
Expt. 2			
Complete system	323	17.7	4.3
Complete system + 0.01M glycine	436	24.7	2.1

Table 4. *Synthesis of [¹⁴C]serine by liver extracts from [2-¹⁴C]glycine and formaldehyde under various conditions*

Incubations performed at 37° in N₂-filled tubes each containing 0.02M formaldehyde or 0.01M formate, 0.02M [2-¹⁴C]glycine ($2.68 \times 10^{-2} \mu\text{C}/\text{mg.}$), 0.05M THAM buffer, pH 7.7, liver extract (dialysed 40 hr. and heated to 53°), and $1.5 \times 10^{-4}\text{M}$ PGA in a total volume of 5.00 ml. Time 6 hr.

Substrates	Specific activity of serine p-hydroxyazobenzene- p'-sulphonate (counts/min./cm. ²)	Serine formed (μmoles)
Glycine, formaldehyde	1371	64
Glycine, formaldehyde*	1177	53
Glycine*	88	3
Glycine, formate*	82	3
Glycine, formaldehyde*†	1475	70
Glycine, formate*†	77	2

* Yeast extract (0.50 ml.) present.

† Liver extract dialysed but not heated to 53°.

formaldehyde as shown in Table 4, where a synthesis of about 12 μmoles of serine/ml. reaction solution in 6 hr. is indicated. The formation of serine from glycine was also demonstrated by paper chromatography of portions of the reaction solutions removed before addition of carrier serine. As the results of Table 4 indicate, formate was unable to replace formaldehyde for serine synthesis.

Similar results on serine synthesis were obtained when glycine and H¹⁴CHO or H¹⁴COOH were incubated with supplemented liver extracts (Table 5). There was a rapid synthesis of [3-¹⁴C]serine from glycine and H¹⁴CHO, but none from glycine and H¹⁴COOH. Addition of ascorbic acid, homocysteine, yeast extract and PGA, singly and in various combinations, did not initiate serine synthesis from glycine and formate in the presence of either crude or heated (53°) liver extract, even when synthesis from glycine and formaldehyde was rapid.

Role of yeast extract and PGA in [2-¹⁴C]serine formation from L-serine and [2-¹⁴C]glycine

As the results in Tables 1 and 2 indicate, the formation of [2-¹⁴C]serine from [2-¹⁴C]glycine and L-serine in the presence of pigeon-liver enzymes was substantially increased when the enzyme preparation was supplemented with PGA in catalytic amounts and also with a boiled yeast extract. A liver extract made by boiling a minced fresh pigeon liver with twice its weight of water for a few minutes produced an effect similar to that of the yeast extract. Yeast extract alone produced negligible activation of the enzyme, and PGA alone produced only slight activation compared with its effect when yeast extract was also present. The rate of [2-¹⁴C]serine formation by undialysed liver preparations is high even in the absence of added PGA or yeast extract, and addition of these factors produces only a slight increase in rate, but the requirement for PGA and yeast extract is marked after dialysis of the liver extracts, and is greatest

yields of formaldehyde from serine must be accounted for on other grounds besides the loss of formaldehyde by reaction with protein or by oxidation. One possibility investigated is that the formaldehyde formed during the enzymic breakdown of serine is a secondary product arising by reduction of formate produced in the breakdown of serine. It was found, however, that no reduction of formate to formaldehyde occurs under the experimental conditions. The low yields of formaldehyde may be due to side reactions of the 'active formaldehyde' formed from serine with other constituents of the relatively crude enzyme preparations used. The yields of formaldehyde are sufficiently high to permit the conclusion that it is probably the main product of the hydroxymethyl group of serine under the experimental conditions.

Synthesis of serine from glycine and formaldehyde in liver extracts

Suitably supplemented liver extracts rapidly synthesized [2-¹⁴C]serine from [2-¹⁴C]glycine and

Table 5. *Synthesis of [3-¹⁴C]serine by liver extracts from glycine and H¹⁴CHO*

Conditions as for Table 4, except that substrates were 0.02M glycine and 0.01M sodium formate (approx. 0.1 μ C/mg.) or 0.01M formaldehyde (approx. 0.2 μ C/mg.). Enzyme dialysed 20 hr. but not heated to 53°. In all cases 0.01M L-ascorbic acid and 1.5×10^{-4} M PGA were present.

Substrates	Other additions	Specific activity of serine <i>p</i> -hydroxyazobenzene- <i>p'</i> -sulphonate (counts/min./cm. ²)
Glycine, H ¹⁴ COONa	Yeast extract (0.50 ml.), homocysteine (0.01 M)	2
Glycine, H ¹⁴ COONa	Yeast extract (0.50 ml.)	2
Glycine, H ¹⁴ COONa	None	0
Glycine, H ¹⁴ CHO	Yeast extract (0.50 ml.), homocysteine (0.01 M)	585
Glycine, H ¹⁴ CHO	Yeast extract (0.50 ml.)	1135
Glycine, H ¹⁴ CHO	None	900

Table 6. *Effect of various cofactors (other than PGA derivatives) on [2-¹⁴C]serine formation from [2-¹⁴C]glycine and L-serine by liver enzymes*

Incubations were performed at 37° in evacuated Thunberg tubes each containing 0.05M L-serine, 0.02M [2-¹⁴C]glycine (2.68×10^{-2} μ C/mg.), 0.05M THAM buffer, pH 7.7, PGA at the concentration indicated, and enzyme in a total volume of 5.00 ml. Time 6 hr.

Other additions	Specific activity of serine <i>p</i> -hydroxyazobenzene- <i>p'</i> -sulphonate (counts/min./cm. ²)
Expt. 1. Enzyme preparation dialysed 40 hr. 4.5×10^{-5} M PGA present in all cases	
None	28
Cobalamin (100 μ g.)	34
ATP (commercial preparation, 0.001M)	303
Homocysteine (0.001M)	35
Pyridoxal phosphate (0.001M)	11
Yeast extract (0.50 ml.)	775
Yeast extract (0.50 ml.), cobalamin (100 μ g.)	593
Yeast extract (0.50 ml.), ATP (commercial, 0.001M)	698
Yeast extract (0.50 ml.), homocysteine (0.001M)	570
Yeast extract (0.50 ml.), pyridoxal phosphate (0.001M)	375
Expt. 2. Enzyme dialysed 40 hr. and kept at 53° for 20 min. 1.5×10^{-4} M PGA and 0.01M ascorbic acid present in all cases	
None	36
Yeast extract (0.50 ml.)	584
ATP (0.001M)	228
ATP (0.002M)	145
ATP (0.001M), pyridoxal phosphate (10^{-4} M)	193
ATP (0.001M), cobalamin (100 μ g.)	185
ATP (0.001M), homocysteine (0.001M)	278
ATP (0.001M), DPN (7.8×10^{-5} M, 47% pure)	634
ATP (0.001M), TPN (10^{-4} M, 60% pure)	654

after prolonged dialysis. With well-dialysed preparations (40 hr.), the addition of PGA to the otherwise complete system causes from 20 to 200-fold stimulation of [¹⁴C]serine formation. The concentration of PGA required to produce maximum activation under these conditions was of the order of 10^{-4} M (Fig. 1; Expt. 3, Table 2). Addition of yeast extract to the otherwise complete system produced increases in [2-¹⁴C]serine formation of from twofold, with enzyme preparations subjected to a short period of dialysis (Table 1), to 30-fold, with well-dialysed enzyme preparations (Table 6).

Effect of other cofactors (except PGA derivatives) on [2-¹⁴C]serine formation

It has been suggested, on the basis of previous experimental work, that cofactors other than PGA derivatives participate in serine synthesis. Among these suggested activators are cobalamin, pyridoxal phosphate (Lascelles & Woods, 1950) and homocysteine (Deodhar & Sakami, 1953). These and other cofactors have been tested singly and in various combinations for ability to replace yeast extract or to activate further the synthesis of [2-¹⁴C]serine by dialysed liver preparations in

presence of yeast extract. Some of the results are shown in Table 6.

No activating effect was shown under any of the conditions employed to study serine-glycine interconversion by pyridoxal phosphate (10^{-3} to 10^{-4} M), cobalamin (20 μ g./ml.) or homocysteine (10^{-2} to 10^{-3} M). Homocysteine failed to activate enzyme preparations for the synthesis of [14 C]serine from [14 C]glycine and L-serine (Table 6) or from [14 C]glycine and formaldehyde or formate (Table 5), despite several repetitions of these experiments.

Investigation of the activating effect of boiled yeast extract on the otherwise complete enzyme system for [14 C]serine synthesis from [14 C]glycine and L-serine revealed that ATP in low concentrations produced an activation similar to, although smaller than, that of yeast extract (Table 6). Addition of both ATP and yeast extract to the otherwise complete system produced only the same activation as yeast extract alone. A preparation of adenosine tetrphosphate (Marrian, 1953) produced a similar effect to that of ATP, while commercial preparations of adenosine diphosphoric acid (ADP) and adenosine monophosphoric acid (AMP) produced smaller effects. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) in combination with ATP produced greater activation than ATP alone. In some cases a combination of ATP and DPN (50 % purity) equalled or surpassed yeast extract in its activating effect (Table 6). The activating effect of DPN of 83 % purity was somewhat lower but still significant.

Among cofactors which were tested and found inactive in the system were the following: coenzyme III, glutamic acid, *N*-formylglutamic acid, coenzyme A. None of the divalent cations tested produced any effect with the exception of the cupric ion, which was strongly inhibitory. Ethylenediaminetetraacetate (0.5 mg./ml.) did not affect the enzymic reaction. The only compound other than those previously mentioned causing activation of [14 C]serine synthesis was ascorbic acid, which under suitable circumstances doubled [14 C]serine synthesis from [14 C]glycine and L-serine (Table 7).

Since this activating effect of ascorbic acid is presumably due to its reducing properties the effect of oxygen was investigated. Synthesis of [14 C]serine under an oxygen atmosphere was considerably lower than in evacuated vessels, and this in turn was lower than under a nitrogen atmosphere (Table 7).

Effect of PGA and other cofactors on net serine synthesis and breakdown

The activating effects reported above were obtained in well-dialysed liver extracts catalysing [14 C]serine synthesis from [14 C]glycine and L-serine, with little net synthesis or breakdown of serine. It was found that well-dialysed enzyme preparations are also activated consistently by PGA for catalysis of net serine synthesis from glycine and formaldehyde and of net serine breakdown to glycine and formaldehyde. It may be seen from Table 8 that the activating effects of PGA in these cases are just as great as that on [14 C]serine synthesis from [14 C]glycine and L-serine. In contrast to this consistent effect of PGA, it was found that yeast extract, ATP, DPN and ascorbic acid produce only slight effects, or none, on the net synthesis of serine from glycine and formaldehyde and on net serine breakdown (Table 8). The reason for this is discussed later.

Effect of leucovorin on serine-glycine interconversion

The fact that both PGA and yeast extract are required to activate well-dialysed liver enzyme preparations for the synthesis of [14 C]serine from [14 C]glycine and L-serine may be interpreted as indicating the enzymic conversion, in the presence of ATP and DPN or yeast extract and ascorbic acid, of PGA into a derivative which is catalytically active in the serine-glycine interconversion. Since leucovorin (5-formyl-5,6,7,8-tetrahydroPGA) (V) has been suggested as the PGA derivative catalytically active in enzymic transfers of one-carbon units or as representing the derivative more closely than PGA itself (see, for example, Jukes, 1953), it was tested for activation of the systems here investigated.

Table 7. *Effect of ascorbic acid and of oxygen on [14 C]serine formation from [14 C]glycine and L-serine in liver extracts*

Conditions as for Table 6. All tubes contained 1.1×10^{-4} M PGA. Enzyme dialysed 40 hr.

Gas phase	Additions		Specific activity of serine <i>p</i> -hydroxyazobenzene- <i>p</i> '-sulphonate (counts/min./cm. ²)
	Yeast extract (0.30 ml.)	L-Ascorbic acid (0.01 M)	
O ₂	Present	Absent	309
O ₂	Present	Present	479
Air (30 mm. Hg)	Present	Absent	823
Air (30 mm. Hg)	Present	Present	1496
Air (30 mm. Hg)	Absent	Present	123
Air (30 mm. Hg)	Absent	Absent	22
N ₂	Present	Absent	1243

Table 8. Requirement for cofactors for net serine synthesis and breakdown

Conditions for Expts. 1 and 2 as for Table 4, except that 0.02M formaldehyde was present in all cases and PGA was omitted where indicated. Conditions for Expt. 3 as for Table 3, except that PGA and yeast extract were omitted where indicated.

Additions to complete system	Specific activity of serine <i>p</i> -hydroxyazobenzene- <i>p</i> '-sulphonate (counts/min./cm. ²)	Serine formed (μmoles)	
Expt. 1. Serine synthesis from [2- ¹⁴ C]glycine and formaldehyde			
None	1371	64	
Yeast extract (0.50 ml.)	1177	53	
ATP (0.001M)	1456	69	
ATP (0.001M), DPN (2 × 10 ⁻⁴ M), L-ascorbic acid (0.01M)	1326	62	
Expt. 2. Serine synthesis from [2- ¹⁴ C]glycine and formaldehyde			
None	1258	58	
None; PGA omitted	135	5	
Expt. 3. Breakdown of [2- ¹⁴ C]serine to [¹⁴ C]glycine and formaldehyde			
Additions to complete system	Specific activity of DNP-glycine (counts/min./cm. ²)	Glycine formed (μmoles)	Formaldehyde formed (μmoles)
None; PGA and yeast extract omitted	14	0.8	0.2
None; PGA omitted	10	0.5	0.2
None; yeast extract omitted	340	18.5	8.4
None	323	17.7	4.3
ATP (0.001M); yeast extract omitted	350	19.2	8.5
ATP (0.001M), DPN (2 × 10 ⁻⁴ M); yeast extract omitted	347	19.1	7.4
L-Ascorbic acid (0.01M)	276	14.7	14.5

Fig. 1. Effect of PGA and leucovorin concentration on [2-¹⁴C]serine formation from [2-¹⁴C]glycine and L-serine. Conditions as for Table 1.

Using dialysed, unheated liver extracts it was found that leucovorin stimulates [2-¹⁴C]serine synthesis from [2-¹⁴C]glycine and L-serine in a similar way to equimolar amounts of PGA over a wide range of leucovorin and PGA concentrations (Fig. 1). Like PGA, leucovorin requires the presence of yeast extract or its equivalent in order to exhibit its activating effect in this system. In most experiments activation by leucovorin was somewhat less

than by equivalent amounts of PGA, whereas leucovorin should be more effective than PGA if it were identical with the coenzyme or represented it more closely than PGA. A further fact unfavourable to the concept of leucovorin as coenzyme is the fact that during incubation of PGA with yeast extract and the liver enzyme preparation there is negligible synthesis of leucovorin as determined by microbiological assays. Assay with *Leuconostoc citrovorum* indicated the presence of active material equivalent to only 0.1% of the PGA present as assayed by *Lactobacillus casei* and *Strep. faecalis* R.

Dialysed liver enzyme preparations which had been kept at 53° for 20 min. before removal of suspended material by centrifuging were activated to a much smaller extent by leucovorin than by PGA (Table 9). Heating the enzyme preparation to 53° therefore appeared largely to inactivate an enzyme converting leucovorin into the coenzyme. With such heated preparations leucovorin produces only slight activation not only for synthesis of [2-¹⁴C]-serine from [2-¹⁴C]glycine and L-serine, but also for serine breakdown (Table 9, Expt. 2). In all cases PGA produces as great a stimulation with heated as with unheated enzyme. These results constitute further evidence against leucovorin itself being the coenzyme in serine-glycine interconversion.

Table 9. *Activating effect of leucovorin for [2-¹⁴C]serine synthesis from [2-¹⁴C]glycine and L-serine and for net serine breakdown*

Incubations were performed at 37° in evacuated Thunberg tubes each containing 0.05M THAM buffer, pH 7.7, enzyme (dialysed 40 hr. and treated as indicated) and substrates as indicated. Time 6 hr.

Expt. 1. [2-¹⁴C]serine synthesis from [2-¹⁴C]glycine and L-serine.

Each tube contained 0.05M L-serine, 0.02M [2-¹⁴C]glycine (2.68×10^{-3} μ C/mg.) and yeast extract (0.50 ml.)

Enzyme treatment	Pteridine added (1.5×10^{-4} M)	Specific activity of serine <i>p</i> -hydroxyazobenzene- <i>p'</i> -sulphonate (counts/min./cm. ²)
Unheated	PGA	1421
Unheated	Leucovorin	1441
Heated (53°, 20 min.)	PGA	1589
Heated (53°, 20 min.)	Leucovorin	140

Expt. 2. Breakdown of L-[2-¹⁴C]serine to [¹⁴C]glycine and formaldehyde.

Each tube contained 0.02M L-[2-¹⁴C]serine (7.23×10^{-3} μ C/mg.) yeast extract (0.20 ml.) and 0.01M L-ascorbic acid

Enzyme treatment	Pteridine added (1.5×10^{-4} M)	Specific activity of DNP-glycine (counts/min./cm. ²)	Glycine formed (μ moles)	Formaldehyde formed (μ moles)
Heated (53°, 20 min.)	PGA	352	19.3	7.5
Heated (53°, 20 min.)	Leucovorin	58	3.1	0.7

Table 10. *Activating effects of PGA derivatives on liver enzymes for serine-glycine interconversion*

Incubations were performed at 37° in evacuated Thunberg tubes each containing 0.05M THAM buffer, pH 7.7, 0.01M L-ascorbic acid, enzyme (dialysed 40 hr. and kept 53° for 20 min.) and substrates as indicated. Time 6 hr.

Expt. 1. [2-¹⁴C]Serine formation from L-serine and [2-¹⁴C]glycine.

Each tube contained 0.05M L-serine and 0.02M [2-¹⁴C]glycine (2.68μ C/mg.)

Pteridine added (1.5×10^{-4} M)	Yeast extract (0.50 ml.)	Specific activity of serine <i>p</i> -hydroxyazobenzene- <i>p'</i> -sulphonate (counts/min./cm. ²)
None	Absent	8
None	Present	4
PGA	Absent	450
PGA	Present	1601
N ¹⁰ -formylPGA	Absent	36
N ¹⁰ -formylPGA	Present	273
N ¹⁰ -formyltetrahydroPGA	Absent	632
N ¹⁰ -formyltetrahydroPGA	Present	983
TetrahydroPGA	Absent	1818
TetrahydroPGA	Present	1800
Leucovorin	Present	231

Expt. 2. Net serine synthesis from [¹⁴C]glycine and formaldehyde.

Each tube contained 0.02M formaldehyde and 0.02M [2-¹⁴C]glycine (2.68μ C/mg.)

Pteridine added (1.5×10^{-4} M)	Yeast extract (0.50 ml.)	Specific activity of serine <i>p</i> -hydroxyazobenzene- <i>p'</i> -sulphonate (counts/min./cm. ²)	Serine formed (μ moles)
PGA	Absent	1008	44
TetrahydroPGA	Absent	1484	71

Expt. 3. Net [¹⁴C]serine breakdown to [¹⁴C]glycine and formaldehyde.

Each tube contained 0.02M L-[2-¹⁴C]serine (7.23×10^{-3} μ C/mg.)

Pteridine added (1.5×10^{-4} M)	Yeast extract (0.20 ml.)	Specific activity of DNP-glycine (counts/min./cm. ²)	Glycine formed (μ moles)	Formaldehyde formed (μ moles)
None*	Absent	13	0.7	0.3
PGA*	Absent	110	5.6	5.1
PGA*	Present	222	11.6	7.2
TetrahydroPGA	Absent	265	14.0	10.3

* Ascorbic acid omitted.

*Activating effects of other PGA derivatives
on serine-glycine interconversion*

Since leucovorin does not appear to be the PGA derivative active as a coenzyme in these reactions, other PGA derivatives were tested for activating effects. The results appear in Table 10. Of the compounds tested, the only one consistently surpassing PGA in its activating effect on serine-glycine interconversion was tetrahydroPGA (IV). While *N*¹⁰-formyltetrahydroPGA (III) was more effective than PGA in stimulating [2-¹⁴C]serine synthesis from [2-¹⁴C]glycine and serine in absence of yeast extract, *N*¹⁰-formylPGA (II) and its tetrahydro derivative were considerably less effective than PGA when yeast extract was present in the system. A preparation of *N*¹⁰-formyl-tetrahydroPGA which had been briefly aerated in an attempt to produce *N*¹⁰-formyldihydroPGA, acted similarly to the untreated *N*¹⁰-formyltetrahydroPGA in stimulating [2-¹⁴C]serine synthesis. In a single experiment not recorded in Table 10, teropterin (sodium pteroyl-triglutamate) showed an activating effect very similar to that of PGA.

In the absence of yeast extract tetrahydroPGA was by far the most active compound, its activating effect being as great as, or greater than, that of PGA in the presence of yeast extract on [2-¹⁴C]serine synthesis from [2-¹⁴C]glycine and L-serine. The superiority of tetrahydroPGA over PGA as acti-

vator of dialysed enzyme preparations, even when the latter are supplemented with yeast extract, is apparent not only in the synthesis of [2-¹⁴C]serine from [2-¹⁴C]glycine and L-serine, but also in net

Fig. 2. Formation of [2-¹⁴C]serine from [2-¹⁴C]glycine and L-serine in presence of tetrahydroPGA and of PGA and yeast extract respectively. Conditions as for Table 1 except that the enzyme preparation was dialysed 40 hr. and heated at 53° for 20 min.

Table 11. *Inhibition by 4-aminoPGA of [2-¹⁴C]serine formation from [2-¹⁴C]glycine and L-serine*

Conditions as for Table 6, except that yeast extract (0.50 ml.) was present in all cases and pteridines were added as indicated.

Expt. 1. Enzyme dialysed 16 hr.

Pteridine added	4-AminoPGA (4.4 × 10 ⁻⁴ M)	Specific activity of serine p-hydroxyazobenzene- p'-sulphonate (counts/min./cm. ²)
None	Absent	8
PGA (1.1 × 10 ⁻⁴ M)	Absent	1013
PGA (2.2 × 10 ⁻⁴ M)	Absent	947
PGA (1.1 × 10 ⁻⁴ M)	Present	84
PGA (2.2 × 10 ⁻⁴ M)	Present	41
Leucovorin (1.1 × 10 ⁻⁴ M)	Absent	1136
Leucovorin (2.2 × 10 ⁻⁴ M)	Absent	1378
Leucovorin (1.1 × 10 ⁻⁴ M)	Present	896
Leucovorin (2.2 × 10 ⁻⁴ M)	Present	1220

Expt. 2. Enzyme dialysed 40 hr. 0.01 M L-Ascorbic acid present in all cases

Pteridine added (1.5 × 10 ⁻⁴ M)	4-AminoPGA (3 × 10 ⁻⁴ M)	Specific activity of serine p-hydroxyazobenzene- p'-sulphonate (counts/min./cm. ²)
PGA	Absent	1672
PGA	Present	29
<i>N</i> ¹⁰ -formylPGA	Absent	471
<i>N</i> ¹⁰ -formylPGA	Present	39
<i>N</i> ¹⁰ -formyltetrahydroPGA	Absent	1067
<i>N</i> ¹⁰ -formyltetrahydroPGA	Present	534
TetrahydroPGA	Absent	1721
TetrahydroPGA	Present	1536

serine synthesis and breakdown. This superiority of tetrahydroPGA is most evident during shorter periods of incubation, as seen from Fig. 2. [2-¹⁴C]-Serine synthesis in presence of PGA and yeast is low during the first hour, becoming rapid only after the incubation has proceeded for about 2.5 hr. Synthesis in presence of tetrahydroPGA is initially very rapid, however, though the rate appears to diminish because the substrates are approaching their equilibrium radioactivities.

Inhibitory effect of 4-aminoPGA on [2-¹⁴C]serine synthesis from [2-¹⁴C]glycine and L-serine

Experiments by previous investigators on the toxicity of 4-aminoPGA for intact animals indicate that its effect is primarily due to inhibition of the reduction of PGA in the animal body (Nichol & Welch, 1950; Jukes, 1953). If, as the results reported above appear to indicate, PGA is enzymically reduced before becoming catalytically active in the systems here employed, 4-aminoPGA should inhibit [2-¹⁴C]serine synthesis when PGA and yeast extract are added to activate the enzyme system, but not when tetrahydroPGA and possibly other reduced PGA derivatives are used. Results of experiments designed to test whether this is the case are shown in Table 11.

There is a strong inhibition of [2-¹⁴C]serine synthesis when PGA and yeast extract, or N¹⁰-formylPGA and yeast extract, are used as activators. In these experiments, doubling the concentration of PGA did not reverse the inhibition. In the presence of tetrahydroPGA or leucovorin as activators inhibition is slight, however, and is reversed by increasing the concentration of leucovorin. If N¹⁰-formyltetrahydroPGA is used as catalyst, the inhibition produced by 4-aminoPGA is intermediate between that produced with PGA as catalyst and that when tetrahydroPGA is catalyst.

DISCUSSION

It has been demonstrated that soluble pigeon-liver extracts catalyse the synthesis of [2-¹⁴C]serine from [2-¹⁴C]glycine and L-serine. The course of this reaction has been elucidated by the following observations. The liver enzyme preparation catalyses the breakdown of L-serine to glycine and formaldehyde, as demonstrated by accumulation of formaldehyde and formation of [¹⁴C]glycine from L-[2-¹⁴C]serine:

L-Serine is also synthesized by the enzyme from formaldehyde and glycine. This has been demonstrated with isotopic carbon according to the reactions:

and

An obvious mechanism for the formation of [2-¹⁴C]serine from [2-¹⁴C]glycine and L-serine is by way of the reactions:

In reactions (4) and (5), however, 'active formaldehyde' rather than the free substance may participate.

The rate of reactions (1) to (5) was rapid in the presence of the enzyme system. In a 6 hr. period up to 71 μ moles of serine were synthesized from glycine and formaldehyde (Table 10), and up to 29 μ moles of L-serine broken down to glycine (Table 3). These amounts correspond to 14 μ moles of serine synthesized/ml. of reaction medium and 12 μ moles of serine broken down to glycine/ml. of reaction medium. The initial rates are certainly considerably higher than these, since after a few hours the reaction is approaching the equilibrium state where synthesis and breakdown of serine are occurring at similar rates. Comparison of the above rates of breakdown and synthesis indicates that under these similar (though not identical) conditions the rates were nearly the same.

Soluble liver enzyme preparations used in these experiments did not catalyse the incorporation of C-2 of glycine into C-3 of serine reported to occur in intact rats (Sakami, 1949), and even in similar unrecorded experiments with liver homogenates incorporation of ¹⁴C into C-3 of serine did not exceed 20% of the simultaneous incorporation into serine C-2. These observations are consistent with the evidence presented by Arnstein & Neuberger (1953) that the conversion of glycine C-2 into serine C-3 *in vivo* is a function of the glycine content of the diet and is presumably a subsidiary mechanism to deal with excessive amounts of glycine.

Formaldehyde and formate as serine precursors

Under conditions where there is a rapid synthesis of serine from glycine and formaldehyde by the liver enzyme preparation, synthesis from glycine and formate is not significant (Tables 4 and 5). Formaldehyde is therefore closely related to the immediate precursor of C-3 of serine into which it is converted by a pathway not involving formate as an intermediate. These experiments do not exclude the possibility that the introduction of the formate carbon atom into the hydroxymethyl group of serine *in vivo* may occur by a pathway other than reduction of formate to formaldehyde, as suggested

by the results of Siekevitz & Greenberg (1950) and Berg (1951). The significance of the experiments of these authors is doubtful, however, since they were performed with liver slices where permeability barriers and the occurrence of some of the relevant reactions at localized sites in subcellular particles preclude free mixing of radioactive formate and formaldehyde with unlabelled material. The rapidity with which formaldehyde is incorporated into serine suggests, however, that formaldehyde may be more closely related to the immediate precursors of C-3 of serine than is formate. A similar conclusion has also been reached by Mitoma & Greenberg (1952), who found that the methyl group of sarcosine is also a good precursor of serine C-3, via an intermediate closely related to formaldehyde.

The reactive nature of formaldehyde and its inhibitory effect on some enzyme systems make it unlikely as a normal enzyme substrate, though the reactions studied in this investigation do not appear to be adversely affected by formaldehyde even in relatively high concentrations. On the other hand, liver slices form formaldehyde from the methyl groups of sarcosine, methionine, choline and betaine, from C-2 of glycine and C-3 of serine (Siekevitz & Greenberg, 1950; Mackenzie, 1950), so that it is either a normal intermediate of metabolism or closely related to one. It seems probable that the 'active formaldehyde', which is the immediate precursor of serine C-3, can be formed from labile methyl groups as well as from formaldehyde, and *in vitro* breaks down to produce free formaldehyde, but that the latter is not a normal intermediate *in vivo*.

Role of yeast extract, ATP, DPN and ascorbic acid

The pigeon-liver enzymes catalysing serine synthesis and breakdown are inactivated by dialysis, but their activity can be restored by supplementing them with PGA or its derivatives. In the case of [2-¹⁴C]serine synthesis from [2-¹⁴C]glycine and L-serine, complete reactivation of the dialysed enzyme is achieved only when PGA or certain of its derivatives, ascorbic acid and a boiled extract of yeast or fresh liver are present (Tables 1 and 2). Omission of PGA from the otherwise complete system reduces [2-¹⁴C]serine synthesis to as little as 0.5% of its maximum rate, but the effects of omitting yeast extract or ascorbic acid are smaller, causing the incorporation of ¹⁴C into serine to drop to 2–50% and to about 50% of the maximum rate respectively.

Preparations of both ATP and DPN can partly replace yeast extract in reactivating dialysed enzymes for [2-¹⁴C]serine synthesis under these conditions, and ADP, AMP and adenosine tetra-

phosphate preparations have effects similar to or smaller than that of ATP.

The exclusion of oxygen during the reaction increases the rate of [2-¹⁴C]serine synthesis. This, together with the above facts, suggests that the yeast extract, ATP, DPN and ascorbic acid all participate in an essential reduction step, probably the enzymic reduction of PGA to a derivative catalytically active in the serine-glycine interconversion. The accumulation of an essential catalyst is also suggested by the manner in which the rate of synthesis of [2-¹⁴C]serine from [2-¹⁴C]glycine and L-serine increases with time. When PGA and yeast extract are used as activators, serine labelling is initially very slow and there is a considerable lag period before it attains its maximum rate (Fig. 2). Nichol (1952) has previously reported an enzymic reduction of PGA in liver extracts which is increased by ascorbic acid, and the occurrence of this reaction as a necessary preliminary to serine-glycine interconversion seems to explain the results satisfactorily.

Net synthesis of serine from glycine and formaldehyde and the breakdown of serine with accumulation of glycine and formaldehyde are not appreciably affected by the addition to the medium of yeast extract, ATP, DPN or ascorbic acid, the addition of PGA alone being sufficient for full activation of dialysed enzymes for these reactions. This is most plausibly explained by assuming that formaldehyde, added as substrate or accumulated by the initial slow breakdown of serine, can participate in the postulated enzymic reduction of PGA.

Nature of the coenzyme

In view of the evidence for an enzymic reduction of PGA to a catalytically active derivative, it is not surprising that tetrahydroPGA proved the most active of the PGA derivatives tested in stimulating serine synthesis and breakdown (Table 10). Under conditions where PGA and all its other derivatives tested require the presence of yeast extract to effect maximum reactivation of dialysed enzyme preparations, tetrahydroPGA produced maximum reactivation in absence of yeast extract. When the enzyme preparation is activated by tetrahydroPGA the rate of serine labelling is initially high and there is no initial lag such as occurs when the enzyme is activated with PGA and yeast extract (Fig. 2). The activating effect of PGA and its other derivatives is therefore presumably due to their conversion into tetrahydroPGA in the presence of yeast extract or ATP and DPN, ascorbic acid and the enzyme preparation.

Enzymic reduction of PGA is inhibited in the intact animal by administration of 4-aminoPGA (Nichol & Welch, 1950). In accordance with this, 4-aminoPGA added to the enzyme system strongly

inhibited serine-glycine interconversion when it was dependent on production of tetrahydroPGA from PGA and yeast extract, but only slightly when tetrahydroPGA was added directly to the enzyme system. A moderate inhibition of serine-glycine interconversion by 4-aminoPGA occurred when the enzyme system was supplemented with N^{10} -formyltetrahydroPGA. This suggests that N^{10} -formyltetrahydroPGA may be converted into tetrahydroPGA at least partly by a pathway not involving PGA. Leucovorin is converted into tetrahydroPGA by a pathway not involving PGA, since in its presence 4-aminoPGA is only slightly inhibitory. Leucovorin is not itself the catalytic derivative of PGA, since it is less effective than tetrahydroPGA for activating the dialysed enzyme and requires the simultaneous addition of yeast extract to produce activation. Furthermore, after heating the dialysed enzyme preparation to 53° for 20 min., leucovorin is able to reactivate it to only a small extent even in the presence of yeast extract, whereas tetrahydroPGA reactivates it just as readily as before heating. Leucovorin is therefore converted into tetrahydroPGA by an enzyme sensitive to slightly elevated temperatures.

The activating effect of tetrahydroPGA is presumably due to enzymic combination of tetrahydroPGA with formaldehyde to form a reactive intermediate, which in turn may react with glycine to form serine and regenerate tetrahydroPGA. A possible structure for the intermediate (suggested by Dr A. Neuberger, F.R.S.) is the tetrahydroPGA derivative (VI), in which a tetrahydroglyoxaline ring is formed by condensing formaldehyde with the amine groups of the N^5 - and N^{10} -positions of tetrahydroPGA. Crystalline tetrahydroPGA derivatives of this type, containing a glyoxaline or tetrahydroglyoxaline ring, have been produced by acid degradation of leucovorin (Cosulich, Roth, Smith, Hulquist & Parker, 1951). Alternatively, the intermediate may have the structure N^5 -hydroxymethyltetrahydroPGA, though the greater co-enzyme effect of N^{10} -formyltetrahydroPGA than leucovorin (Table 10) and the probably greater instability of the N^5 -hydroxymethyltetrahydroPGA are less consistent with this view.

The activating effect of leucovorin on unheated dialysed enzyme preparations can be readily explained in terms of its enzymic reduction to the postulated tetrahydroglyoxaline intermediate, and the occurrence of such a rapid transformation would account for the effectiveness of leucovorin in overcoming the toxic effect of 4-aminoPGA *in vivo*, since it bypasses tetrahydroPGA formation from PGA. TetrahydroPGA has only about one-third the activity of leucovorin in reversing the toxicity of 4-aminoPGA for mice, and about 0.025 the growth-promoting activity of leucovorin for *Ln. citrovorum*

(Broquist, Fahrenbach, Brockman, Stockstad & Jukes, 1951). This low activity of tetrahydroPGA *in vivo* compared with leucovorin may be explained by the fact that, while tetrahydroPGA is readily oxidized, leucovorin is stabilized by its N^5 -formyl group against attack by oxygen.

The concentrations of PGA and tetrahydroPGA employed in these experiments (about 10^{-4} M) are very much higher than the concentration of PGA or leucovorin required to promote optimum growth of micro-organisms dependent upon them for growth. At concentrations below 10^{-4} M PGA causes slower enzymic serine-glycine interconversion (Fig. 1). The high concentration of PGA necessary under these conditions is presumably due to the necessity for its conversion into tetrahydroPGA prior to catalysing the serine-glycine reaction. Determination of the concentration of tetrahydroPGA required for maximum serine-glycine interconversion has been hindered by the difficulty of preventing re-oxidation at very low concentrations and the impurity of the enzyme at present employed. It will be the subject of further investigations with a purified enzyme.

Besides its reaction with glycine to give serine, the reactive intermediate must be capable of hydrolysis to formaldehyde and tetrahydroPGA. This is evident from the fact that during net serine breakdown, formaldehyde is produced and the intermediate regenerated so that it acts catalytically. The turnover of the intermediate in this system can be determined from the amount of radioactive glycine formed from $[2-^{14}\text{C}]$ serine. The radioactivity of this glycine, and therefore the turnover of the intermediate, is increased by about 50% by adding non-labelled glycine to the system (Table 3). From this it may be inferred that the reaction of the intermediate with glycine to give serine is somewhat faster than its reaction with water to give formaldehyde. The scheme proposed need not be inconsistent with the observation of Elwyn, Weissbach & Sprinson (1951) that the conversion of C-3 of serine into labile methyl groups occurs without the loss of the carbon-bound hydrogen atoms.

These postulated relationships of PGA derivatives to the serine-glycine interconversion are represented diagrammatically on p. 460.

Among cofactors tested and found inactive in these experiments is pyridoxal phosphate, despite the fact that this compound stimulates serine synthesis by washed cells of *Strep. faecalis* R and enables *Ln. mesenteroides* to grow on glycine instead of serine, provided a CO_2 -rich atmosphere is used (Lascelles & Woods, 1950). The indifference of serine synthesis in liver extracts to addition of pyridoxal phosphate may be due to the presence of closely bound pyridoxal phosphate in the enzyme preparation, in the same way that the presence of

bound pyridoxal phosphate in some transaminase preparation obviates the need to activate them with added pyridoxal phosphate (O'Kane & Gunsalus, 1947).

Homocysteine has been reported to produce considerable activation of the incorporation of labelled formate into serine in liver extracts (Deodhar &

Sakami, 1953; Berg, 1953). This has been interpreted as indicating that homocysteine has a catalytic role in one-carbon transfer, for example, by forming a reactive thio-ether (Berg, 1953). It has been found in the present work, however, that homocysteine is inactive or inhibitory in the enzyme system incorporating formaldehyde into

serine. This raises some doubt whether the effect of homocysteine in formate incorporation may not be due to participation of homocysteine in the specific enzymic reduction of formate to formaldehyde or of leucovorin to the tetrahydroglyoxaline (VI) or a similar derivative.

SUMMARY

1. A soluble enzyme preparation from pigeon liver catalyses the formation of [2-¹⁴C]serine from L-serine and [2-¹⁴C]glycine. D-Serine is inactive in the reaction and no ¹⁴C is incorporated into C-3 of serine.

2. The mechanism of this reaction has been elucidated by the demonstration of net synthesis of [¹⁴C]serine from formaldehyde and [¹⁴C]glycine, and of net conversion of [2-¹⁴C]serine into [¹⁴C]glycine and formaldehyde in presence of the enzyme.

3. In presence of the enzyme, H¹⁴CHO and glycine are converted into [3-¹⁴C]serine, but no synthesis of serine occurs when formate is substituted for formaldehyde.

4. For synthesis of [2-¹⁴C]serine from [2-¹⁴C]glycine and L-serine, well-dialysed enzyme preparations must be activated by the addition of pteroylglutamic acid (PGA) and a boiled extract of yeast or liver. The yeast extract may be replaced by a combination of ATP and DPN.

5. Ascorbic acid increases the formation of [2-¹⁴C]serine from [2-¹⁴C]glycine and L-serine by the liver enzyme system, and the reaction is much faster under anaerobic conditions than in the presence of oxygen.

6. For net serine synthesis from glycine and formaldehyde and for net serine degradation to glycine and formaldehyde, dialysed enzyme preparations are fully activated by the addition of PGA alone.

7. The effect of leucovorin (*N*⁵-formyltetrahydro-PGA) in activating the crude dialysed enzyme for [2-¹⁴C]serine formation from [2-¹⁴C]glycine and L-serine is similar to that of PGA; simultaneous addition of yeast extract is necessary for full activation. Heating dialysed enzyme preparations to 53° does not diminish their activity when supplemented by PGA and yeast extract, but activity in presence of leucovorin is decreased to 10–20 % of its former value.

8. Of the PGA derivatives tested, tetrahydro-PGA is the most effective in activating dialysed enzyme for serine-glycine interconversion, and it activates without addition of yeast extract or other factors. Moreover, serine-glycine interconversion catalysed by the enzyme and tetrahydroPGA commences at a high rate, but when PGA and yeast extract activate the enzyme there is a lag of over an hour before appreciable serine-glycine intercon-

version occurs. During this period enzymic reduction of PGA to tetrahydroPGA is believed to occur.

9. 4-AminoPGA inhibits serine-glycine interconversion when the enzyme preparation is supplemented with PGA or *N*¹⁰-formylPGA, but only slightly when leucovorin or tetrahydroPGA are added as supplements.

10. The significance of these results is discussed and a scheme describing the role of PGA derivatives in one-carbon metabolism proposed, in which it is suggested that tetrahydroPGA plays a central part in the transfer on one-carbon units.

I am indebted to Roche Products, Ltd., and Lederle Laboratories for gifts of chemicals already mentioned; also to Dr T. P. Singer for a gift of coenzyme III, to Dr D. H. Marrian for gifts of adenosine tetraphosphate and ATP purified by chromatography on ion-exchange resin, to Dr D. D. Woods for a gift of leucovorin, to Dr H. R. V. Arnstein for samples of L-serine and [¹⁴C]formaldehyde, and to Dr A. Neuberger, F.R.S., for the *p*-hydroxyazobenzene-*p*-sulphonic acid. I also wish to thank Dr M. J. Cross and Dr J. Lascelles for performing the microbiological assays, Mr B. S. Michaels for technical assistance, and Dr A. Neuberger for valuable advice and criticism.

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Baicalinase, a Plant β -Glucuronidase

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(Received 19 January 1954)

Miwa (1932, 1935) showed that the roots of *Scutellaria baicalensis* contain an enzyme, baicalinase, that decomposes baicalin and scutellarin, glucuronides of 5:6:7-trihydroxyflavone and 5:6:7:4'-tetrahydroxyflavone respectively. Baicalin is present in the roots and scutellarin in the foliage of *S. baicalensis* (Shibata, Iwata & Nakamura, 1923). After incubating each glucuronide with baicalinase, Miwa was able to isolate D-glucuronic acid and the aglycone, proving that the enzyme is purely hydrolytic in character. By measuring the reducing sugar liberated after several hours' incubation at 30°, he found that the pH for optimum enzymic hydrolysis was 6.0 in the case of baicalin and 5.7 in the case of scutellarin.

On the evidence available, it was impossible to decide whether or not the flavone glucuronides were β -D-glucopyranuronides and baicalinase a β -glucuronidase, analogous to the corresponding compounds and enzyme of animal origin. The fact that they have high negative rotations suggested that baicalin and scutellarin were at least β -glucuronides. On the other hand, Miwa (1932) could observe no action of baicalinase on menthyl β -D-glucopyranuronide. From details given in his paper, however, it is evident that his enzyme preparation was contaminated with baicalin, and that his measurements of reducing sugar were subject to high and variable blanks.

After earlier unsuccessful attempts (Miwa, 1932), Miwa (1936) was able to obtain preparations of almond emulsin that hydrolysed baicalin and scutellarin. The enzyme responsible was shown to be distinct from β -glucosidase, but its action on menthyl β -D-glucuronide was not examined. It was not therefore possible to say whether the

enzyme acting on the flavone glucuronides was identical with the enzyme in emulsin that hydrolyses menthyl β -glucuronide (Helfferich & Sparmberg, 1933).

In connexion with a study of plant glucuronides, it was decided to examine the action of baicalinase on glucuronides of known structure. The experiments were limited by the fact that *S. baicalensis* was difficult to cultivate in Aberdeenshire, but sufficient material was obtained to establish the two main points, that baicalinase is a β -glucuronidase, similar in specificity to the enzyme in animal tissues (Levy & Marsh, 1952), and that baicalin is a β -D-glucopyranuronide. The glucuronide of chrysin (5:7-dihydroxyflavone), recently isolated from the foliage of *S. galericulata* (Marsh, private communication), was also shown to be a β -D-glucopyranuronide.

EXPERIMENTAL AND RESULTS

Materials

Chrysin glucuronide was isolated from the leaves of *S. galericulata* and scutellarin from the leaves of *Centaurea scabiosa* (Charaux & Rabaté, 1940). These compounds were supplied by Dr C. A. Marsh, as were also (-)-menthyl α -D-glucopyranuronide and methyl α - and β -D-galactopyranuronides, prepared by catalytic oxidation of the hexosides (Marsh, 1952). Baicalin was presented by Professor S. Hattori. Methyl β -D-glucopyranuronide was obtained in solution by treating its 3:6-lactone with dilute NaOH. This lactone and saccharo-1:4-lactone were gifts from Professor F. Smith. α -Ethylhexanoyl glucuronide (potassium salt) was presented by Professor R. T. Williams, and veratroyl glucuronide by Dr H. G. Bray. Potassium hydrogen saccharate and glucono-1:5-lactone were purchased from the British Drug Houses Ltd., and phenyl β -D-glucoside