from the amounts ingested in the diet are sufficient criteria on which to base judgment of vitamin E status. Much more must be discovered about the actual concentrations of tocopherol in the specific affected sites of vitamin E deficiency in animals, particularly in conditions of dietary or metabolic stress.

SUMMARY

1. Four groups of female rabbits were maintained on various diets in order to study the distribution of vitamin E in their tissues. After a test period of 15 weeks, seven tissues were analysed for tocopherol, vitamin A, ubiquinone and ubichromenol.

2. Skeletal muscle in the rabbit contained little tocopherol and became depleted to exceptionally low levels. This has been related to the muscular dystrophy that occurs in this species. In contrast with the rat uterus, the uterus in the rabbit is little affected by tocopherol. This reflects the difference in the vitamin E-deficiency syndrome in the two species.

3. Vitamin E-deficient rabbits have lower concentrations of ubiquinone in heart, liver and skeletal muscle than animals on the same diet supplemented with α-tocopheryl acetate. In most tissues except nerve and brain, ubichromenol was also lower. Administration of single doses of vitamin E to deficient animals increased ubiquinone in all tissues examined except fat and also decreased ubichromenol. There appears to be a relationship between α-tocopherol, ubiquinone and ubichromenol in the tissues of the rabbit.

4. Rabbits kept on a vitamin E-deficient diet for 15 weeks still contain large reserves of vitamin E in their adipose tissue. Deficiency states can apparently be produced by local deprivation of tocopherol from a specific tissue.

Our thanks are due to Mrs W. S. Miller and Miss Janice Robinson for their great help with the analyses.

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O-Sulphate Esters of L-Serine, L-Threonine and L-Hydroxyproline

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The hydroxy amino acids, with the exception of tyrosine, have not so far been shown to occur naturally as *O*-sulphate conjugates. The presence of tyrosine *O*-sulphate in one of the two peptides which are liberated from bovine fibrinogen by the action of thrombin was first noted by Bettelheim (1954). This ester has also been detected in similar peptides prepared from rabbit and pig fibrinogens (Blombäck, Boström & Vestermark, 1960; Blombäck & Sjöquist, 1960) and in human and horse fibrins (Bettelheim-Jevons, 1958). These findings raise the possibility that other hydroxy amino acids may occur in proteins as *O*-sulphate conjugates. The wide distribution of the *O*-phosphate conjugates of serine and threonine in phosphoproteins is well known (see Perlmann, 1955).

Attempts to detect *O*-sulphate esters of hydroxylated amino acids in tissues have been preceded by the study of the preparation and properties of the *O*-sulphate esters of L-serine, L-threonine and L-hydroxyproline which is reported here.

EXPERIMENTAL

Preparation of amino acid O*-sulphates*

Potassium L-*serine* O-*sulphate*. The *O*-sulphate esters of tyrosine and tyrosine derivatives have been prepared by Dodgson, Rose & Tudball (1959) with relatively mild conditions of sulphation. A similar method gave a poor yield of potassium L-serine *O*-sulphate. The method of Reitz, Ferrel, Fraenkel-Conrat & Olcott (1946) for the preparation of *O*-DL-serine acid sulphate, when used with L-serine, yielded a product which was contaminated with inorganic SO_4^{2-} ions and unchanged starting material. A satisfactory product was obtained by modifying this method.

L-Serine (1 g.) was added at room temperature to 5 ml. of H_2SO_4 (sp.gr. 1·84) and the mixture stirred until the material dissolved (usually 10 min.). The reaction mixture was kept *in vacuo* for the remainder of 1 hr., when it was poured into 400 ml. of dry ether at −17°. After 15 min. at this temperature the supernatant was decanted and the residue washed (×3) with 200 ml. portions of ether at −17°, the supernatant being decanted after each washing.

The sticky white residue was dissolved in 100 ml. of ice-cold water and inorganic SO_4^{2-} ions were removed by adding an ice-cold saturated solution of $Ba(OH)_2,8H_2O$ until the solution was slightly alkaline. Precipitated $BaSO_4$ was removed by centrifuging and the excess of Ba^{2+} ion in the clear supernatant was precipitated as $BaCO_3$ by passing CO_2 through the cold solution. The whole was clarified by centrifuging and concentrated to dryness *in vacuo* at 38°. The residue was extracted with 20 ml. of ice-cold water and, after centrifuging, the clear extract was passed through a column (7 cm. × 1 cm.) of Dowex 50 ion-exchange resin (Dow Chemical Co., Michigan, U.S.A.; 20–50 mesh; H^+ form). The column was washed with 20 ml. of water and the combined eluate and washings were adjusted to pH 7·8 with aqueous 5% (w/v) KOH before concentrating to dryness *in vacuo* at 38°. The residue was dissolved in the minimum amount of cold water and precipitated by the dropwise addition of ice-cold ethanol. The white crystals were separated at the pump, washed well with ethanol followed by ether and dried *in vacuo* over $CaCl_2$. Yield, 810 mg.; $[\alpha]_D^{16}$ −9·5° in water (*c*, 5·0); $[\alpha]_D^{16}$ +8·7 in N-HCl (*c*, 3·4) (Found: ester SO_4^{2-} ion, 40·9; amino N (Van Slyke), 6·1; amino N (formol titration), 6·0; loss at 110° *in vacuo*, 3·7%. $C_3H_6O_6NSK,\frac{1}{2}H_2O$ requires ester SO_4^{2-} ion, 41·4; amino N, 6·0; H_2O, 3·9%).

Potassium L-*threonine* O-*sulphate*. This preparation was identical with that for potassium L-serine *O*-sulphate except that after keeping for 1 hr. the reaction mixture was poured into 400 ml. of butan-2-one. The final product was precipitated from aqueous solution with ether–butan-2-one (1:1). Before drying, the crystals were washed with butan-2-one followed by ether. Yield, 0·85 g.; $[\alpha]_D^{18}$ −20·6 in water (*c*, 9·7) (Found: ester SO_4^{2-} ion, 38·8; amino N (Van Slyke), 5·4; amino N (formol titration), 5·1; loss at 110° *in vacuo*, 7·1%. $C_4H_8O_6NSK,H_2O$ requires ester SO_4^{2-} ion, 37·6; amino N, 5·5; H_2O, 7·1%).

Potassium L-*hydroxyproline* O-*sulphate*. This preparation was identical with that of L-serine except that the reaction mixture was poured into 300 ml. of crushed ice and water containing 50 g. of $Ba(OH)_2,8H_2O$ rather than into ether. The final product was crystallized by dropwise addition of ethanol–ether (1:1, v/v). Yield, 0·6 g.; $[\alpha]_D^{18}$ −26·1° in water (*c*, 10·5); $[\alpha]_D^{18}$ −12° in N-HCl (*c*, 9·8) (Found: ester SO_4^{2-} ion, 39·7; imino N (formol titration), 5·2%. $C_5H_8O_6NSK$ requires ester SO_4^{2-} ion, 38·5; imino N, 5·6%).

It is clear from the analytical results that *O*-sulphation rather than *N*-sulphation has occurred. Formol and Van Slyke determinations indicate the presence of free amino (or imino) groupings; moreover, all the esters reacted with ninhydrin and isatin. With the L-serine *O*-sulphate preparation no reaction occurred on treatment of the ester with periodate according to the procedure of Frisell & Mackenzie (1958), suggesting that the terminal hydroxyl group was blocked. Further evidence that *O*-sulphation had occurred in all cases was provided by infrared spectroscopy (see below).

From each amino acid, the same sulphated product was obtained by either the method described or the method of Dodgson *et al.* (1959), although the latter gave lower yields. During the course of many preparations of potassium L-serine *O*-sulphate it has been noted that the water content of the crystals can vary and, in some instances, is absent. In this respect the ester resembles the *O*-phosphate ester of L-serine, which is also known to crystallize in different hydrated forms (cf. Fölsch & Melander, 1957).

Paper chromatography. The homogeneity of all the preparations was checked by descending paper chromatography on Whatman no. 1 with the following solvent systems: I, butan-1-ol–acetic acid–water (50:12:25); II, 2-methylpropan-2-ol–formic acid–water (8:3:4); III, propan-1-ol–ammonia (25% soln.)–water (6:3:1). Chromatograms were usually run overnight and were subsequently detected by spraying with a 0·1% solution of ninhydrin in acetone or, with a 0·2% solution of isatin in acetone, followed by heating at 85° for 5 min. Alternatively, ester sulphate groupings were detected by the method of Lloyd (1960). All the preparations ran as single spots, the areas staining with ninhydrin or isatin coinciding exactly with those giving a positive reaction for ester sulphate. Table 1 shows the chromatographic mobilities of the various esters and parent compounds in the three solvent systems.

Paper electrophoresis. All the esters ran as single homogeneous spots when subjected to horizontal paper electrophoresis on Whatman no. 1 paper in 0·1 M-ammonium acetate–acetic acid buffers (ranging from pH 4·0 to 7·0). Electrophoresis was for 2 hr. at a potential of 12 v/cm. Materials were located on the strips as described in the preceding section.

Infrared spectroscopy. The spectra of the esters were determined with the Perkin–Elmer Infracord recording spectrophotometer, the esters being examined as mulls in Nujol (liquid paraffin). The distribution of spectral bands confirmed that the two methods of preparation yielded the same product. Comparison of the spectra with those of a series of alkyl *O*-sulphate esters of known structure provided additional evidence that the amino acids were *O*-sulphated.

X-ray diffraction. X-ray-diffraction patterns of the crystalline powders were kindly prepared by Mr B. Delf of the Department of Physics and confirmed the common identities of the products of the two methods of preparation.

Acid and alkaline hydrolysis

The rates of acid and alkaline hydrolysis of the amino acid *O*-sulphates were determined by estimating the liberated inorganic SO_4^{2-} ions. Weighed samples (20–30 mg.) of the esters were dissolved in 20 ml. of 4% (w/v) trichloroacetic acid (acid hydrolysis) or 20 ml. of N-NaOH (alkaline

Table 1. *Chromatographic mobilities of* L-*serine*, L-*threonine*, L-*hydroxyproline and their corresponding* O-*sulphate esters*

Solvent systems: I, butan-1-ol–acetic acid–water (50:12:25); II, 2-methylpropan-2-ol–formic acid–water (8:3:4); III, propan-1-ol–ammonia (25% soln.)–water (6:3:1). Experimental details are given in the text.

	R_F		
Compound	Solvent I	Solvent II	Solvent III
L-Serine	0·20	0·60	0·51
L-Serine *O*-sulphate	0·12	0·46	0·47
L-Threonine	0·25	0·65	0·57
L-Threonine *O*-sulphate	0·15	0·52	0·49
L-Hydroxyproline	0·25	0·63	0·53
L-Hydroxyproline *O*-sulphate	0·15	0·50	0·49

hydrolysis). Portions (1·5 ml.) were transferred to Pyrex tubes (15 cm. × 1·5 cm.) which were then sealed and placed in an oven at 135° (acid hydrolysis) or, except when otherwise stated, at 100° (alkaline hydrolysis). Zero time was taken as being 2 min. after the tubes were placed in the oven. Tubes were removed from the oven at suitable time intervals and were placed immediately in the deep-freeze (−17°). The cooled tubes were shaken before opening, and samples (0·2 ml.) were withdrawn for determination of inorganic SO_4^{2-} ions by the method of Dodgson (1961*a*, method A).

Enzyme experiments

Biosynthesis of amino acid O-*sulphates*. Spencer (1960) indicated that free L-serine, L-threonine and L-hydroxyproline were not sulphated when added, at a concentration of 0·01 M and at pH 7·4, to a rat-liver preparation that was capable of sulphating simple alcohols and other hydroxylated compounds. Experimental conditions of pH and acceptor concentration were varied in further attempts to achieve enzymic sulphation of the three amino acids.

A particle-free supernatant fraction (30 µl.) of a rat-liver suspension, prepared according to the directions of Spencer (1960), was incubated for 1 hr. at 38° with 10 µl. of an aqueous solution of the amino acid (adjusted to the appropriate pH with a trace of 0·05 N-NaOH), 10 µc (approx. 1 µl.) of $Na_2{}^{35}SO_4$ (code SJS 1, The Radiochemical Centre, Amersham, Bucks) and 10 µl. of a solution containing 1 µmole of adenosine triphosphate, 1 µmole of KH_2PO_4 and 0·3 µmole of $MgCl_2$, the solution being adjusted to the appropriate pH with N-NaOH. Control experiments were made, in which water was substituted for the amino acid solution. After incubation the reaction tubes were placed in a boiling-water bath for 2 min. before centrifuging. Samples (10 µl.) of the clear supernatants were subjected to descending paper chromatography on Whatman no. 1 paper with solvent systems I and II. After drying, radioautographs were prepared by placing the papers in contact with Ilford Industrial B X-ray film for 7 days. The appearance on the test radioautographs of a new radioactive spot in the position which the particular amino acid O-sulphate would be expected to occupy was considered to indicate enzymic sulphation.

Enzymic desulphation. Limited searches (e.g. Neuberg & Wagner, 1925; Roy, 1953; Dodgson, 1961*b*) have failed to detect the presence, in Nature, of an enzyme capable of desulphating relatively simple alkyl sulphate esters. Although it therefore seemed unlikely that the three amino acid O-sulphates would undergo enzymic desulphation by tissue preparations, the possibility was checked as follows. Aqueous suspensions (4%, w/v) of rat tissues were prepared with a glass homogenizer. A portion (0·1 ml.) of the suspension was incubated at 38° for 18 hr. with an equal volume of a 0·02 M solution of each amino acid O-sulphate in 0·25 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid buffer, pH 6·0, 7·0 and 8·0. After incubation, 0·8 ml. of ethanol was added and liberated sulphate was determined by the chloranilate procedure of Lloyd (1959). Control determinations were made in which enzyme and substrate were incubated separately and mixed only just before adding the ethanol. Preliminary experiments showed that inorganic SO_4^{2-} ions (K_2SO_4) could be recovered quantitatively under these conditions.

RESULTS

Acid and alkaline hydrolysis

Figs. 1 and 2 show the rates of liberation of inorganic SO_4^{2-} ions from the three amino acid O-sulphates. The stability of the three esters under

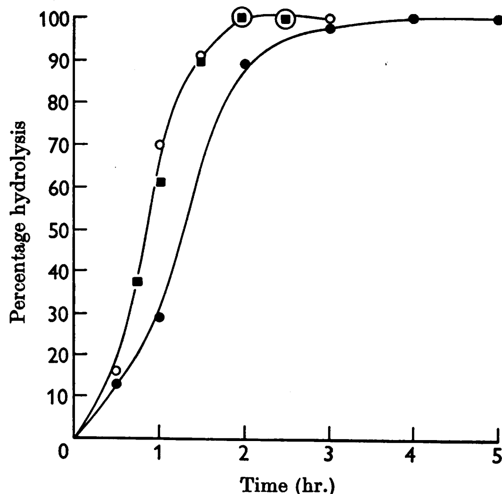

Fig. 1. Rates of hydrolysis of: ●, L-serine O-sulphate (5 mM); ○, L-threonine O-sulphate (4 mM); ■, L-hydroxyproline O-sulphate (5·8 mM), by 4% (w/v) trichloroacetic acid at 135°. Experimental details are given in the text.

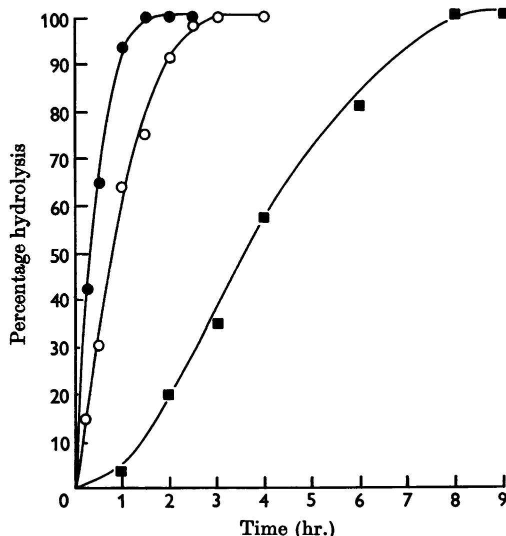

Fig. 2. Rates of hydrolysis of: ●, L-serine O-sulphate (4·9 mM); ○, L-threonine O-sulphate (3·8 mM); ■, L-hydroxyproline O-sulphate (6·5 mM), by N-NaOH. Hydrolysis of L-serine O-sulphate and L-threonine O-sulphate was carried out at 100°; hydrolysis of L-hydroxyproline O-sulphate was at 115°.

acidic conditions is very similar. Under alkaline conditions the O-sulphate esters of L-serine and L-threonine are relatively unstable, complete hydrolysis being achieved in 90 and 180 min. respectively. In contrast, L-hydroxyproline O-sulphate was relatively stable to alkali. The behaviour of L-serine O-sulphate is not unexpected in view of the known alkaline instability of the corresponding O-phosphate ester (see Perlmann, 1955). Separate experiments showed that slight release of SO_4^{2-} ion from L-serine O-sulphate occurred (approx. 4% in 24 hr.) at room temperature in the presence of N-NaOH. No measurable hydrolysis of the ester occurred under the same conditions in the presence of trichloroacetic acid.

Attempted sulphation of L-serine, L-threonine and L-hydroxyproline by rat-liver preparations

No evidence was obtained that the three amino acids, at final concentrations of 0·01M and 0·066M, could undergo sulphation in the presence of rat-liver-supernatant preparations at pH 7·4. All test and control radioautographs showed the presence of many ^{35}S-containing compounds. Spencer (1960) has shown that these compounds are ester sulphates arising from the sulphation of endogenous hydroxylated acceptors. Additional spots did not appear on the test radioautographs when L-serine, L-threonine or L-hydroxyproline was present in the incubation mixtures.

During attempts to achieve the enzymic sulphation of various tyrosine derivatives, Segal & Mologne (1959) showed that, in certain cases, a high pH (9·3) favoured the sulphation. They suggested that suppression of the ionization of the α-amino grouping might be partly responsible for this effect. An attempt was therefore made to sulphate L-serine, L-threonine and L-hydroxyproline with liver preparations at pH 9·3, two concentrations of acceptor being used as before. Negative results were obtained in all cases.

Enzymic desulphation of the amino acid O-sulphates

No significant liberation of inorganic SO_4^{2-} ions occurred when the three amino acid O-sulphates were incubated with suspensions of rat pancreas, spleen, heart, kidney and lung. With liver preparations some release of SO_4^{2-} ions from L-serine O-sulphate was observed. In the most active preparation tested, the release of SO_4^{2-} ions at pH 6·0, 7·0 and 8·0 corresponded respectively to 2, 25 and 15% hydrolysis of the substrate over a period of 18 hr. Liver preparations had no effect on the O-sulphates of L-threonine and L-hydroxyproline under similar conditions. A pH of 7·0 was used in all subsequent experiments with L-serine O-sulphate.

In attempts to obtain greater enzyme activity a more concentrated preparation was obtained as follows. Fresh rat liver was squeezed through a fine mesh to remove connective tissue, and 13 g. of the resultant pulp was suspended in water at 4° with a glass homogenizer, and made up to 100 ml. with water. The suspension was then alternately frozen and thawed three times and subsequently resuspended with a glass homogenizer. A portion (25 ml.) of the suspension was withdrawn and kept at 4° (fraction 1) and the remainder was dialysed against 150 vol. of water at 4° for 4 hr. After dialysis, the contents of the dialysis bag were resuspended as usual and a portion (25 ml.) was withdrawn and stored at 4° (fraction 2). The remainder was centrifuged at 2500 *g* and 2° for 20 min., and the faintly cloudy supernatant was separated and kept at 4° (fraction 3). The activities of the three fractions towards 0·01M-L-serine O-sulphate in the presence of 0·25M-tris–acetic acid buffer, pH 7·0, was then determined. The slight increase in volume which occurred as a result of the dialysis procedure was ignored in comparing the activities of the three fractions.

The results suggested that the bulk of the activity which was present in the original suspension (fraction 1) was still present in the final supernatant fraction (fraction 3). However, it became apparent from several experiments that recoveries of inorganic SO_4^{2-} ions from these various fractions were poor and inconsistent. To overcome this difficulty a further series of experiments were made with L-serine O-sulphate that had been labelled with ^{35}S. This material was prepared from 0·15 g. of L-serine and 0·5 ml. of $H_2{}^{35}SO_4$ (sp.gr. 1·84; specific activity 2·64 mc/m-mole). The final preparation had a specific activity of approx. 11 μc/mg. and was mixed with unlabelled L-serine O-sulphate in the proportions 1:2.

L-Serine O-[^{35}S]-sulphate (0·1 ml. of a 0·02M-solution in 0·5M-tris–acetic acid buffer, pH 7·0) was incubated at 38° with 0·1 ml. of the rat-liver supernatant (fraction 3) for 1, 2, 8 and 18 hr. At the end of the appropriate incubation period the reaction tubes were placed in boiling water for 2 min. After cooling, coagulated protein was removed by centrifuging and 20 μl. of the clear supernatant was applied to Whatman no. 1 paper (32 cm. × 11 cm.) and subjected to horizontal paper electrophoresis in aqueous 0·1M-ammonium acetate for 2 hr. at a potential gradient of 12 v/cm. Control determinations were made in which the liver preparation was added to the substrate and the whole immediately placed in a boiling-water bath for 2 min. After cooling, the controls were incubated at 38° for the appropriate period and were then treated exactly as described for the test determinations except that heating to 100° after incubation was omitted. Solutions of L-serine O-[^{35}S]sulphate and inorganic

$^{35}SO_4^{2-}$ ion ($Na_2{}^{35}SO_4$) were run on the same paper as markers. The paper sheets were air-dried and cut into appropriate strips (1 in. wide). The radioactive zones were located and the relative radioactivity of these zones was measured with the C. 100 Actigraph automatic strip-scanner (Nuclear–Chicago Corp., Ill., U.S.A.). Strips were scanned at a speed of 12 in./hr. with a slit width of 0·125 in., an integration time of 50 sec. and a scale setting of 1000 counts/min. Fig. 3 shows the relative amounts of liberated inorganic SO_4^{2-} ions and residual L-serine O-[^{35}S]sulphate for the various incubation periods. The areas of the various peaks of radioactivity on the scanning chart were used in calculating the approximate rate of liberation of inorganic $^{35}SO_4^{2-}$ ion, which over periods of 1, 2, 8 and 18 hr. corresponded to a percentage hydrolysis of 28·7, 47·4, 54·0 and 61·0 respectively. Although it seemed clear from these experiments that inorganic $^{35}SO_4^{2-}$ ion was being liberated from L-serine O-[^{35}S]sulphate, the possibility that the ester was being metabolized so as to give a sulphated product that moved on paper electrophoresis at the same rate as inorganic $^{35}SO_4^{2-}$ ion was eliminated in the following way. L-Serine O-[^{35}S]sulphate (40 μl. of a 0·02M-solution in 0·5M-tris–acetic acid buffer, pH 7·0) was incubated at 38° for 3 hr. with 40 μl. of the rat-liver preparation (fraction 3). After incubation, the reaction tube was placed in a boiling-water bath for 2 min. A control determination was made as described above. After cooling, precipitated protein was removed by centrifuging, and 60 μl. of the clear supernatant was withdrawn and added to a mixture of 1·5 ml. of aqueous (0·15M) carrier K_2SO_4, 4 ml. of 2·5N-HCl and 30 ml. of water. After mixing, inorganic sulphate was precipitated by the dropwise addition of 4 ml. of an aqueous 10% solution of $BaCl_2$. Precipitated $BaSO_4$ was separated by centrifuging and washed (with intermediate centrifuging) with three 40 ml. portions of water followed by 40 ml. of acetone. After drying at 110° the $BaSO_4$ was plated and radioactivity measured (at infinite thickness) with a thin mica end-window Geiger–Müller tube. Preliminary experiments showed that L-serine O-[^{35}S]sulphate was not hydrolysed or co-precipitated with $BaSO_4$ during the acidic precipitation procedure. The radioactivity by the $BaSO_4$ obtained in the test determination was approx. six times as great as that obtained in the control, confirming that enzymic liberation of inorganic $^{35}SO_4^{2-}$ ion had occurred.

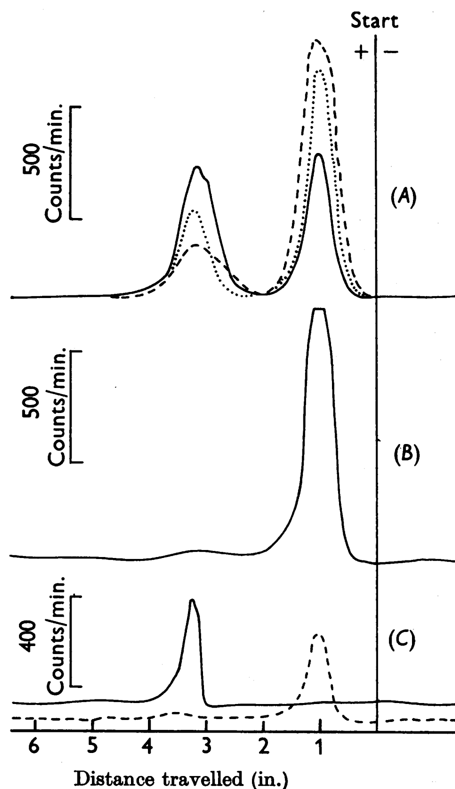

Fig. 3. Continuous scanning records of radioactive zones obtained by paper electrophoresis after incubating L-serine O-[^{35}S]sulphate with a rat-liver preparation (fraction 3) in the presence of 0·25M-tris–acetic acid buffer, pH 7·0, for various periods at 38°. (A) Superimposed test determinations: 1 hr. incubation period (broken line); 2 hr. incubation period (dotted line); 8 hr. incubation period (solid line). (B) A typical control experiment with boiled enzyme. (C) Samples of inorganic [^{35}S]sulphate (solid line) and L-serine O-[^{35}S]sulphate (broken line) run separately on the same electrophoresis papers as the incubation mixtures. The scans have been stylized and the curve for the 18 hr. incubation period is omitted for clarity. See text for experimental details.

In a separate experiment the relative enzyme activities of fractions 1, 2 and 3 over an incubation period of 6 hr. were compared as described above. The relative activities of the fractions were similar (Fig. 4) and corresponded to a percentage hydrolysis of 47·0, 46·6 and 48·5 respectively.

The activity of fraction 1 was also checked in the presence of 0·25M-sodium acetate–acetic acid buffer, pH 7·0, and in the presence of a 0·25M-KH_2PO_4 solution that had been adjusted to pH 7·0 with 10% (w/v) NaOH. The enzyme activities of the fraction in the presence of acetate and phosphate over a period of 6 hr. corresponded to a percentage hydrolysis of 38·4 and 39·6 respectively.

Fig. 5 shows the effect of pH on the rate of liberation of inorganic $^{35}SO_4^{2-}$ ion from 0·01M-L-serine O-[^{35}S]sulphate in the presence of 0·25M-tris–acetic acid buffer. A preparation of fraction 3

was used as the enzyme source, incubation was for 3 hr. at 38°, and the liberated inorganic $^{35}SO_4^{2-}$ ion was determined as before.

DISCUSSION

Previous work with L-tyrosine and related derivatives (Dodgson *et al.* 1959) has shown that relatively mild treatment with sulphuric acid results in the sulphation of the hydroxyl group and does not affect the amino grouping or the benzene ring. More drastic conditions of sulphation can give rise to ring sulphonates, and sulphation with chlorosulphonic acid leads to the formation of sulphamates. Reitz *et al.* (1946) have reported that mild treatment of proteins with sulphuric acid leads to the sulphation of hydroxylated amino acids, but prolonged treatment leads to some pronounced change which is accompanied by sulphate elimination. This could be interpreted by postulating a N → O shift. Elliot (1952) showed that sulphuric acid caused this type of shift about the serine and threonine residues in silk fibroin and lysozyme, and similar findings have been made for the serine residues of poly-DL-serine (Fasman, 1960). In the present work, the mild treatment in the method of Dodgson *et al.* (1959) and the relatively severe treatment in the method described both yielded *O*-sulphate esters only.

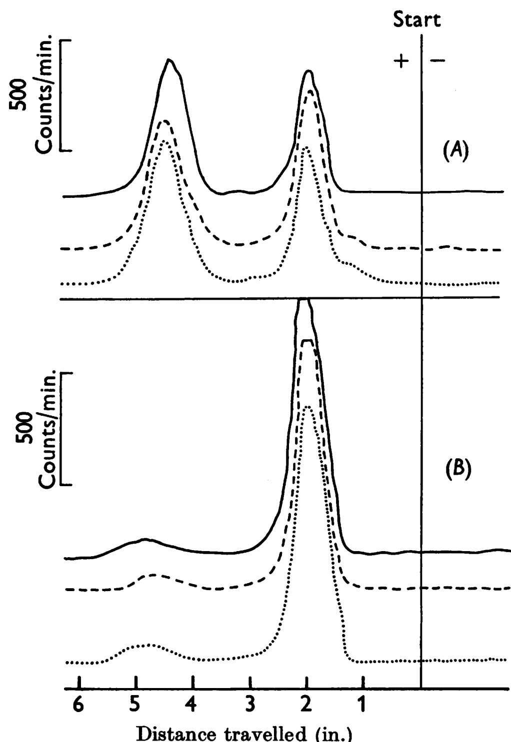

Fig. 4. Continuous scanning records of radioactive zones obtained by paper electrophoresis after incubating L-serine *O*-[^{35}S]sulphate with rat-liver fractions 1–3 (see text for details). Incubation was for 6 hr. at 38° in the presence of 0·25M-tris–acetic acid, pH 7·0. (*A*) Test determinations: fraction 1 (dotted line); fraction 2 (broken line); fraction 3 (solid line). (*B*) Control determinations (designated as for test determinations). The curves have been stylized and displaced vertically to facilitate comparison.

The present work confirms and extends the findings of Spencer (1960) that free L-serine, L-threonine and L-hydroxyproline do not undergo sulphation by fortified liver-supernatant preparations which are able to sulphate other hydroxylated compounds. A number of workers have made similar findings with L-tyrosine (see Dodgson, 1958), and Segal & Mologne (1959) have produced evidence which indicates that, before L-tyrosine can be enzymically sulphated, the carboxyl group must be blocked and the amino group present in an undissociated form. Similar conditions may well apply to the enzymic sulphation of L-serine, L-threonine and L-hydroxyproline, and the negative results reported here should not necessarily be taken as indicating that L-serine *O*-sulphate cannot be present in proteins.

The findings that rat liver contains an enzyme system capable of achieving the desulphation of the *O*-sulphate ester of L-serine but not the corresponding esters of L-threonine and L-hydroxyproline, raises some interesting points. The presence of an enzymic mechanism for the desulphation of

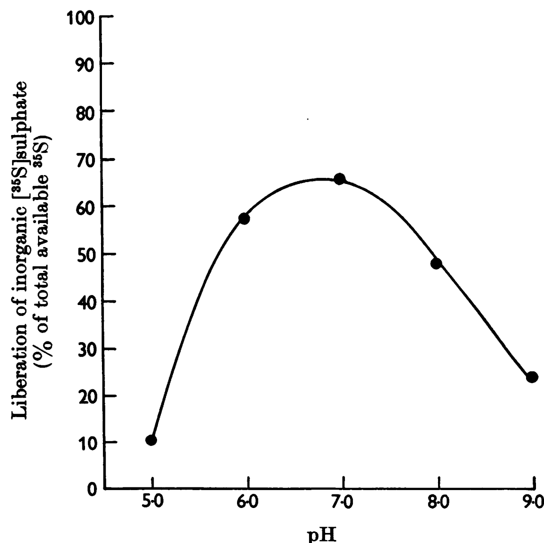

Fig. 5. Effect of pH on the liberation of inorganic [^{35}S]-sulphate from 0·01M-L-serine *O*-[^{35}S]sulphate by a rat-liver preparation (fraction 3). Incubation was for 3 hr. at 38° in 0·25M-tris–acetic acid buffer.

L-serine O-sulphate may indicate that this ester is of some significance in metabolism. However, it has not yet been established that a simple hydrolase (a sulphatase) is involved and the appearance of inorganic SO_4^{2-} ion may merely reflect the activity of an entirely different type of enzyme system. For example, the replacement of the α-amino grouping of L-serine O-sulphate with a keto grouping by an enzyme system analogous to that responsible for the conversion of 3-phosphoserine into 3-phosphohydroxypyruvate (Ichihara & Greenberg, 1957) would yield the O-sulphate ester of 3-hydroxypyruvic acid. Such a compound might well be unstable and tend to lose SO_4^{2-} ion spontaneously. Other similar possibilities could be argued, and under such circumstances the actual release of SO_4^{2-} ion, although in itself a non-enzymic process, would appear to be enzymic in experiments such as those described above. Reaction sequences of this type might be expected to be cofactor-dependent and it must be borne in mind that fraction 3 of the liver preparation used in this work has been dialysed, although for a limited period (4 hr.) only.

A sulphatase could be responsible for the desulphation of L-serine O-sulphate although, with one exception (the type-1 arylsulphatases), all the sulphatases known at present are strongly (often completely) inhibited by phosphate. Desulphation of L-serine O-sulphate still proceeds at an appreciable rate in the presence of phosphate. Dodgson (1961b) has noted that partially purified extracts of the digestive organs of the periwinkle (*Littorina littorea*), which contain arylsulphatase, 3β-steroid sulphatase, glycosulphatase and cortisone 21-sulphatase, are without effect on L-serine O-sulphate. The digestive juice of *Helix pomatia* (containing arylsulphatase, 3β-steroid sulphatase and cortisone 21-sulphatase) was also unable to desulphate the ester.

Although the quantitative experiments suggest that the rat-liver enzyme system is only a moderately active one, preliminary experiments (unpublished) have shown that L-serine O-[^{35}S]sulphate undergoes appreciable desulphation when injected into rats.

SUMMARY

1. Methods have been described for the preparation of the O-sulphate esters of L-serine, L-threonine and L-hydroxyproline.

2. The esters exhibit a similar stability towards acid, but L-serine and L-threonine O-sulphates are much less stable to alkali than the corresponding L-hydroxyproline derivative.

3. Enzymic sulphation of the three amino acids by a rat-liver system known to be capable of sulphating other hydroxylated compounds could not be achieved.

4. Rat liver contains an enzyme system, the activity of which results in the liberation of inorganic sulphate from L-serine O-sulphate. Maximum enzyme activity is obtained in the region of pH 7·0.

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