3. The viscosity-reducing action is due to a sulphydryl-activated enzyme, which has been named a tropomyosinase, whose action is inhibited by homologous antisera.

4. The enzyme does not attack collagen, nor haemolyse the red blood cells of sheep and horses. It is activated by cysteine, glutathione and thiolacetate, but not by thiosulphate. It is not inhibited by soya-bean trypsin inhibitor.

5. By neutralization tests with selective antisera, this tropomyosinase was identified as a specific antigen which has been designated, in accordance with the nomenclature of Oakley *et al.* (1947), as *Cl. oedematiens* η-antigen. It has been found in crude toxins of *Cl. oedematiens* type B and *Clostridium haemolyticum*, but not in *Cl. oedematiens* type A nor in *Clostridium welchii*, *Clostridium septicum* or *Corynebacterium diphtheriae* toxins.

I wish to express my thanks to my colleagues Mr Weitz and Dr Dolby, to Miss M. Barr, Dr D. A. Long, Dr D. G. Evans and especially to Dr G. H. Warrack of the Wellcome Research Laboratories for gifts of toxins and antitoxins, to Miss S. Jolley for her assistance, and to Mr H. Murray for the ultracentrifugal examinations.

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The Interconversion of Serine and Glycine: Participation of Pyridoxal Phosphate

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The enzyme system of avian and mammalian liver which catalyses the reversible synthesis of serine from glycine and formaldehyde has been shown to require tetrahydropteroylglutamic acid (tetrahydroPGA) as a coenzyme (Blakley, 1954*b*, *d*; Kisliuk & Sakami, 1954). In well dialysed, unfractionated liver extracts pteroylglutamic acid (PGA) and some other derivatives of it besides tetrahydroPGA also stimulate serine synthesis (Blakley, 1954*a*, *d*), but the evidence indicates that in such crude enzyme preparations PGA and derivatives of it other than tetrahydroPGA are first converted enzymically into the latter compound before participating in serine biosynthesis (Blakley, 1954*d*). When crude enzyme preparations are used diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) in conjunction with PGA also stimulate serine–glycine interconversion, but the effect of ATP and DPN appears to be concerned with the transformation of PGA into tetrahydroPGA (Blakley, 1954*d*). Thus the only coenzyme of serine biosynthesis, for which *in vitro* studies by the isotope method have provided conclusive evidence, is tetrahydroPGA. *In vivo* experiments have suggested, however, that pyridoxal phosphate also participates in serine synthesis (Deodhar & Sakami, 1953).

Serine biosynthesis in the presence of a partially purified enzyme system has now been studied by a manometric method, and under appropriate conditions a marked activation by pyridoxal phosphate has been observed (Blakley, 1954*c*). In the present communication the evidence for the activation of enzymic serine synthesis by pyridoxal phosphate is presented in detail, together with data concerning the effect on the reaction of other pyridoxine derivatives.

EXPERIMENTAL

Reagents. TetrahydroPGA was prepared by catalytic hydrogenation of neutral aqueous solutions of commercial preparations of PGA (O'Dell, Vandenbelt, Bloom & Pfiffner, 1947). Pyridoxine derivatives were gifts: crystalline pyridoxal phosphate (4-formyl-3-hydroxy-2-methylpyrid-5-ylmethyl dihydrogen phosphate) was received from Dr A. L. Morrison of Roche Products and crystalline pyridoxamine 5-phosphate (4-aminomethyl-3-hydroxy-2-methylpyrid-5-ylmethyl dihydrogen phosphate), pyridoxine 5-phosphate (3-hydroxy-4-hydroxymethyl-2-methylpyrid-5-ylmethyl dihydrogen phosphate) and deoxypyridoxine 5-phosphate (3-hydroxy-2:4-dimethylpyrid-5-ylmethyl dihydrogen phosphate) from Dr H. A. Sober.

Animals. Various strains of rabbit were used, though the majority were the wild type. Some had been infected with myxomatosis and had recovered. The enzyme content of the liver varied considerably and appeared to be dependent on the general condition of the animal, but not on the particular strain of animal used. Animals were kept on a stock diet before use, the period on the diet ranging from 24 hr. to many months.

METHODS

Determination of enzyme activity. Under the experimental conditions employed, serine synthesis proceeded at a constant rate, proportional to the amount of enzyme added, until the formaldehyde (added in smaller amount than glycine) was almost exhausted (unpublished observations). Enzymic activity was therefore assayed by estimating the serine formed in 1 hr. under standard conditions. The enzyme preparation in the presence of tetrahydroPGA (0·0013 M) and bicarbonate (0·01 M) was equilibrated with $N_2 + CO_2$ (95:5) (v/v) at 37° in Warburg vessels. When equilibration was complete glycine and formaldehyde were tipped in from the side arm to give a final concentration of 0·1 and 0·01 M respectively. After 1 hr. the manometers were removed from the bath, the water was blotted from around the necks of the flasks and the side-arm stoppers, the flasks were removed from the manometers and a glass sphere was placed in the neck of each flask. The flasks were immediately placed in a boiling-water bath and kept there for 2 min. to inactivate the enzyme. When the flasks had cooled, 0·3 ml. of 0·5 M sodium periodate (adjusted to pH 6·0) was placed in the side arm of each. The flasks were then replaced on their manometers, flushed with pure CO_2 and equilibrated at 37°. When the periodate was tipped into the main compartment, serine was oxidized with the evolution of 1 mole CO_2/mole of serine (Fig. 1). The reaction was completed in 20 min. Only small amounts of CO_2 (about 3 μmoles) were evolved by reactions other than the oxidation of serine by periodate, and an estimate of this 'blank' CO_2 production was obtained by carrying out the determination with the omission of tetrahydroPGA. When crude enzyme preparations were used some serine was produced even in the absence of added tetrahydroPGA. In such cases both formaldehyde and tetrahydroPGA were omitted from the control flask, but even under these conditions the 'blank' was often too high, owing to some serine synthesis proceeding even in the absence of tetrahydroPGA and formaldehyde. Consequently, values for the activity of crude enzyme preparations were frequently low.

From 1 to 30 μmoles of serine added to the enzyme-assay system could be recovered with an accuracy of $\pm 5\%$ (Fig. 1). Specific activity of the enzyme is expressed as μmoles serine formed/hr./mg. protein under standard conditions. A unit of enzyme is taken as that amount forming 1 μmole serine/hr. under standard conditions.

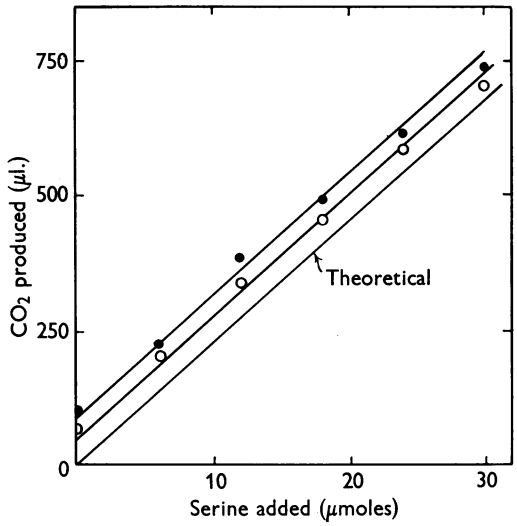

Fig. 1. Production of CO_2 under standard assay conditions by periodate oxidation of pure serine added to complete enzyme system, ●; and of serine added to the same system with enzyme omitted, ○.

Preparation of the enzyme. The rabbits were killed by a blow on the head and the livers quickly removed and dispersed in acetone as previously described (Blakley, 1954*d*). After drying at room temperature and about 3×10^{-2} mm. Hg for 2 hr. over P_2O_5 the powder was extracted with 15 times its weight of cold 0·01 M potassium phosphate buffer (pH 7·5) for 1 min. in a Waring Blendor. The extract was allowed to stand for 30 min. at 5° before centrifuging (1 hr. at 4300 *g*) at 2°. The supernatant was brought to 53° within 5 min., held at this temperature for a further 10 min. and then rapidly cooled in ice water. Centrifuging at 4300 *g* for 30 min. produced a clear supernatant, which was fractionated with ammonium sulphate (adjusted to pH 7·5). The fraction precipitating between 1·47 and 2·06 M ammonium sulphate was collected separately, dissolved in a small volume of 0·01 M phosphate (pH 7·5) and dialysed for 3 hr. in a rocking dialyser in the cold (5°) against 100 vol. 0·02 M potassium phosphate buffer (pH 7·5) and then for a further 3 hr. against 0·001 M phosphate (pH 6·3). Attempts at further purification of the enzyme by the usual methods were attended by considerable losses, and difficulty was experienced in reproducing the fractionation from one batch of enzyme to another. The best results from ethanol fractionation were obtained if a trial fractionation of a small portion of the enzyme was carried out first. A small portion of the dialysed solution was adjusted to pH 6·2 with 1 M acetate (pH 5·0), diluted to give a protein concentration of about 12 mg./ml. and fractionated with ethanol, the temperature being lowered

Table 1. *Summary of yields and specific activities of fractions obtained during the purification of serine-synthesizing enzyme from rabbit-liver–acetone powder*

The details given are for a preparation from 53·5 g. acetone powder. Units of activity are expressed as μmoles serine/hr. Specific activity is expressed as μmoles serine/hr./mg. protein.

Fraction	Volume (ml.)	Total protein (mg.)	Total units	Specific activity
Phosphate-buffer extract	644	18 900	54 000*	1·9*
Supernatant after heating at 53°	504	10 900	47 500*	2·8*
Ammonium sulphate precipitate	63	3 270	78 200	16·5
Ethanol-precipitated fraction	57	608	27 400	27·4

* Estimation of activity in these fractions gave low values, probably because some synthesis occurred in the absence of added tetrahydroPGA and formaldehyde, and hence was included in the blank.

from 0 to −3° during addition of ethanol. Five fractions were collected over the range of ethanol concentration 0–25% (v/v), and each fraction was tested for enzymic activity. The remainder of the enzyme was then fractionated with ethanol in the same way and the most active fraction, as previously determined in the trial, was dissolved in cold 0·01 M phosphate buffer (pH 7·5). To preserve activity it was found necessary to precipitate the enzyme immediately with 2·21 M ammonium sulphate (adjusted to pH 7·5), centrifuge after standing 10 min. at 0°, and redissolve the precipitate in dilute phosphate buffer (pH 7·5). With some preparations the enzymic material could be further purified by ammonium sulphate fractionation at pH 7·5, the material precipitating at ammonium sulphate concentration 1·54–2·02 M being collected separately and dissolved in a small volume of cold 0·01 M phosphate (pH 7·5). At any stage in the preparation the enzyme solution could be kept frozen without great loss in activity, but considerable and variable losses frequently occurred when preparations were kept for a period and frequently frozen and thawed. Data for a typical preparation are shown in Table 1.

Estimation of protein. The protein concentration of enzyme solutions was determined by measurement of the optical density at 280 mμ. after appropriate dilution with 0·1 N-NaOH. Under these conditions a solution containing 1 mg. protein/ml. was found to have a density of 1·5.

Estimation of pH. This was performed with a pH meter, employing a glass electrode.

RESULTS

Unfractionated extracts of rabbit-liver–acetone powder showed little increase in the rate of serine synthesis upon addition of pyridoxal phosphate to the otherwise complete reaction medium. After purification of the enzyme by heat treatment and by ammonium sulphate fractionation, however, stimulation of serine synthesis by added pyridoxal phosphate became greater, and at the stage of the ammonium sulphate-fractionated enzyme pyridoxal phosphate usually produced about a twofold increase in the rate of serine synthesis (Table 2). Pre-incubation of the enzyme with pyridoxal phosphate before addition of substrates caused no greater activation than addition of pyridoxal phosphate to the enzyme with the substrates. The

Table 2. *Activation of serine synthesis by pyridoxal phosphate at various stages of enzyme fractionation*

The enzyme was tested under standard conditions. Pyridoxal phosphate when present was added to give a concentration of 5×10^{-4} M. Specific activity of the enzyme is expressed as μmoles serine synthesized/hr./mg. protein.

Enzyme preparation	Specific activity: Without pyridoxal phosphate	Specific activity: With pyridoxal phosphate
Phosphate extract of acetone powder	2·73	3·78
Supernatant after heat treatment	4·78	7·50
Fractionated with ammonium sulphate	10·00	20·90

Table 3. *Effect of various concentrations of pyridoxal phosphate on serine synthesis by ammonium sulphate-fractionated enzyme*

Enzyme purified to first ammonium sulphate fractionation: specific activity 17 μmoles/hr./mg. protein. Tested under standard conditions.

Concn. of added pyridoxal phosphate (M)	Serine synthesized (μmoles)
None	12·9
2×10^{-5}	15·2
10^{-4}	18·5
5×10^{-4}	26·8
$2·5 \times 10^{-3}$	17·4

concentration of pyridoxal phosphate required to produce maximum increase of synthesis with such enzyme preparations was rather high, usually about 5×10^{-4} M, as may be seen from Table 3.

Enzyme preparations at this or the later stage of purification did not show any increased requirement for added pyridoxal phosphate after dialysis for several days. Although considerable loss in enzymic activity occurred during prolonged dialysis, addition of pyridoxal phosphate to the dialysed preparations did not stimulate serine

synthesis any more than with undialysed preparations. Most other attempts to produce an enzyme preparation with an absolute requirement for pyridoxal phosphate, for example by treating the enzyme with high salt concentrations even at temperatures up to 60°, followed by dialysis, were unsuccessful.

Effect of mildly alkaline pH *on pyridoxal phosphate requirement*

All enzyme preparations were found to undergo considerable inactivation at pH lower than 6 or higher than 8. After keeping the enzyme system at pH 8·6–9·6 and 37° for 30 min. synthetic activity was largely lost, but a large proportion of the activity could be restored to enzyme preparations thus inactivated by subsequently equilibrating the solution with 0·01 N-$NaHCO_3$ under N_2+CO_2 (95:5) at 37°, when the pH of the solution was quickly adjusted to 7·2, and then incubating the enzyme with pyridoxal phosphate for a short period before addition of substrate (Table 4). At higher pH values the enzyme was inactivated more completely, but recovery of activity during incubation with pyridoxal phosphate was lower. A trial was carried out with each batch of enzyme to determine the pH at which the enzyme should be incubated in order to obtain subsequently the greatest activating effect with pyridoxal phosphate. This was usually about pH 9.

Table 4. *Effect of incubating the enzyme at alkaline* pH *on requirement for pyridoxal phosphate*

Enzyme was purified to the stage of ammonium sulphate precipitation; specific activity 22 μmoles/hr./mg. protein; 138 units/ml. Four samples of equal volume were diluted with 27·3 vol. water, adjusted with 0·1 N-NaOH to the respective pH values shown and kept at 37° for 30 min. Samples of these solutions corresponding to 0·06 ml. original solution, or 8·3 enzyme units, were used for activity tests. Samples were quickly equilibrated with 0·01 M-$NaHCO_3$ under N_2+CO_2 (95:5) at 37° both in the presence and absence of 5×10^{-4} M pyridoxal phosphate, when the solutions rapidly reached pH 7·2. After 30 min. at 37° substrates were added from the side arms and the assay was continued as normally.

pH of enzyme during 30 min. incubation	Serine synthesized (μmoles) Without pyridoxal phosphate	With pyridoxal phosphate
6·6	5·36	9·48
8·6	2·01	6·12
9·1	0·00	4·50
9·6	0·13	1·43

Some experiments described below were performed with undialysed solutions of the ammonium sulphate-precipitated enzyme, but for most experiments ethanol-precipitated preparations were used. Unless otherwise stated, experiments referred to in tables and figures were performed with enzyme of specific activity 15–30 μmoles serine/hr./mg. protein which had been purified by the complete procedure. Some work was performed with preparations which had been stored some time or which had been frequently frozen and thawed, and which possessed specific activity 2–10 μmoles serine/hr./mg. protein. Enzyme preparations at the ammonium sulphate stage were found to behave in the same manner as ethanol-precipitated enzyme,

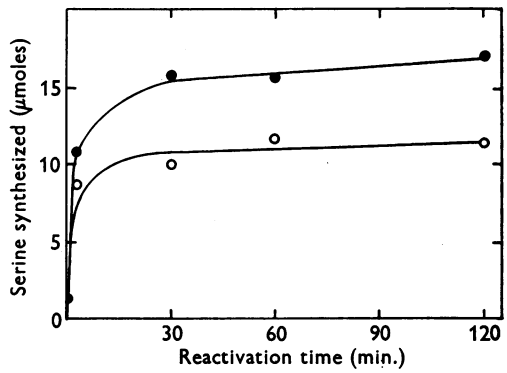

Fig. 2. Effect of incubating inactivated enzyme with pyridoxal phosphate for varying periods of time. Enzyme, 33·2 units/flask, was inactivated by holding at pH 9·1 and 37° for 30 min., then incubated at pH 7·2 and 37° with 2×10^{-4} M pyridoxal phosphate, ●; or with 10^{-5} M pyridoxal phosphate, ○, before adding substrates. Serine synthesis determined under standard conditions.

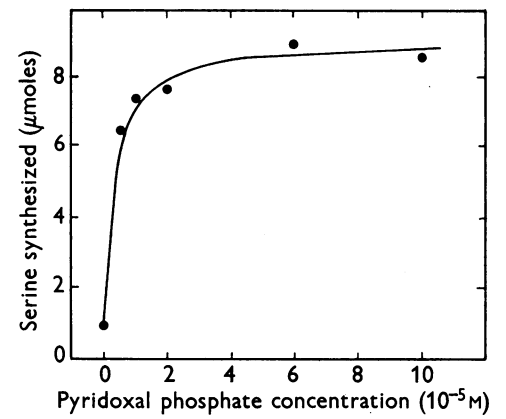

Fig. 3. Effect of incubating inactivated enzyme with pyridoxal phosphate of various concentrations. Enzyme, 18·5 units/flask, was inactivated at pH 9·1 and 37° for 30 min., then incubated at pH 7·2 and 37° with pyridoxal phosphate of various concentrations for 30 min. before adding substrates and measuring serine synthesis under standard conditions.

and no difference in behaviour was observed in preparations partly inactivated during storage or by freezing and thawing.

Reactivation by pyridoxal phosphate of enzyme treated at mildly alkaline pH was very rapid. Thus 70% of the maximum activity was obtained when the substrates were added to the enzyme only a few minutes after the addition of pyridoxal phosphate (Fig. 2). If the enzyme was incubated with pyridoxal phosphate for longer periods before addition of substrates, slightly greater activity was obtained, the maximum reactivation being achieved by pre-incubating the enzyme with pyridoxal phosphate for about 2 hr. (Fig. 2).

The concentration of pyridoxal phosphate required to produce maximum reactivation of the enzyme was quite low. Thus it may be seen by interpolation in Fig. 3 that incubation of the enzyme with 10^{-6} M pyridoxal phosphate produced about half the maximum rate of synthesis.

Effect of pyridoxal phosphate on serine breakdown

Since the enzyme system catalyses both serine synthesis and breakdown, addition of pyridoxal phosphate to enzyme inactivated at pH 9 should stimulate serine breakdown. In preliminary experiments designed to test this, serine breakdown was followed by measuring formaldehyde production, but beyond providing a qualitative demonstration of pyridoxal phosphate activation of serine breakdown the results were unsatisfactory. Recovery of formaldehyde was erratic, and tetrahydroPGA and pyridoxal phosphate interfere with its colorimetric determination. Moreover, the equilibrium appears to favour synthesis, so that only a small proportion of the serine added is converted into glycine and formaldehyde. Determination of serine disappearance gave more consistent results.

Portions of enzyme inactivated by incubation for 30 min. at pH 9·2 and 37° were placed in Warburg vessels. Sodium bicarbonate and tetrahydroPGA were added so that their final concentration was 0·01 and 0·0013 M respectively, and water was added so that the final volume on addition of serine was 3 ml. After equilibrating the mixtures with $N_2 + CO_2$ (95:5) (v/v) at 37° for 15 min. in the absence and presence of pyridoxal phosphate respectively, the reaction was started by addition of 25 μmoles L-serine from the side arms of the vessels. The reaction was stopped after 1 hr., and determination of residual serine by the usual method showed that enzyme equilibrated with 5×10^{-5} M pyridoxal phosphate had catalysed the disappearance of 4·1 μmoles serine, while in absence of pyridoxal phosphate only 0·7 μmole had disappeared. Residual serine in both cases was compared with a control in which tetrahydroPGA was omitted.

Specificity of the requirement for pyridoxal phosphate

Enzyme inactivated at pH 9 has a specific requirement for pyridoxal phosphate (Table 5). Pyridoxamine 5-phosphate, pyridoxine 5-phosphate and deoxypyridoxine 5-phosphate caused negligible reactivation of the enzyme under the same conditions in which pyridoxal phosphate produced its effect.

Table 5. *Specificity of the requirement for pyridoxal phosphate*

Enzyme inactivated for 30 min. at pH 9·1 and 37°; incubated with compound under test for 30 min. at pH 7·2 before addition of substrates. In the first experiment 33·2 units ammonium sulphate-precipitated enzyme, specific activity 22 μmoles/hr./mg. protein, were used per flask. In the second experiment 48·3 units ethanol-precipitated enzyme were used per flask. Activity tested under standard conditions.

Activator added	Serine synthesized (μmoles)
Expt. 1	
None	1·9
Pyridoxal 5-phosphate (10^{-4} M)	14·4
Pyridoxamine 5-phosphate (10^{-4} M)	3·3
Pyridoxamine 5-phosphate (5×10^{-4} M)	3·5
Expt. 2	
None	1·9
Pyridoxal 5-phosphate (5×10^{-5} M)	20·7
Pyridoxine 5-phosphate (2×10^{-4} M)	3·1
2-Deoxypyridoxine 5-phosphate (2×10^{-4} M)	2·8

Inhibition of serine synthesis by pyridoxine phosphate and deoxypyridoxine phosphate

It may be seen from Fig. 4 that deoxypyridoxine 5-phosphate is a strong inhibitor of the enzyme. If both deoxypyridoxine phosphate and pyridoxal phosphate were incubated together with the inactivated enzyme for 15 min. before the addition of substrates, much less serine synthesis occurred than when the enzyme was incubated with pyridoxal phosphate alone. Thus when pyridoxal phosphate and deoxypyridoxine phosphate, each at a concentration of 5×10^{-5} M, were incubated with the enzyme before addition of substrates, serine synthesis was inhibited about 35%. If the inactivated enzyme was first incubated with pyridoxal phosphate for 15 min. and then deoxypyridoxine phosphate added and the mixture kept a further 15 min. at 37° before addition of substrates, inhibition was considerably less at all concentrations of deoxypyridoxine phosphate than in the former case. Very marked inhibition was produced if the procedure was reversed, i.e. the inactivated enzyme was incubated for 15 min. with deoxypyridoxine phosphate before the 15 min.

reactivation with pyridoxal phosphate. In this case 67 % inhibition resulted when pyridoxal phosphate and deoxypyridoxine phosphate were both added at a concentration of 5×10^{-5} M. With the last procedure 50 % inhibition was produced by about 2×10^{-5} M deoxypyridoxine phosphate.

Pyridoxine phosphate was a weaker inhibitor of the enzyme than deoxypyridoxine phosphate (Fig. 4). Even when incubated with the enzyme before reactivation by pyridoxal phosphate an inhibitor concentration of 2×10^{-4} M produced only 25 % inhibition.

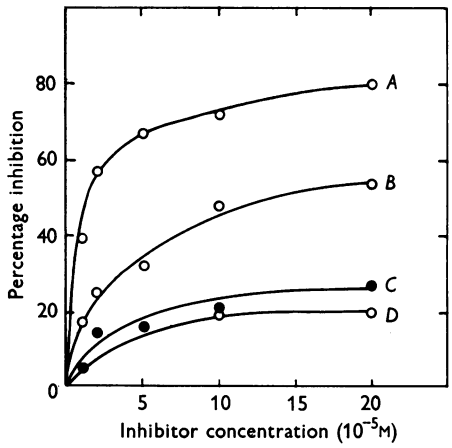

Fig. 4. Inhibition of serine synthesis by deoxypyridoxine phosphate or pyridoxine phosphate. Enzyme, 20–25 units/flask, inactivated by incubating at pH 9·1 and 37° for 30 min. Curve *A*: inactivated enzyme incubated with various concentrations of deoxypyridoxine phosphate for 15 min. at 37° and pH 7·2; pyridoxal phosphate then added and the mixture incubated for a further 15 min. before adding substrates. Curve *B*: inactivated enzyme incubated with both pyridoxal phosphate and various concentrations of deoxypyridoxine phosphate for 15 min. at 37° and pH 7·2 before adding substrates. Curve *C* (●): as for curve *A*, but pyridoxine phosphate used instead of deoxypyridoxine phosphate. Curve *D*: inactivated enzyme incubated with pyridoxal phosphate for 15 min. at 37° and pH 7·2; various concentrations of deoxypyridoxine phosphate then added and the mixture incubated a further 15 min. before adding substrates. Activity tested under standard conditions in all cases. Pyridoxal phosphate concentration 5×10^{-5} M in all cases.

Effect of cations on serine synthesis

In previous studies none of the bivalent cations tested was found to stimulate serine–glycine interconversion, and ethylenediaminetetraacetate (0·01 M) did not inhibit the reaction (Blakley, 1954*d*). This was interpreted as evidence against the participation of multivalent cations in the reaction. Studies on a non-enzymic cleavage of serine to glycine and formaldehyde with pyridoxal as catalyst have demonstrated, however, that in this non-enzymic reaction the presence of certain cations is essential (Metzler, Longenecker & Snell, 1954). On this and other evidence a general mechanism has been proposed for the action of pyridoxal and related compounds in both enzymic and non-enzymic reactions (Metzler, Ikawa & Snell, 1954), and in the proposed scheme the chelation of a cation plays an important part.

In view of the discrepancy between results with the enzymic and non-enzymic systems respectively, the effect of cations on the partially purified enzyme system has been investigated. The results, shown in Table 6, are in agreement with previous

Table 6. *Effect of cations on the enzymic synthesis of serine*

Serine synthesis measured under standard conditions either with purified enzyme, 10–20 units/flask, in the presence of 5×10^{-4} M pyridoxal phosphate or with the same enzyme after inactivation at pH 9, 37° for 15 min. and reactivation with 5×10^{-5} M pyridoxal phosphate at 37°, pH 7·2 for 15 min. Metals added as chlorides except Fe^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+} and Al^{3+}, which were added as sulphates, and Zn^{2+}, which was added as acetate.

Cation added (0·001 M)	Inhibition (%)	
	Normal enzyme	Reactivated enzyme
Cu^{2+}	96	95
Zn^{2+}	87	84
Ni^{2+}	99	73
Mn^{2+}	39	78
Cd^{2+}	71	39
Co^{2+}	61	38
Fe^{2+}	62	19
Fe^{3+}	41	2
Al^{3+}	33	0
Mg^{2+}	9	16
Ca^{2+}	8	0

findings, for, while they demonstrate inhibition by many cations in varying degree, none of the cations tested was an activator of enzymic serine synthesis either with the normal or reactivated enzyme. Among cations showing an inhibitory effect, Cu^{2+}, Zn^{2+}, Mn^{2+} and Ni^{2+} showed particularly strong effects. Ethylenediaminetetraacetate (0·01 M) was again found to produce no inhibition of serine synthesis either with normal or reactivated enzyme.

Spectrophotometric evidence for Schiff's-base formation

Schiff's-base formation between pyridoxal phosphate and glycine is generally assumed to be the first step in serine synthesis. It was noticed during this work that addition of glycine to a solution of pyridoxal phosphate in bicarbonate buffer causes

a marked and rapid increase in the intensity of the yellow colour of the solution. Determination of the absorption spectrum of pyridoxal phosphate in the presence and absence of glycine revealed a marked alteration in the spectrum on addition of glycine, consistent with Schiff's-base formation (Fig. 5). A similar change in the absorption spectrum of a pyridoxal phosphate solution resulted when serine was added instead of glycine, the two absorption maxima of the new spectrum appearing at the same wavelengths and having almost identical extinctions at the same concentration of amino acid. Pyridoxal phosphate alone had a single band with maximum at 388 mμ. ($\epsilon_{max.}$ 5700). In the presence of 0·01 M amino acid, maxima appeared at 277 mμ. ($\epsilon_{max.}$ 5000) and 405 mμ. ($\epsilon_{max.}$ 5800); 0·1 M amino acid produced maxima at 279 mμ. ($\epsilon_{max.}$ 5700) and 413 mμ. ($\epsilon_{max.}$ 6600). Addition of glycine to a solution of pyridoxamine phosphate did not produce such a change in spectrum.

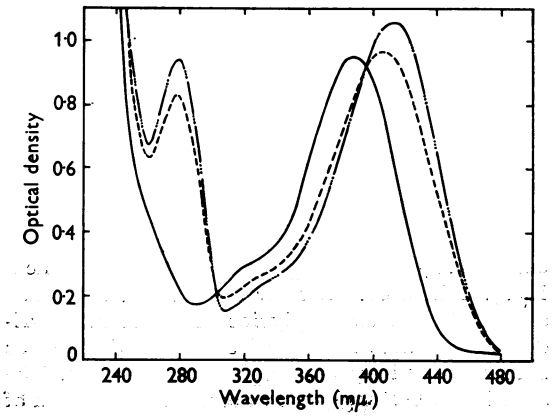

Fig. 5. Absorption spectra of pyridoxal phosphate ($1{\cdot}67 \times 10^{-4}$ M) in $NaHCO_3$–CO_2 buffer ($NaHCO_3$ 0·01 M, equilibrated with $N_2 + CO_2$ (95:5), pH 7·2). ——, Alone; – – –, in the presence of 0·01 M glycine, (adjusted to pH 7·2); —·—·—, in the presence of 0·10 M glycine (adjusted to pH 7·2).

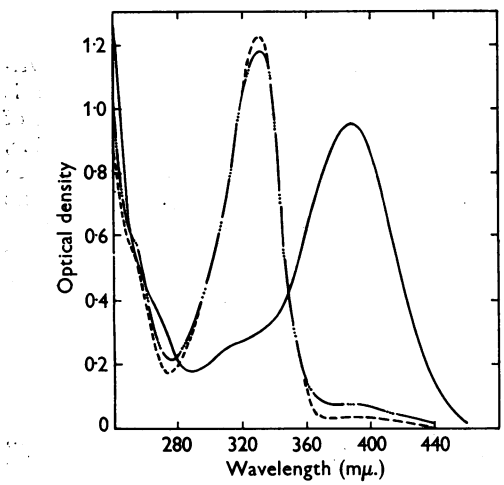

Fig. 6. Absorption spectra of pyridoxal phosphate ($1{\cdot}67 \times 10^{-4}$ M) in $NaHCO_3$ buffer (0·01 M, equilibrated with $N_2 + CO_2$ (95:5), pH 7·2). ——, Alone; – – –, in the presence of 0·0067 M-$NaHSO_3$; —·—·—, in the presence of 0·0067 M-$NaHSO_3$ and 0·01 M glycine (adjusted to pH 7·2).

When bisulphite was added to a pyridoxal phosphate solution the solution became colourless, the single absorption band shifting to 330 mμ. ($\epsilon_{max.}$ 7400) (Fig. 6). Addition of 0·01 M glycine to this solution caused only minor changes in the absorption spectrum (Fig. 6), providing further evidence that it was the Schiff's base which was formed between glycine and pyridoxal phosphate in the absence of bisulphite.

DISCUSSION

Pyridoxal phosphate has been shown to stimulate the synthesis of serine by enzyme preparations from rabbit-liver–acetone powder, the activating effect of pyridoxal phosphate becoming marked after ammonium sulphate fractionation of the enzyme (Table 2). The maximum effect of pyridoxal phosphate was obtained at a concentration of 5×10^{-4} M, which caused a doubling of the rate of serine synthesis (Table 3).

When the enzyme was maintained at about pH 9 and a temperature of 37° for 15–30 min. before testing its activity, the rate of serine synthesis was found to fall to a very low value. Such enzyme preparations were markedly reactivated by short incubation with pyridoxal phosphate before addition of substrates, 50% or more of the activity being recovered (Table 4). For this reactivation process pyridoxal phosphate produced half its maximum effect at about 2×10^{-6} M (Fig. 3). Investigation of the effect of pyridoxal phosphate on the breakdown of serine presents technical difficulties, but a stimulation of serine disappearance by added pyridoxal phosphate has been demonstrated.

These results are in good agreement with the observation that liver extracts from pyridoxine-deficient pigeons show a reduced ability to synthesize serine from formate (Deodhar & Sakami, 1953), and with the evidence that pyridoxal phosphate, together with PGA or a derivative, is essential for serine synthesis in *Streptococcus faecalis* (Lascelles & Woods, 1954).

The very slight activation of the unfractionated enzyme by added pyridoxal phosphate is most easily explained by assuming that the unfractionated enzyme possesses strongly bound pyridoxal

phosphate. Although no direct evidence has been adduced for this hypothesis, it is in agreement with all the results, and this has been shown for bacterial and mammalian transaminase (O'Kane & Gunsalus, 1947; Schlenk & Snell, 1945; Lichstein, Gunsalus & Umbreit, 1945). As with pig-heart transaminase (O'Kane & Gunsalus, 1947), fractionation of the serine-synthesizing enzyme system with ammonium sulphate increases the activation produced by added pyridoxal phosphate, although in these circumstances the activation by pyridoxal phosphate was not found to cause an increase in serine synthesis appreciably in excess of 100%.

The loss of enzymic activity at pH 9 and concomitant reactivation by added pyridoxal phosphate is presumably due to a modification, at mildly alkaline pH, of the linkages by which pyridoxal phosphate is bound by the enzyme. Even without removing the pyridoxal phosphate, presumed to have been released from the enzyme by treatment at pH 9, the enzymic activity was reduced to a very low figure in the absence of added pyridoxal phosphate. The enzyme cannot therefore recapture any but a small fraction of the released pyridoxal phosphate, even when the solution is again adjusted to pH 7·2. This is probably due to non-specific adsorption of the released pyridoxal phosphate by inert protein present in the relatively impure enzyme preparations used so far. It is noteworthy, however, that after the enzyme had been inactivated at pH 9·0 pyridoxal phosphate at a concentration as low as 2×10^{-6} M produced 50% of the maximum reactivation, indicating a high affinity of the enzyme for free pyridoxal phosphate.

It was desirable to test whether pyridoxal phosphate added to the inactivated enzyme became strongly bound at neutral pH. Experiments designed to test this point, in which enzyme reactivated with added pyridoxal phosphate was dialysed before its synthetic activity was tested in the presence and absence of added pyridoxal phosphate, were inconclusive. Large irreversible losses of enzymic activity occurred during dialysis and these obscured a possible loss of activity due to removal of free pyridoxal phosphate.

Serine synthesis by the reactivated enzyme was inhibited by deoxypyridoxine phosphate and pyridoxine phosphate in a manner parallel in many respects to the inhibition of transaminase by these compounds (Meister, Sober & Peterson, 1954). Thus deoxypyridoxine phosphate inhibits serine synthesis most strongly when the enzyme is incubated with the inhibitor before reactivation of the enzyme by pyridoxal phosphate, less markedly when the pyridoxal phosphate and inhibitor are added simultaneously to the enzyme, and only slightly when the enzyme is reactivated by pyridoxal phosphate before addition of inhibitor (Fig. 4).

The serine-synthesizing enzyme and transaminase appear to differ in their relative affinities for pyridoxal phosphate, deoxypyridoxine phosphate and pyridoxine phosphate respectively. Thus the serine-synthesizing enzyme is inhibited to a much greater extent by deoxypyridoxine phosphate than by pyridoxine phosphate (Fig. 4), whereas transaminase is inhibited similarly by these compounds (Meister *et al.* 1954). The affinity of the serine-synthesizing enzyme for deoxypyridoxine phosphate is very similar to its affinity for pyridoxal phosphate (Fig. 4), whereas transaminase has a considerably stronger affinity for pyridoxal phosphate.

Mechanism of the reaction

Schiff's-base formation between the 4-formyl group of pyridoxal phosphate and the α-amino group of amino acids is generally supposed to be involved in the mechanism of enzymic reactions catalysed by pyridoxal phosphate (Metzler & Snell, 1952). Direct evidence for the formation of such a compound between glycine or serine and pyridoxal phosphate in neutral aqueous solution has been provided by spectrophotometric data. The addition of 0·01 M glycine or serine to a solution of pyridoxal phosphate ($1{\cdot}67 \times 10^{-4}$ M) in bicarbonate buffer pH 7·2 caused a shift in the absorption band from 388 to 413 mμ. (Fig. 5). At the same time a new absorption band appeared with maximum at 279 mμ. A similar and somewhat greater effect was produced by addition of 0·10 M glycine or serine to the pyridoxal phosphate solution, but the change in spectrum was very largely complete at an amino acid concentration of 0·01 M.

The change in the absorption spectrum of pyridoxal phosphate in the presence of glycine is similar to that which occurs in the spectrum of salicylaldehyde on addition of ethylenediamine, where a shift of an absorption band from 324 to 408 mμ. occurs (Kiss & Auer, 1941). In both cases it may be presumed that Schiff's-base formation causes the shift of the absorption band towards the visible by providing a double bond conjugated with the unsaturated ring system. Further evidence that glycine and pyridoxal phosphate form a Schiff's base was provided by the fact that bisulphite, by suppressing resonance between the aldehyde group and the ring, shifted the absorption band of pyridoxal phosphate from 388 to 330 mμ., and that in the presence of 0·0067 M bisulphite 0·01 M glycine produced no further change in the spectrum of pyridoxal phosphate (Fig. 6).

In recent discussions of the mechanism of pyridoxal phosphate-catalysed enzyme reactions it has been proposed that Schiff's bases formed by pyridoxal phosphate and amino acids are stabilized by

chelating a multivalent cation (Baddiley, 1952; Metzler, Ikawa & Snell, 1954), the chelate ring maintaining planarity of the conjugate ring system. In addition, it has been suggested that the chelated metal ion acts by providing an additional electron-attracting group that operates in the same direction as the nitrogen atom of the pyridyl ring, thus increasing electron displacements from the α-carbon atom of the amino acid (Metzler, Ikawa & Snell, 1954). On the other hand, enzymic synthesis of serine was not stimulated by any of the cations tested with either normal or reactivated enzyme. Ca^{2+} and Mg^{2+} had no significant effect; Co^{2+}, Fe^{2+}, Al^{3+} and Fe^{3+} were slightly inhibitory; and Zn^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+} and Ni^{2+} produced marked inhibition (Table 6). Ethylenediaminetetraacetate (0·01 M) caused no inhibition. Similarly, purified transaminase appears not to require cations for activity (Meister *et al.* 1954).

The view that chelation of a metal ion is essential to enzymic reactions involving pyridoxal phosphate is perhaps tenable, despite failure of added cations to stimulate serine synthesis, on the assumption that a metal is an integral part of the enzyme. Evidence for this must await further purification of the enzyme. Alternatively, it is conceivable that the binding of pyridoxal phosphate and the amino acid to the enzyme surface is sufficient to maintain planarity of the system, and that interaction of the carboxyl group, phenolic hydroxyl, amino nitrogen atom and pyridyl nitrogen respectively with groups on the enzyme surface is even more favourable than chelation of a cation to the requisite electromeric displacement of electrons.

SUMMARY

1. A manometric method is presented for the estimation of the activity of the enzyme system synthesizing serine from glycine and formaldehyde.

2. The enzyme system has been partially purified by heat treatment, ammonium sulphate fractionation and ethanol fractionation.

3. During enzyme fractionation the requirement for pyridoxal phosphate increased. A twofold increase in the activity of the partially purified enzyme was produced by addition of 5×10^{-4} M pyridoxal phosphate to the reaction mixture.

4. Enzyme incubated at about pH 9 and 37° for 15–30 min. was largely inactivated, but a substantial proportion of the activity could be recovered by incubating the enzyme with pyridoxal phosphate at pH 7·2 for a short period.

5. Half the maximum reactivation was produced by 10^{-6} M pyridoxal phosphate. Pyridoxamine phosphate, pyridoxine phosphate and deoxypyridoxine phosphate produced negligible reactivation of the enzyme.

6. Deoxypyridoxine phosphate and, to a lesser extent, pyridoxine phosphate inhibited the reactivated enzyme, particularly when added to the inactivated enzyme before its reactivation with pyridoxal phosphate.

7. Ethylenediaminetetraacetate (0·01 M) did not inhibit serine synthesis by either normal or reactivated enzyme. Bi- and ter-valent cations did not stimulate serine synthesis; on the contrary, many cations were found to be strong inhibitors.

8. Spectrophotometric evidence is presented for the formation of Schiff's bases between pyridoxal phosphate and amino acids in neutral aqueous solution. These results are discussed in relationship to proposed mechanisms of enzymic reactions involving pyridoxal phosphate.

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