Studies on the Enzymic Degradation of L-Serine O-Sulphate by a Rat Liver Preparation

By J. H. THOMAS AND N. TUDBALL

Department of Biochemistry, University College, St Andrew's Place, Cardiff

(Received 21 April 1967)

1. The enzyme system of rat liver responsible for the degradation of L-serine O-sulphate was purified 300-fold and the optimum conditions for the activity were determined. 2. Inorganic sulphate, pyruvate and ammonia were found to be the products of enzyme action on L-serine O-sulphate, being formed in equivalent amounts under all conditions examined. No free L-serine was detected as a product of enzyme action. 3. The enzyme preparation was free from other serine-metabolizing systems such as O-phospho-L-serine phosphatase and L-serine dehydratase. 4. The enzyme has a very narrow substrate specificity and is inactive towards a wide variety of related sulphate esters and amino acids. 5. Pyridoxal 5'-phosphate is capable of catalysing the non-enzymic breakdown of L-serine O-sulphate in the presence of metal salts to yield inorganic sulphate, pyruvate and ammonia as products. 6. The possible role of pyridoxal 5'-phosphate as a coenzyme in the enzymic degradation of L-serine O-sulphate is discussed.

Previous studies in these Laboratories (Dodgson, Lloyd & Tudball, 1961) have revealed the presence in rat liver of a non-particulate enzyme system capable of liberating inorganic [^{35}S]sulphate from L-serine O[^{35}S]-sulphate. Other studies (Dodgson & Tudball, 1961; Harada, 1964; Noda, 1965) have shown that the enzyme system is widely distributed in Nature. Evidence has been obtained that the enzyme can act effectively *in vivo*, since the injection into rats of free L-serine O[^{35}S]-sulphate and of dipeptides containing the labelled sulphate ester is followed by the appearance of large amounts of the radioactivity in the urine as inorganic [^{35}S]-sulphate (Tudball, 1962; Tudball, Noda & Dodgson, 1964, 1965).

Information about the mechanism whereby L-serine O-sulphate is desulphated is very limited and Dodgson *et al.* (1961) have pointed out that the process may not necessarily be due to a simple hydrolase. To obtain further information about the mechanism of desulphation the rat-liver enzyme has now been considerably purified and a study made of its properties.

A preliminary account of this work has already been published (Tudball & Thomas, 1965).

MATERIALS AND METHODS

Chemicals. The O-sulphate esters of L-serine, D-serine, DL- α -methylserine, DL-homoserine, ethanolamine, glycollic acid and 3-hydroxypropionic acid were prepared as potassium salts by sulphation of the parent compounds with conc.

H_2SO_4 under the conditions described by Tudball (1962) for the O-sulphation of L-serine.

Potassium L-threonine O-sulphate and potassium L-hydroxyproline O-sulphate were prepared by the method of Dodgson *et al.* (1961). Potassium glycyl-L-serine O-sulphate was prepared according to the directions of Tudball *et al.* (1964), and the potassium salts of the sulphate esters of L-seryl-glycine, L-seryl-L-alanine, L-seryl-L-leucine, L-seryl-L-valine and L-seryl-L-phenylalanine were prepared by the method of Tudball *et al.* (1965). Paper-chromatographic analysis of the sulphate esters prepared showed that the preparations were homogeneous and free of the parent compounds. The infrared spectra of the sulphate esters were marked by the appearance of strong absorption bands in the ranges 1210–1250 and 770–810 cm^{-1} , characteristic of the spectra obtained with a series of alkyl sulphates (Lloyd, Tudball & Dodgson, 1961). There was no absorption at 3320 cm^{-1} , a region ascribed to vibrations involving the unsubstituted hydroxyl group.

L-Serine N-sulphate was a gift from Dr G. Embury, β -chloro-L-alanine was prepared from L-serine methyl ester hydrochloride by the method of Fischer & Raske (1907) and O-acetyl-L-serine by the method of Sakami & Toennies (1942). O-Phospho-L-serine was purchased from Sigma (London) Chemical Co., London, S.W. 6, and O-methyl-DL-serine from Mann Research Laboratories Inc., New York, N.Y., U.S.A.

Experimental animals. Male M.R.C. hooded rats, 2.5–4 months old, were used throughout.

Analytical methods. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) and (for stages 6 and 7 of the enzyme purification) by the spectrophotometric procedure of Warburg & Christian (1941). Sulphate was measured by the method of Dodgson (1961) and keto acids by the procedure of Friedemann & Haugen

(1943) as modified by Sayre & Greenberg (1956). Serine was determined by the chromotropic acid method of Frisell & Mackenzie (1958) and ammonia by the boric acid-HCl micro-diffusion method of Conway (1962).

Assay of the desulphating enzyme system. The assay mixture contained 0.1 ml. of suitably diluted enzyme solution in a total volume of 0.22 ml. containing a final concentration of 0.05 M-L-serine O-sulphate, 5.0 mM-2-mercaptoethanol and 0.1 M-tris that had been adjusted to pH 7.0 with 2 N-HCl. After incubation at 37° for 40 min. the reaction was stopped by adding 30 μ l. of 25% (w/v) trichloroacetic acid; precipitated protein was removed by centrifuging at 1000g for 10 min. at 4° and a portion (200 μ l.) of the supernatant used for determination of liberated SO_4^{2-} ion. Suitable control determinations were made in which L-serine O-sulphate was added only at the end of the incubation period. Preliminary experiments established that the rate of SO_4^{2-} ion liberation was linearly proportional to the enzyme concentration under the conditions described for enzyme assay.

A unit of enzyme activity is defined as that amount of enzyme which liberates 1 μ mole of SO_4^{2-} ion from L-serine O-sulphate in 1 hr. under the above conditions. The specific activity of the enzyme is expressed as the number of enzyme units/mg. of protein.

Assay of other enzyme activities. O-Phospho-L-serine phosphatase (phosphoserine phosphohydrolase, EC 3.1.3.3) was assayed as described by Borkenhagen & Kennedy (1959). L-Serine dehydratase [L-serine hydro-lyase (deaminating), EC 4.2.1.13] activity was measured by the method of Selim & Greenberg (1959). L-Threonine dehydratase [L-threonine hydro-lyase (deaminating), EC 4.2.1.16] was measured by the procedure of Sayre & Greenberg (1956), DL-homoserine dehydratase [DL-homoserine hydro-lyase (deaminating), EC 4.2.1.15] by the method of Matsuo & Greenberg (1958), cystathione synthetase [L-serine hydro-lyase (adding L-homocysteine), EC 4.2.1.21] by the method of Selim & Greenberg (1959) and cysteine desulphhydrase [L-cysteine hydrogen sulphide-lyase (deaminating), EC 4.4.1.1] by determining liberated pyruvate by employing the incubation conditions described by Smythe (1955).

EXPERIMENTAL AND RESULTS

Purification of the enzyme

Unless otherwise stated, all extraction and purification steps were carried out at 4°. The buffer used throughout was 0.1 M-tris, adjusted to pH 7.0 with 2 N-hydrochloric acid and containing 5.0 mM-2-mercaptoethanol. All centrifuging operations were carried out at 0°.

Stage 1. Rats were killed by dislocation of the neck. Livers were removed as quickly as possible and cooled in crushed ice for 5 min. before being dried by blotting. The livers (usually 150 g. total wt.), were then finely diced before macerating (MSE top-drive macerator for 3 min. at half-speed) in sufficient 1.2% (w/v) potassium chloride, containing 5.0 mM-EDTA, to give a final concentration of liver suspension of 15% (w/v).

Stage 2. The crude suspension from stage 1 was

clarified by centrifuging at 10000g for 30 min. and the precipitate was discarded.

Stage 3. The supernatant was transferred in batches (approx. 220 ml./batch) to a 500 ml. round-bottomed flask equipped with a thermometer, and the flask was swirled in a water bath at 55° until the temperature of the preparation reached 50°. The flask and contents were then placed in a second water bath at 50° for 10 min. After being cooled rapidly to 10–15° in an ice bath the suspension was centrifuged for 20 min. at 4000g and the precipitate discarded.

Stage 4. The supernatants from the several batches were combined and the whole adjusted to pH 7.0 with 2 N-ammonia before adding sufficient ammonium sulphate (mechanical stirring) to give a 30% saturated solution (at 4°). The pH was maintained at 7.0 by the addition of 2 N-ammonia. After 3 hr. the precipitate was removed by centrifuging at 6000g for 20 min. and sufficient ammonium sulphate was added to the supernatant to give a 50% saturated solution. After 3 hr. the dark-yellow precipitate was separated by centrifuging, dissolved in the minimum volume of the pH 7.0 buffer and the solution dialysed for 16 hr. against two changes of the same buffer (total volume 12 l.).

Stage 5. Calcium phosphate gel was prepared according to the method of Keilin & Hartree (1938) and was stored in the dark for 1 month before use. The gel was added to the stage 4 preparation (1.75 g. dry wt. of gel/100 ml. of preparation containing approx. 3.5 g. of protein) and the mixture was stirred mechanically for 30 min. before centrifuging at 8000g for 10 min. to remove the gel. The gel procedure was then repeated on the clear supernatant, the gel again removed by centrifuging and the clear supernatant stored at –20°.

Stage 6. The volume of the stage 5 supernatant (containing approx. 2.0 mg. of protein/ml.) was reduced tenfold by means of the LKB 6300 A Ultrafilter (LKB-Produkter A.B., Stockholm, Sweden) and applied to a column (60 cm. $\times$ 3.6 cm.) of Sephadex G-200 that had been equilibrated with the pH 7.0 buffer. The column was developed with the same buffer at a flow rate of 30 ml./hr. and the eluate was collected in 5 ml. fractions. Fractions were assayed for protein and enzyme activity and those containing the bulk of the enzyme activity (usually in the range fractions 60–85) were combined and concentrated threefold by ultrafiltration.

Stage 7. DEAE-Sephadex A-50 (medium grade) was allowed to swell in a large excess of 0.01 M-tris-hydrochloric acid buffer, pH 7.0 (containing a final concentration of 5.0 mM-2-mercaptoethanol), for 24 hr., the buffer being renewed three times. A column (10 cm. $\times$ 2 cm.) of the DEAE-Sephadex was equilibrated with the buffer. The stage 6 preparation was dialysed for 12 hr. against two

Table 1. *Purification of the L-serine O-sulphate-degrading system from rat liver*

Stage	Vol. (ml.)	Total protein (mg.)	Total activity (units)	Recovery (%)	Sp. activity (units/mg. of protein)	Purification	O-Phospho- L-serine phosphatase activity (units)
(1) 15% (w/v) homogenate	825	56760	17080	100	0.30	1	3423
(2) High-speed supernatant	680	30192	16420	97	0.55	1.83	3264
(3) 50°-treated extract	665	20320	15130	89	0.74	2.46	3111
(4) (NH ₄) ₂ SO ₄ precipitate (30–50% saturation)	210	6174	10530	62.3	1.73	5.76	924
(5) Ca ₃ (PO ₄) ₂ gel adsorption	225	468	8325	48.4	17.6	58.6	63
(6) Gel filtration (Sephadex G-200 fractions 64–84)	240	168	5530	32.0	35.3	117	—
(7) DEAE-Sephadex (fractions 96–106)	24	21	1860	10.9	88.5	295	—

changes (total volume 41.) of the buffer before being applied to the column. Stepwise elution of the column was achieved by washing with 75 ml. of buffer followed by successive 75 ml. portions of the buffer containing the following concentrations of sodium chloride: 0.025 M, 0.05 M, 0.15 M and 0.45 M. A flow-rate of 30 ml./hr. was maintained by means of a peristaltic pump and the eluate was collected in 2.5 ml. fractions. The enzyme was eluted when the sodium chloride concentration had reached 0.15 M. Those fractions (usually nos. 95–105) containing enzyme of the greatest specific activity were pooled and the enzyme was precipitated by adding ammonium sulphate to give a final concentration equivalent to 70% saturation. After keeping for 3 hr. the precipitate was separated by centrifuging, dissolved in the minimum volume of pH 7.0 buffer and dialysed against the same buffer until free of SO₄²⁻ ions. The volume of the diffusate was then adjusted to give a protein concentration of approx. 2 mg./ml. and the whole was stored at –20°. Under these conditions the enzyme remained stable for several weeks.

In a typical experiment (see Table 1) a 295-fold purification of the enzyme was achieved by this procedure, 11% of the total original activity being recovered.

Separation of O-phospho-L-serine phosphatase activity. During their early studies on the L-serine O-sulphate-desulphating enzyme, Dodgson & Tudball (1961) pointed to the possibility that the enzyme might be identical with O-phospho-L-serine phosphatase. This possibility may now be eliminated since the phosphatase is almost totally removed at the calcium phosphate gel stage (Table 1), being readily adsorbed on to the gel, leaving the L-serine O-sulphate-degrading activity in solution. The phosphatase could be recovered from the gel by

washing with 0.5 M-ammonium sulphate (see Borkenhagen & Kennedy, 1959).

Optimum conditions for desulphating activity

Prolonged dialysis (4 days) of the purified enzyme preparation against 0.1 M-tris, adjusted to pH 7.0 with 2 N-hydrochloric acid, resulted in an appreciable loss of activity, but addition of 2-mercaptoethanol (to give a final concentration of 0.1 mM) prevented this loss. A study of the effect of 2-mercaptoethanol on enzyme activity was therefore made and revealed that maximum activity was obtained when 2-mercaptoethanol was present in the incubation mixtures at a final concentration of 5.0 mM; 2-mercaptoethanol was subsequently included in all incubation mixtures.

The system showed optimum activity with a final substrate concentration of 0.05 M and at pH 7.0 in the presence of 0.1 M-tris, adjusted to pH 7.0 with 2 N-hydrochloric acid, and 5.0 mM-2-mercaptoethanol. The K_m for the reaction, calculated from the double-reciprocal plot of Lineweaver & Burk (1934), was 2.3×10^{-3} M.

Identification of the products of the enzymic degradation of L-serine O-sulphate

By analogy with the sulphatase group of enzymes (sulphohydrolases, EC 3.1.6.–) the enzyme catalysing the desulphation of L-serine O-sulphate should yield L-serine and inorganic SO₄²⁻ ions as products if the enzyme is a simple hydrolase. However, an extensive search for L-serine as a reaction product was unsuccessful, in spite of the fact that added L-serine could be recovered quantitatively from the incubation mixtures.

Pyruvate was subsequently identified as a product of the enzymic degradation as follows. The

enzyme (2.0 ml.) was incubated for 3 hr. at 37° with an equal volume of a 0.1 M solution of L-serine O-sulphate in the usual tris buffer, pH 7.0, and 200 μ l. of a solution of 0.1 M-2-mercaptoethanol in the same buffer. Enzyme action was stopped by adding 0.6 ml. of 25% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifuging and 8 ml. of a 0.2% (w/v) solution of 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid was added to the supernatant. The solution was left for 20 min. at 25° and the precipitated hydrazone was separated by filtration. After recrystallization from ethyl acetate, 2.4 mg. of the hydrazone was obtained. The hydrazone gave a melting point (214–215°) and mixed melting point identical with that of authentic pyruvate 2,4-dinitrophenylhydrazone. Paper-chromatographic mobilities in butan-1-ol-ethanol-0.5N-ammonia (7:1:2, by vol.) were also identical.

The demonstration that pyruvate and sulphate are products of the enzymic degradation of L-serine O-sulphate implies that the reaction also involves a deamination. Ammonia was detected as the other product with the aid of Conway micro-diffusion units.

The results of an experiment designed to compare the quantitative production of sulphate, pyruvate and ammonia from L-serine O-sulphate are given in Table 2. Under all conditions employed these three products were found in equivalent amounts. No apparent time-lag occurred between the appearance of pyruvate, ammonia and sulphate, suggesting that deamination occurs concomitantly with sulphate release.

Table 2. *Formation of pyruvate, sulphate and ammonia from L-serine O-sulphate by the purified rat liver enzyme*

A series of incubation mixtures (total vol. 0.8 ml.) was set up. The final concentrations of the reactants were 0.5 M-L-serine O-sulphate, 5.0 mM-2-mercaptoethanol, 0.1 M-tris-HCl buffer, pH 7.0, and 120 μ g. of enzyme protein. Incubations were at 37° for various times. The reaction was stopped by the addition of 200 μ l. of 25% (w/v) trichloroacetic acid and portions (200 μ l.) of the deproteinized supernatant were assayed for the products liberated.

Time (min.)	Products liberated (μ moles/200 μ l. of deproteinized incubation mixtures)		
	Pyruvate	SO ₄ ²⁻	NH ₄ ⁺
5	0.10	0.08	0.09
10	0.17	0.16	0.16
15	0.25	0.23	0.25
30	0.46	0.45	0.41
45	0.64	0.63	0.61
60	0.80	0.74	0.78

The production of pyruvate from L-serine O-sulphate under normal conditions was identical with the activity observed when experiments were performed anaerobically in the presence of nitrogen, which excludes the possibility of involvement of an amino acid oxidase.

Substrate specificity

The ability of the enzyme to utilize compounds related to L-serine O-sulphate was examined. Enzyme activity towards sulphate esters was determined by measuring liberated SO₄²⁻ ion, except with DL-homoserine O-sulphate, when the amount of keto acid liberated was determined. This was necessary since DL-homoserine O-sulphate forms an insoluble barium salt that interferes with the estimation of sulphate. When non-sulphated substrates were used, enzyme activity was determined by measuring liberated keto acid, except with O-phospho-L-serine, when inorganic PO₄³⁻ ions were determined.

The results (Table 3) suggest that the enzyme has a relatively high specificity. All compounds that could serve as substrates, with the exception of L-threonine O-sulphate and β -chloro-L-alanine, contained a L-serine O-sulphate residue with a free amino group. The enzyme is apparently stereo-

Table 3. *Substrate specificity of the L-serine O-sulphate-degrading system of rat liver*

The final concentration of substrate was 0.05 M. Details of the assay method are given in the text.

Substrate	Product liberated (μ moles)
L-Serine O-sulphate	1.08
D-Serine O-sulphate	0.03
DL- α -Methylserine O-sulphate	0.00
Glycyl-L-serine O-sulphate	0.00
L-Serylglycine O-sulphate	0.25
L-Seryl-L-alanine O-sulphate	0.30
L-Seryl-L-valine O-sulphate	0.12
L-Seryl-L-leucine O-sulphate	0.05
L-Seryl-L-phenylalanine O-sulphate	0.01
L-Threonine O-sulphate	0.02
L-Hydroxyproline O-sulphate	0.00
DL-Homoserine O-sulphate	0.00
Ethanolamine O-sulphate	0.00
Glycollic acid sulphate	0.00
3-Hydroxypropionic acid sulphate	0.00
L-Serine N-sulphate	0.00
O-Methyl-DL-serine	0.00
O-Acetyl-L-serine	0.00
O-Phospho-L-serine	0.00
β -Chloro-L-alanine	1.37
L-Serine	0.00
L-Threonine	0.00
DL-Homoserine	0.00

specific, since only feeble activity was shown towards D-serine O-sulphate. The products resulting from the action of the enzyme on β -chloro-L-alanine were established as being pyruvate, ammonia, and chloride.

Effect of inhibitors

The effects of possible inhibitors on enzyme activity were tested, particularly those of thiol-specific agents in view of the protection afforded to the enzyme by 2-mercaptoethanol. Carbonyl reagents were also examined, since they are known to have marked inhibitory effects on many pyridoxal 5'-phosphate-dependent systems. The enzyme preparation and inhibitor were preincubated together for various times before the addition of L-serine O-sulphate. Inhibition was practically complete with 1.0 mM *p*-chloromercuribenzoate or 1.0 mM iodoacetic acid (Table 4). Inhibition by 0.1 mM *p*-chloromercuribenzoate could be partly abolished by addition of 5.0 mM 2-mercaptoethanol, after removal of excess of *p*-chloromercuribenzoate by dialysis. The carbonyl reagents hydroxylamine, hydrazine, cyanide and isonicotinic acid hydrazide (isoniazid) had little or no effect on enzyme activity. D-Serine O-sulphate and DL-homoserine O-sulphate acted as weak competitive inhibitors, whereas O-phospho-L-serine and O-acetyl-L-serine acted as potent non-competitive inhibitors.

Effect of pyridoxal 5'-phosphate on enzyme activity

The finding that pyruvate and ammonia are products of the enzymic desulphation of L-serine O-sulphate could indicate that the mechanism involves an $\alpha\beta$ -elimination reaction. The enzyme preparation was thus examined for enzymes catalysing $\alpha\beta$ -elimination reactions of amino acids related to L-serine O-sulphate to check whether the activity towards this ester could be attributed to an enzyme system already well documented. Results showed the preparation to be free from the activities of L-serine dehydratase, L-threonine dehydratase, DL-homoserine dehydratase, cystathionine synthetase and L-cysteine desulphhydrase.

All known enzyme systems catalysing $\alpha\beta$ -elimination reactions of amino acids require pyridoxal 5'-phosphate as a coenzyme (see Meister, 1965). It became apparent during these investigations that pyridoxal 5'-phosphate was able to bring about the non-enzymic degradation of L-serine O-sulphate, yielding the same products obtained in the enzyme-catalysed reaction. Thus when pyridoxal 5'-phosphate (at concentrations of 1.0 mM, or greater) was incubated with L-serine O-sulphate at 37° in the presence of cupric chloride (0.2 mM) at pH values varying from 6.5 to 8.5, inorganic SO_4^{2-} , pyruvate and ammonia were produced.

Table 4. *Inhibition of the L-serine O-sulphate-degrading enzyme*

The enzyme was assayed as described in the text after prior incubation at 37° with the compounds listed.

Added compound	Concn. (mM)	Pre-incubation time (min.)	Inhibition (%)
<i>p</i> -Chloromercuribenzoic acid	0.01	10	18
	0.1	10	61
	1	10	94
Iodoacetic acid	0.01	30	17
	0.1	30	76
	1	30	95
Isonicotinic acid hydrazide	0.1	15	0
	1	15	0
	10	15	0
KCN	0.1	15	0
	1	15	0
	10	15	0
Hydroxylamine hydrochloride	0.1	15	8
	1	15	13
Hydrazine hydrochloride	0.1	15	0
	1	15	15
O-Phospho-L-serine	5	0	44
	10	0	98
O-Acetyl-L-serine	4	0	53
	10	0	96
O-Methyl-DL-serine	10	0	0
D-Serine O-sulphate	10	0	19
DL-Homoserine O-sulphate	10	0	11

The ability of pyridoxal 5'-phosphate to activate the enzymic degradation of L-serine O-sulphate was examined. Pyridoxal 5'-phosphate, at final concentrations from 0.01 to 10 mM, was incorporated into the usual incubation mixture at pH values ranging from 6.5 to 8.5. However, no significant increase in activity was obtained under any of the conditions used.

DISCUSSION

The products of the enzymic degradation of L-serine O-sulphate have been established as pyruvate, sulphate and ammonia, implying that the enzyme is not a simple sulphotase. The possibility that pyruvate and ammonia could have arisen from L-serine liberated from the parent sulphate ester is negated by the fact that added L-serine is not degraded by the enzyme system. Moreover, the three products of enzyme action seem to be produced simultaneously.

The finding that the enzyme preparation shows activity towards β -chloro-L-alanine is noteworthy in that, in common with L-serine O-sulphate, this substrate possesses a large electronegative group on

the β -carbon atom. At least two reports of the presence in rat liver of an enzyme able to degrade β -chloro-L-alanine to pyruvate, ammonia and chloride have been made (Greggerman & Christensen, 1956; Nagabhushanam & Greenberg, 1965). However, these rat-liver systems are not specific for β -chloro-L-alanine but contain homoserine dehydratase (Greggerman & Christensen, 1956), cystathionine synthetase, L-serine dehydratase and L-threonine dehydratase activities (Nagabhushanam & Greenberg, 1965). These other activities have been shown to be absent from the purified enzyme preparation employed in the present work.

L-Serine O-sulphate can be degraded enzymically and non-enzymically, the latter process being catalysed by pyridoxal 5'-phosphate and metal ions. Pyruvate, ammonia and sulphate are formed in each case. The non-enzymic breakdown of serine and its O-esters by pyridoxal and metal ions giving rise to pyruvate, ammonia and an anion has been known for many years (Metzler & Snell, 1952; Longenecker & Snell, 1957), and the degradation of L-serine O-sulphate appears to be consistent with the mechanism proposed for these former degradations. The question arises whether the enzymic process requires pyridoxal 5'-phosphate as coenzyme. Addition of pyridoxal 5'-phosphate to incubation mixtures under a variety of conditions failed to affect enzyme activity in any way. Further, several known carbonyl reagents failed to inhibit enzyme activity. However, the former observations might merely indicate that one or more pyridoxal 5'-phosphate molecules are firmly covalently bound to the enzyme molecule, whereas the effectiveness of carbonyl reagents as inhibitors depends on the type of bonding involved between the coenzymes and protein (e.g. see Meister, 1965). At the present time therefore it is not possible to state with certainty whether pyridoxal 5'-phosphate is involved in the enzymic reaction.

It has been suggested (Tudball *et al.* 1964, 1965) that the inorganic [^{35}S]sulphate that is liberated *in vivo* after the injection of dipeptides containing L-serine O[^{35}S]sulphate originates from free L-serine O[^{35}S]sulphate, liberated as a result of dipeptidase action. This must certainly be true for glycyl-L-serine O-sulphate, which has now been shown to be incapable of acting as a substrate for the desulphating enzyme.

Finally, no obvious physiological function can yet be assigned to the enzyme system. However, the present work clearly disposes of the early suggestion of Tudball (1962) that the enzyme might be involved in maintaining the integrity of serine hydroxyl groups in proteins by protecting such groups from biological sulphation. Clearly, such an enzyme would need to be a simple sulphatase show-

ing high activity towards peptide-bound L-serine O-sulphate residues.

This work was supported by a grant to Professor K. S. Dodgson, of this Department, from the U.S. Public Health Service (Grant no. AM01982 from the Arthritis and Metabolic Diseases Division). J. H. T. is grateful to the Medical Research Council for a studentship.

REFERENCES

- Borkenhagen, L. F. & Kennedy, E. P. (1959). *J. biol. Chem.* **234**, 849.
- Conway, E. G. (1962). *Microdiffusion Analysis and Volumetric Error*, 5th ed., p. 98. London: Crosby, Lockwood and Son Ltd.
- Dodgson, K. S. (1961). *Biochem. J.* **78**, 312.
- Dodgson, K. S., Lloyd, A. G. & Tudball, N. (1961). *Biochem. J.* **79**, 111.
- Dodgson, K. S. & Tudball, N. (1961). *Biochem. J.* **81**, 68.
- Fischer, E. & Raske, K. (1907). *Ber. deutsch. chem. Ges.* **40**, 3717.
- Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Frisell, W. R. & Mackenzie, C. G. (1958). *Meth. biochem. Anal.* **6**, 63.
- Greggerman, R. I. & Christensen, H. N. (1956). *J. biol. Chem.* **220**, 765.
- Harada, T. (1964). *Biochim. biophys. Acta*, **81**, 193.
- Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc. B*, **124**, 397.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Lloyd, A. G., Tudball, N. & Dodgson, K. S. (1961). *Biochim. biophys. Acta*, **52**, 413.
- Longenecker, J. B. & Snell, E. E. (1957). *J. biol. Chem.* **225**, 409.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Matsuo, Y. & Greenberg, D. M. (1958). *J. biol. Chem.* **230**, 545.
- Meister, A. (1965). *Biochemistry of the Amino Acids*, vol. 1, p. 319. New York: Academic Press Inc.
- Metzler, D. E. & Snell, E. E. (1952). *J. biol. Chem.* **198**, 353.
- Nagabhushanam, A. & Greenberg, D. M. (1965). *J. biol. Chem.* **240**, 3002.
- Noda, Y. (1965). *J. Biochem., Tokyo*, **57**, 709.
- Sakami, W. & Toennies, G. (1942). *J. biol. Chem.* **144**, 203.
- Sayre, F. W. & Greenberg, D. M. (1956). *J. biol. Chem.* **220**, 787.
- Selim, A. S. M. & Greenberg, D. M. (1959). *J. biol. Chem.* **234**, 1474.
- Smythe, C. V. (1955). In *Methods in Enzymology*, vol. 2, p. 315. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Tudball, N. (1962). *Biochem. J.* **85**, 456.
- Tudball, N., Noda, Y. & Dodgson, K. S. (1964). *Biochem. J.* **90**, 439.
- Tudball, N., Noda, Y. & Dodgson, K. S. (1965). *Biochem. J.* **95**, 678.
- Tudball, N. & Thomas, J. H. (1965). *Abstr. 2nd. Meet. Fed. Europ. biochem. Soc., Vienna*, p. 201.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.