REFERENCES

Alexander, N. & Greenberg, D. M. (1955). *J. biol. Chem.* **214**, 821.
Blakley, R. L. (1954). *Biochem. J.* **58**, 448.
Blakley, R. L. (1955). *Biochem. J.* **61**, 315.
Blakley, R. L. (1957). *Biochem. J.* **65**, 342.
Cosulich, D. B., Roth, B., Smith, J. M., Hultquist, M. E. & Parker, R. P. (1951). *J. Amer. chem. Soc.* **73**, 5006.
Dalgliesh, C. E. (1952). *Biochem. J.* **52**, 3.
Kaselis, R. A., Liebmann, W., Seaman, W., Sickels, J. P., Stearns, E. I. & Woods, J. T. (1951). *Analyt. Chem.* **23**, 746.
Kisliuk, R. & Sakami, W. (1955). *J. biol. Chem.* **214**, 47.
May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G. L. & Shive, W. (1951). *J. Amer. chem. Soc.* **73**, 3067.
O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S. & Pfiffner, J. J. (1947). *J. Amer. chem. Soc.* **69**, 250.
Pohland, A., Flynn, E. H., Jones, R. G. & Shive, W. (1951). *J. Amer. chem. Soc.* **73**, 3247.

The Interconversion of Serine and Glycine: some Further Properties of the Enzyme System

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The partial purification from rabbit liver of a soluble enzyme system which catalyses the net synthesis of serine from glycine and formaldehyde has been previously reported (Blakley, 1955), together with evidence for the participation of pyridoxal phosphate. Further work has had as its chief aim elucidation of the role of tetrahydropteroylglutamic acid (tetrahydroPGA) in this enzymic reaction, but in the course of these investigations certain properties of the enzyme system have been determined. It is the purpose of this paper to present these results, the most important of which concern the specificity of the enzyme system for tetrahydroPGA as the coenzyme.

EXPERIMENTAL

Materials

Pteridines. Commercial samples of pteroylglutamic acid (PGA) were purified as previously described (Blakley, 1957). N^{10}-FormylPGA and calcium leucovorin (calcium N^5-formyl-5:6:7:8-tetrahydropteroylglutamate) were gifts from Dr H. P. Broquist of Lederle Laboratories. Pteroic acid, 4-amino-4-deoxyPGA (aminopterin), sodium pteroyltriglutamate (teropterin) and anhydroleucovorin A were gifts from Dr T. H. Jukes of Lederle Laboratories. 2-DeaminoPGA was a gift from Professor A. Albert and Dr D. J. Brown of the Department of Medical Chemistry of this University. Citrovorum factor was kindly supplied as the crystalline barium salt by Drs Silverman and Keresztesy of the National Institutes of Health, Bethesda, U.S.A. Pteroylheptaglutamic acid was a gift from Dr J. J. Pfiffner of Parke, Davis and Co., Detroit, Michigan, U.S.A., obtained through the courtesy of Dr H. P. Broquist. The 5:6:7:8-tetrahydro derivatives of PGA, N^{10}-formylPGA, 4-amino-4-deoxyPGA, pteroyltriglutamic acid and pteroylheptaglutamic acid and dihydro-2-deaminoPGA were prepared by catalytic hydrogenation in neutral aqueous solution as previously described (Blakley, 1957). DihydroPGA was prepared by hydrogenation in 0·1 N-NaOH (Blakley, 1957), the solution being neutralized before use by shaking under $N_2 + CO_2$ (95:5, v/v).

DL-[3-^{14}C]Serine was obtained from the Radiochemical Centre, Amersham, and contained 0·1 mc in 11·3 mg.

Pyridoxal phosphate monohydrate. A generous gift of the crystalline compound was received from Dr A. L. Morrison of Roche Products Ltd., Welwyn Garden City, England.

METHODS

Enzyme. This was prepared as previously described (Blakley, 1955). All experiments were performed with enzyme purified by the complete procedure and having specific activity 12–25 μmoles of serine/hr./mg. of protein.

Determination of enzyme activity. The manometric method, depending on serine estimation by oxidation with periodate, was used as reported earlier (Blakley, 1955). The standard enzyme system consisted of enzyme (to give 10–20 μmoles of serine/hr. under optimum conditions), 0·01 M-$NaHCO_3$, 5×10^{-4} M pyridoxal phosphate, 1·3 mM tetrahydroPGA and water to bring the final volume to 3·0 ml. Glycine and formaldehyde were added from the side arm after equilibrating the solution at 37° under $N_2 + CO_2$ (95:5, v/v) so that their final concentrations were glycine, 0·1 M and formaldehyde, 0·01 M. Any departure from these conditions is indicated in the legends to tables and figures.

RESULTS

Proportionality of serine formed to reaction time and amount of enzyme

With the standard-assay system and about 0·5 mg. of enzyme the rate of serine synthesis proceeded uniformly for a period of more than an hour (Fig. 1).

Under the standard conditions serine synthesis, in a period of 1 hr., was found to be proportional to the amount of enzyme added within a wide range of enzyme concentrations (Fig. 2). These results were the basis for regarding serine synthesis during a period of 1 hr. as a measure of enzyme activity in this and previous work (Blakley, 1955).

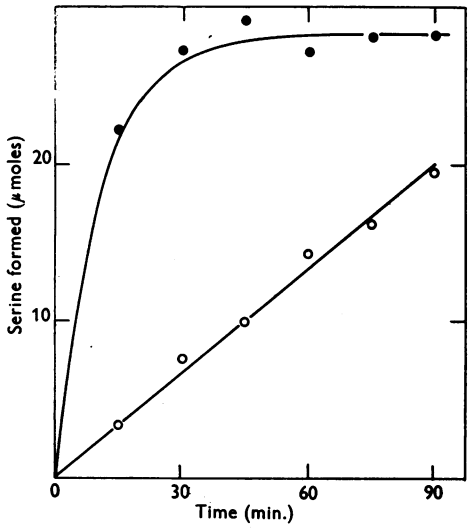

Fig. 1. Effect of period of incubation on serine synthesis. The standard enzyme system was incubated for various periods of time before heat-inactivation of the enzyme and determination of serine; ○, 0·36 mg. of protein/flask, ●, 1·8 mg. of protein/flask.

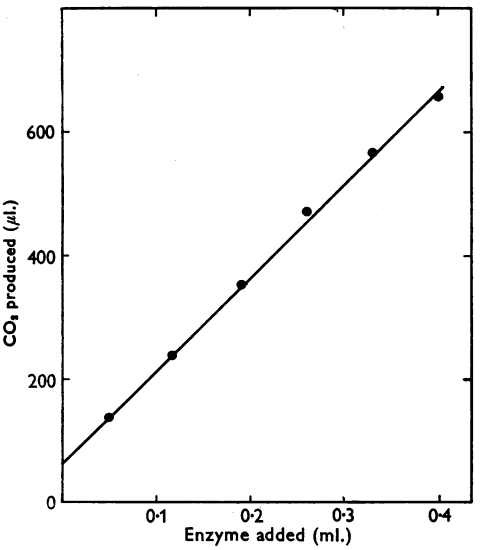

Fig. 2. Effect of the amount of enzyme on serine synthesis. Serine synthesis was measured manometrically as CO_2 after periodate oxidation, as described in the text.

With an excess of enzyme the reaction reached an equilibrium corresponding to the formation of about 28·2 μmoles of serine (Fig. 1). This result enables an approximate estimation of the equilibrium constant for the overall reaction:

$$HO.CH_2.CH(NH_2).CO_2H \rightleftharpoons H_2N.CH_2.CO_2H + H.CHO$$

defined by

$$K = [\text{glycine}]\,[H.CHO]/[\text{serine}]$$

of value 5·8 mM.

More accurate determination of the equilibrium constant by studying the reaction in the reverse direction has not been accomplished because of analytical problems.

Effect of substrate concentration on reaction rates

The effect of glycine concentration on reaction velocity is shown in Fig. 3, where the results are plotted according to the method of Lineweaver & Burk (1934). It may be seen that they conform to

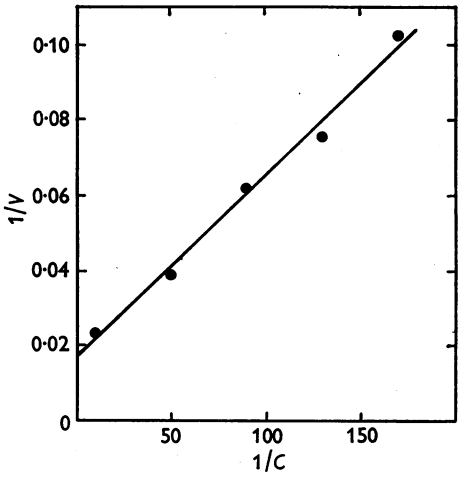

Fig. 3. Effect of glycine concentration on serine synthesis. The results are plotted according to the method of Lineweaver & Burk (1934). *C*, Molar concentration of glycine; *V*, μmoles of serine formed under standard conditions in 1 hr.

Table 1. *Effect of formaldehyde concentration on rate of serine synthesis*

The reaction was performed under standard conditions, except that the concentration of formaldehyde was varied as shown.

Formaldehyde concn. (M)	Serine synthesized (μmoles/hr.)
0·0025	4·6
0·0050	11·9
0·0100	22·1
0·0150	17·3
0·0200	15·6
0·0250	12·6

the requirements of a simple Michaelis–Menten relation over the concentrations used, giving an apparent Michaelis constant with the unusually high value of $2{\cdot}8 \times 10^{-2}$.

As may be seen from Table 1, the rate of serine synthesis increased with formaldehyde concentration up to 0·01 M, but further increase of formaldehyde concentration diminished the synthesis of serine.

Table 2. *Substrate specificity of the enzyme system for serine synthesis*

The reaction was performed under standard conditions (see text), except that the substrates were 0·10 M glycine and those shown below.

Substrate	β-Hydroxy-α-amino acid synthesized (μmoles/hr.)
Expt. 1	
Formaldehyde (0·01 M)	12·8
Acetaldehyde (0·01 M)	0·2
Sodium formate (0·01 M)	0·0
Expt. 2	
Formaldehyde (0·01 M)	23·7
Acetaldehyde (0·025 M)	0
Formaldehyde (0·01 M) + acetaldehyde (0·025 M)	22·8

Table 3. *Activating effect on serine synthesis of pteridines related to PGA*

Pteridines were tested at the concentration shown in the standard enzyme system with an excess of enzyme present.

Expt. no.	Pteridine	Concn. (mM)	Serine synthesized (μmoles/hr.)
1	None	—	0
	Citrovorum factor	1·88	0
	Leucovorin	1·88	0
	2-DeaminoPGA	1·88	0·1
	4-Deoxy-4-aminoPGA	1·88	0
	Pteroyltriglutamic acid	1·88	0
	N^{10}-FormylPGA	1·88	1·1
	TetrahydroPGA	1·88	12·0
2	None	—	0
	Tetrahydro-2-deaminoPGA	2·50	0
	Anhydroleucovorin A	2·50	0
	PGA	2·50	0
	Tetrahydro-4-amino-4-deoxyPGA	2·50	0
	TetrahydroPGA	2·50	12·5
3	None	—	0
	TetrahydroPGA	0·33	16·2
	Tetrahydro-N^{10}-formylPGA	0·33	5·0
	Tetrahydropteroyltri-glutamic acid	0·33	11·5
4	None	—	0
	TetrahydroPGA	0·33	8·6
	Pteroic acid	0·33	0·2
5	None	—	0
	Pteroylheptaglutamic acid	0·093	3·4
	Pteroylheptaglutamic acid	0·038	2·9
	TetrahydroPGA	0·33	24·1

Substrate specificity

Acetaldehyde and formate were unable to replace formaldehyde in the reaction (Table 2). The volatility of acetaldehyde introduces some uncertainty as far as its concentration in solution is concerned, but its lack of activity is in accord with previous reports (Vilenkina, 1952; Blakley, 1954). When added at a concentration of 0·025 M, acetaldehyde did not produce any inhibition of serine synthesis from glycine and formaldehyde.

No serine synthesis occurred when sarcosine (0·1 M) was used in place of glycine in the purified enzyme system.

Specificity of the enzyme for tetrahydroPGA

As reported previously (Blakley, 1957), the enzyme is activated both by dihydroPGA and tetrahydroPGA. Since several other related pteridines have been suggested as possessing coenzyme properties a number of these have been tested with the rabbit-liver enzyme system. The only substances tested which consistently caused significant activation of the enzyme were tetrahydroPGA (and dihydroPGA), tetrahydro-N^{10}-formylPGA, and the tetrahydro derivatives of pteroyltriglutamic and pteroylheptaglutamic acids (Table 3). Consequently the enzymic synthesis of serine does not involve formation of anhydroleucovorin A, leucovorin or the citrovorum factor, and only hydrogenated derivatives of PGA are active. The activity of the hydrogenated PGA derivatives containing several glutamic acid residues in the molecule is noteworthy.

Michaelis constants for dihydroPGA, tetrahydroPGA and tetrahydropteroyltriglutamic acid

When rigid precautions were taken to prevent their reoxidation during the experiment, dihydroPGA, tetrahydroPGA and tetrahydropteroyltriglutamic acid gave results conforming to the simple Michaelis–Menten relation as seen in Figs. 4 and 5. The values for the Michaelis constants were approximately $2{\cdot}9 \times 10^{-4}$ M, $1{\cdot}8 \times 10^{-4}$ M and $8{\cdot}3 \times 10^{-5}$ M respectively.

Inhibition of serine synthesis by analogues of PGA

It is well known that 4-amino-4-deoxyPGA and 2-deaminoPGA are antagonists of PGA *in vivo* (Jukes, 1953; Nimmo-Smith & Brown, 1953). The hydrogenated derivatives of these compounds were therefore tested as inhibitors for the enzymic synthesis of serine, with the results shown in Table 4. In accordance with previous results (Blakley, 1954) tetrahydro-4-amino-4-deoxyPGA did not inhibit the action of tetrahydroPGA in the enzyme reactions, the antagonism of 4-amino-4-deoxyPGA to PGA *in vivo* being due rather to inhibition of the

enzyme system responsible for reducing PGA to its hydrogenated derivative. Dihydro-2-deaminoPGA produced significant inhibition of the synthesis of serine, however, and preliminary results from data plotted according to the method of Lineweaver & Burk (1934) indicate that the inhibition is competitive.

Pteridine intermediate in serine synthesis

The role of tetrahydroPGA in serine synthesis presumably lies in the activation of formaldehyde. Evidence has been presented to indicate that no stable compound is formed by non-enzymic combination of formaldehyde and tetrahydroPGA, although it is possible that readily dissociating compounds, such as those containing hydroxymethyl groups, may be formed (Blakley, 1957). It is conceivable, however, that in the presence of a component of the enzyme system, formaldehyde and tetrahydroPGA could combine to form a long-lived intermediate, stable to oxidation.

Two methods have been used to assist the possible accumulation of such an intermediate: (*a*) incubation of enzyme, formaldehyde (30 μmoles) and tetrahydroPGA (10 μmoles) under N_2, and (*b*) incubation of enzyme, serine (0·1–0·4 M) and tetrahydroPGA (10 μmoles) under N_2. In both cases the amount of enzyme and the period of incubation chosen were such that in previous tests with the complete serine-synthesizing system they resulted in the production of 20 μmoles of serine. When method (*a*) was used the enzyme was inactivated after incubation by heating the mixture under N_2 in a boiling-water bath for 2 min. In method (*b*), after incubation the enzyme was precipitated by 3 vol. of ethanol (99 %, v/v) and the pteridines were precipitated from the supernatant by the addition of 40 μmoles of barium acetate and another 1 vol. of ethanol. The flocculent precipitate was collected

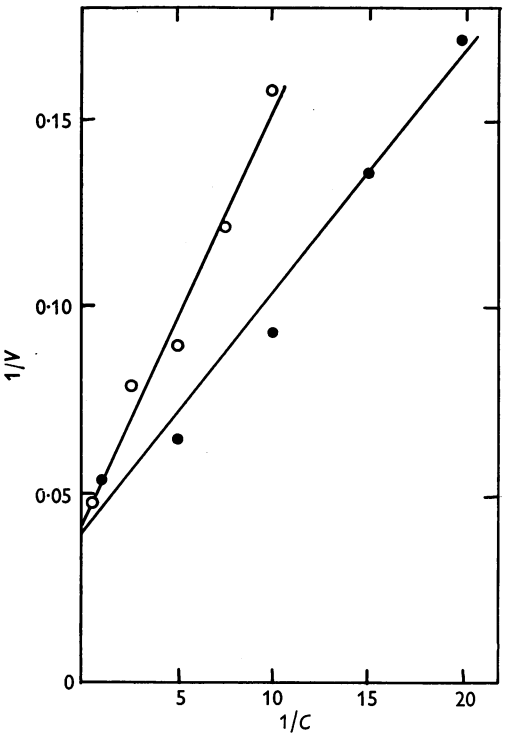

Fig. 4. Effect of dihydroPGA and tetrahydroPGA concentration on serine synthesis. The results are plotted according to the method of Lineweaver & Burk (1934). *C*, 900 × Pteridine concentration (M); *V*, μmoles of serine formed under standard conditions in 30 min.; ○, with dihydroPGA; ●, with tetrahydroPGA.

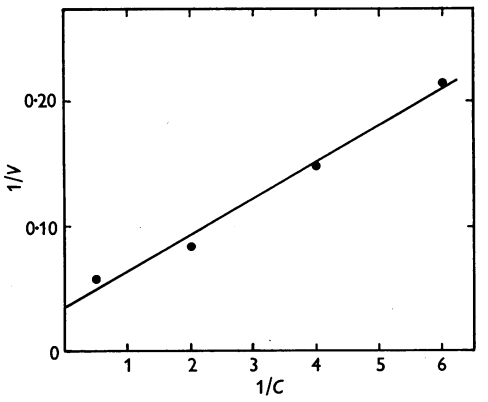

Fig. 5. Effect of tetrahydropteroyltriglutamate concentration on serine synthesis. The results are plotted according to the method of Lineweaver & Burk (1934). *C*, Tetrahydropteroyltriglutamate concentration (10^{-4} M); *V*, μmoles of serine formed under standard conditions in 1 hr.

Table 4. *Effect of tetrahydro-4-amino-4-deoxyPGA and dihydro-2-deaminoPGA on serine synthesis*

Standard conditions were used except that the concentration of tetrahydroPGA was $2·5 \times 10^{-4}$ M.

Inhibitor	Concn.	Serine synthesized (μmoles/hr.)	Inhibition (%)
None	—	14·8	—
Tetrahydro-4-amino-4-deoxyPGA	$7·5 \times 10^{-4}$ M	15·1	0
Dihydro-2-deaminoPGA	$7·5 \times 10^{-4}$ M	6·6	55

by centrifuging under N_2 in the cold, washed with ethanol and dried *in vacuo*.

The reaction medium from method (*a*) and solutions of the barium salt from method (*b*) were examined by paper chromatography for evidence of a new pteridine component. The detection of the postulated intermediate on chromatograms was complicated by the presence of numerous fluorescent compounds formed by oxidative degradation of tetrahydroPGA (Blakley, 1957), and no new compound could be found. Separation of some of the components of the barium salt from method (*b*) by chromatography on Solka Flok (powdered cellulose) columns after removal of Ba^{2+} ions yielded no coenzyme-active compounds which were stable to oxidation. Although some compounds obtained by chromatography on Solka Flok showed a slight coenzyme activity as isolated and considerable coenzyme activity on rehydrogenation, the same was true of solutions of oxidized tetrahydroPGA (Blakley, 1957).

To check this point further the coenzyme activity of a solution obtained by method (*a*) was tested after vigorous shaking in air for various periods. The results, shown in Fig. 6, do not differ appreciably from those obtained with a solution of tetrahydroPGA and formaldehyde (Blakley, 1957).

Effect of incubating [3-^{14}C]*serine with enzyme and tetrahydroPGA*

As a further test for the formation of a stable pteridine intermediate in serine–glycine interconversion, [3-^{14}C]serine has been incubated with enzyme and tetrahydroPGA and the pteridines isolated, chromatographed and examined for radioactivity. DL-[3-^{14}C]Serine (4·5 mg., 40 μC), L-serine (24 mg.), dihydroPGA (9·5 mg.) and dialysed enzyme in a total volume of 3·5 ml. were incubated together under $N_2 + CO_2$ (95:5, v/v) in a Warburg flask at 37° for 20 min. The amount of enzyme used was sufficient to form 40 μmoles of serine in the standard system in this time. At the end of the incubation period the pteridines were separated as their barium salts by the procedure used in method (*b*) above. After removal of Ba^{2+} ions the solution was freeze-dried and a portion of the resultant yellow powder counted on lens tissue on a 1 cm.^{2} polythene disk under a conventional end-window Geiger–Müller tube. The result corresponded to 76 300 counts/min./cm.^{2} at infinite thickness, under conditions in which the L-serine present in the medium would have given 450 000 counts/min./cm.^{2} at infinite thickness.

A portion of the material was dissolved and chromatographed by the ascending technique on Whatman no. 4 paper with 0·1 M-K_2HPO_4 as solvent. Over ultraviolet light three blue-fluorescing areas were visible with R_F values of 0·17, 0·39 and 0·57, and in addition there was a yellow-fluorescing area of R_F 0·05. A radioautograph revealed no radioactivity in the two slowest-moving fluorescent spots but showed areas of weak radioactivity corresponding to the fluorescing areas of R_F 0·39 and 0·57. In addition, strong radioactivity was indicated at a non-fluorescent area of R_F 0·93, which was shown by control chromatograms to correspond to the position of serine. Under an end-window counter the areas of R_F 0·39, 0·57 and 0·93 had radioactivity of 15, 29 and 973 counts/min. above background, so that almost all the radioactivity was present in the fast-moving compound. This was probably a trace of serine which had been co-precipitated with the barium salt; it did not appear to be a pteridine since it was non-fluorescent and did not absorb ultraviolet light.

The significance of the very small amount of radioactivity associated with the fluorescent areas is doubtful, but the radioactivity was far below that expected for an intermediate in equilibrium with the serine β-carbon.

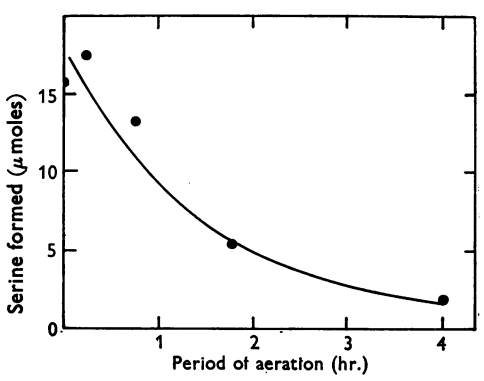

Fig. 6. Effect of aeration on the coenzyme activity of tetrahydroPGA after incubating with enzyme and formaldehyde. The reaction mixture, containing 3·33 mM tetrahydroPGA, 10^{-2}M formaldehyde and enzyme, was incubated under N_2 at 37° and then boiled. It was transferred to a test tube and shaken vigorously under air. At intervals, 0·3 ml. samples were removed and tested with the standard enzyme system. For further details, see text.

DISCUSSION

The enzyme system responsible for serine synthesis showed a normal Michaelis–Menten relationship between glycine concentration and reaction velocity except that the Michaelis constant K_m was found to have the rather high value of $2·8 \times 10^{-2}$M. However, glycine forms a Schiff's base with pyridoxal phosphate before, or perhaps in the process of, combining

with the enzyme (Blakley, 1955), so that this result was perhaps not surprising. The reaction velocity did not increase uniformly with formaldehyde concentration, but diminished with increase of the formaldehyde concentration above 0·01 M (Table 1). Since formaldehyde may inactivate enzymes by combining with them and since tetrahydroPGA is also inactivated by excessive amounts of formaldehyde (Blakley, 1957), this result also conformed to expectation.

Although some liver preparations will convert sarcosine into serine, especially in the presence of added glycine (Mitoma & Greenberg, 1952), no synthesis of serine occurred when sarcosine was substituted for glycine in the enzyme system studied in the present work. It is possible that an entirely different enzyme system is responsible for the conversion of sarcosine into serine, but it seems likely that in crude preparations sarcosine is first converted into glycine and formaldehyde which then react in the system described in this paper.

Results on the specificity of the enzyme for tetrahydroPGA indicate that the 2-amino and 4-hydroxy substituents on the pteridine ring are essential for coenzyme activity. Although slight activity was obtained with 4-amino-4-deoxyPGA this was almost certainly due to contaminating PGA, an impurity difficult to remove. The glutamic acid residue of PGA is essential for activity and the results also suggest that reduced 'conjugates' of PGA possessing three or more glutamic residues are even more active than is tetrahydroPGA itself. Thus with tetrahydropteroyltriglutamate, as with tetrahydroPGA, the results conformed to the simple Michaelis–Menten relation and the respective values of K_m were $8{\cdot}3 \times 10^{-5}$ M and $1{\cdot}8 \times 10^{-4}$ M. Only a small quantity of tetrahydropteroylheptaglutamate was available for testing, but this also had good activity. It seems possible that the extended peptide chain of glutamic acid residues increases the rate of reaction of the coenzyme with the enzyme to form an active complex.

Experiments designed to demonstrate the existence of a pteridine intermediate in which tetrahydroPGA is combined with a one-carbon group which can be converted enzymically into the β-carbon of serine or into formaldehyde have been rather inconclusive. Paper chromatography revealed no new fluorescent or ultraviolet-light-absorbing compound, but the many compounds formed by oxidation of tetrahydroPGA may have obscured such a substance on the chromatogram. Solutions of the products from the incubation of enzyme and tetrahydroPGA with either formaldehyde or serine lose their coenzyme activity during aeration almost as rapidly as a simple solution of tetrahydroPGA and formaldehyde. Only slight amounts of radioactivity were incorporated into fluorescent pteridines when [3-^{14}C]serine was incubated with enzyme and dihydroPGA.

On the whole, therefore, the evidence favours the view that during serine synthesis by the enzyme system tetrahydroPGA forms only readily dissociating compounds, unstable to oxidation, such as hydroxymethyl derivatives.

SUMMARY

1. The rate of synthesis of serine from glycine and formaldehyde by a partially purified enzyme from rabbit liver was found to be proportional to enzyme concentration and time of incubation.

2. The reaction ultimately reached an equilibrium corresponding to a value of the constant [glycine] [formaldehyde]/[serine] of approximately 5·8 mM.

3. The relation between glycine concentration and reaction velocity conformed to the requirements of the simple Michaelis–Menten equation, giving a K_m of $2{\cdot}8 \times 10^{-2}$ M. The optimum formaldehyde concentration was 0·01 M, the reaction velocity diminishing at higher concentrations.

4. Hydrogenated pteroylglutamic acid (PGA), N^{10}-formylPGA, pteroyltriglutamate and pteroylheptaglutamate were the only pteridines active as intermediates among those tested. The 2-amino and 4-hydroxy groups, the hydrogenated pyrazine ring and one or more glutamic acid residues are essential for activity. Leucovorin, citrovorum factor and anhydroleucovorin A are all inactive.

5. Dihydro-2-deaminoPGA, but not tetrahydro-4-amino-4-deoxyPGA, is an inhibitor of serine synthesis with tetrahydroPGA as coenzyme.

6. The relations between reaction velocity and the concentration of dihydroPGA, tetrahydroPGA and tetrahydropteroyltriglutamate respectively conform to the requirements for simple Michaelis–Menten equations giving K_m values of $2{\cdot}9 \times 10^{-4}$ M, $1{\cdot}8 \times 10^{-4}$ M and $8{\cdot}3 \times 10^{-5}$ M respectively.

7. The reaction products from incubation of enzyme and tetrahydroPGA with serine or formaldehyde do not contain any new pteridine detectable by chromatography, and are inactivated by aeration at about the same rate as a simple mixture of solutions of tetrahydroPGA and formaldehyde.

8. Incubation of enzyme, dihydroPGA and [3-^{14}C]serine results in only very slight incorporation of radiocarbon into pteridines.

9. The nature of the pteridine intermediate of enzymic serine synthesis is discussed in terms of these results.

It is a pleasure to express my indebtedness to those mentioned above who supplied essential compounds for this work. In addition I wish to thank Professor A. H. Ennor for helpful advice and criticism.

REFERENCES

Blakley, R. L. (1954). *Biochem. J.* **58**, 448.
Blakley, R. L. (1955). *Biochem. J.* **61**, 315.
Blakley, R. L. (1957). *Biochem. J.* **65**, 331.
Jukes, T. H. (1953). *Fed. Proc.* **12**, 633.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Mitoma, C. & Greenberg, D. M. (1952). *J. biol. Chem.* **196**, 599.
Nimmo-Smith, R. H. & Brown, D. J. (1953). *J. gen. Microbiol.* **9**, 536.
Vilenkina, G. Y. (1952). *C.R. Acad. Sci. U.R.S.S.* **84**, 559.

Biosynthesis of Fatty Acids in Cell-free Preparations

4. SYNTHESIS OF FATTY ACIDS FROM ACETATE BY A PARTIALLY PURIFIED ENZYME SYSTEM FROM RABBIT MAMMARY GLAND*

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It was shown in previous papers of this series (Popják & Tietz, 1955; Tietz & Popják, 1955) that cell-free extracts prepared from lactating-rat mammary gland were able to synthesize long- and short-chain fatty acids from [^{14}C]acetate. This synthesis was dependent on the addition of adenosine triphosphate (ATP), the concomitant oxidation of pyruvate, oxaloacetate or α-oxoglutarate, and the presence of coenzyme A (CoA). This last requirement could be demonstrated only if the extracts were depleted of endogenous CoA by treatment with Dowex-I resin.

Before commencing a detailed study of the steps involved in fatty acid synthesis, it was necessary to simplify the original assay conditions used by Popják & Tietz (1955), and to attempt the purification of the mammary gland extracts. In this communication the preparation of a partially purified enzyme system from rabbit mammary gland is described. This system can synthesize fatty acids from acetate in the presence of stoicheiometric amounts of reduced diphosphopyridine nucleotide (DPNH) as electron donor. The results have been presented in a preliminary form (Hele & Popják, 1955).

MATERIALS AND METHODS

Preparation of the fatty acid-synthesizing fraction from rabbit mammary gland

Lactating rabbits, about 17 days after parturition, were killed by the intravenous injection of Nembutal (10%, w/v; 1·5 ml./kg.) and the mammary glands excised. The average yield was 100 g./animal. The glands were cooled in crushed ice, freed from fat and connective tissue, and finely minced with scissors. The mince was homogenized for 2 min. in a blender (M.S.E. Ato-mix 800), run at top speed, with 2 vol. of buffer (0·154 M-KCl, 100 parts; 0·154 M-$MgCl_2$, 10 parts; 0·1 M potassium phosphate buffer, pH 7·4, 34 parts). This and all subsequent operations were carried out at 0–4°. The homogenate was centrifuged at 400 *g* for 10 min., and the supernatant collected with filtration through a pad of gauze to remove fat. This supernatant was centrifuged at 44 000 *g* for 1 hr. in the Spinco model L preparative ultracentrifuge. In the earliest preparations the supernatant was centrifuged once again, this time at 100 000 *g* for 1 hr., before precipitation with $(NH_4)_2SO_4$ was carried out. In later preparations, when much larger volumes of material were being handled, this second centrifuging was dispensed with and precipitation with $(NH_4)_2SO_4$ carried out in the following manner with the material centrifuged at 44 000 *g*.

The supernatant was brought to 90% saturation by the addition of 70 g. of $(NH_4)_2SO_4$/100 ml. at 0–4°. The precipitate (F 90) was collected by filtration on a large fluted paper (Whatman no. 12) overnight, followed by press-drying between filter papers the next day. This precipitate could be stored in solid CO_2 for at least 6 months with no loss of fatty acid-synthesizing activity.

Refractionation of this material was carried out as follows. Sufficient cold water was added to each 21 g. of F 90 precipitate to bring the volume to 100 ml., and saturated with $(NH_4)_2SO_4$ to about 21%. A smooth suspension was achieved by mechanical stirring. Ammonium sulphate (9·8 g./100 ml.) was added to give a final saturation of about 35%; the precipitate was removed by centrifuging at 100 000 *g* for 10 min. in the Spinco ultracentrifuge. This high-speed centrifuging was necessary for the removal of finely particulate material which could not be removed in 20 min. at 40 000 *g*.

A clear supernatant was obtained, which was brought to about 60% saturation by the addition of 17·4 g. of $(NH_4)_2SO_4$/100 ml. of supernatant, and the precipitate collected by centrifuging at 22 000 *g* for 10 min. This precipitate, which

* Part 3: Tietz & Popják (1955).

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