The Biological Sulphation of L-Tyrosyl Peptides

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1. A rat-liver supernatant preparation can achieve the biological *O*-sulphation of L-tyrosylglycine and L-tyrosyl-L-alanine at pH 7.0. 2. The optimum concentrations of L-tyrosylglycine and L-tyrosyl-L-alanine in this system are 50mM and 60mM respectively. 3. L-Tyrosylglycine yields two sulphated products, whereas L-tyrosyl-L-alanine yields three sulphated products, when used as acceptor for sulphate in the rat-liver system. 4. With both substrates, one of the sulphated products has been identified as the *O*-sulphate ester of the corresponding parent peptide.

L-Tyrosine *O*-sulphate has been identified as a component of mammalian urines (see John, Rose, Wusteman & Dodgson, 1965), of mammalian fibrinogens (see Doolittle & Blombäck, 1964) and of gastrin II isolated from hog antral mucosa (Gregory, Hardy, Jones, Kenner & Sheppard, 1964). Attempts made to achieve the biological sulphation of free L-tyrosine have all failed (Dodgson, Rose & Tudball, 1959; Nose & Lipmann, 1958; Segal & Mologne, 1959; Suzuki & Strominger, 1960; Vestermark & Boström, 1959). However, it has been established that the methyl and ethyl esters of L-tyrosine will act as sulphate acceptors in a rat-liver sulphating system (Segal & Mologne, 1959; Jones & Dodgson, 1965). Thus it has been suggested that naturally occurring carboxyl-substituted derivatives of L-tyrosine may act as sulphate acceptors *in vivo* and that small peptide fragments with L-tyrosine in the *N*-terminal position may well act in this capacity. Segal & Mologne (1959) measured the transfer of sulphate from *p*-nitrophenyl sulphate via adenosine 3',5'-diphosphate to L-tyrosylglycine by using a partially purified phenol-sulphotransferase preparation from rat liver. They detected weak transferase activity at pH 9.3 but none at pH 7.8. The present paper demonstrates the sulphation of L-tyrosylglycine and L-tyrosyl-L-alanine in a rat-liver supernatant preparation at pH 7.0, with inorganic sulphate and ATP as sources of sulphate and energy respectively. Preliminary accounts of this work have already been given (Jones, Scotland & Dodgson, 1965*a,b*).

MATERIALS AND METHODS

Sulphate esters. The potassium salt of L-tyrosine $O^{[35]S}$ -sulphate was prepared according to the method described by Dodgson, Powell, Rose & Tudball (1961). L-Tyrosyl-

glycine (500mg.; Yeda Research and Development Co. Ltd., Rehovoth, Israel) was sulphated by a method similar to that described by Dodgson *et al.* (1959) for the sulphation of glycyl-L-tyrosine. However, the total reaction time was reduced from 40 min. to 15 min. and the final product was precipitated by dissolving it in 100 μ l. of water and adding the solution to 5 ml. of ethanol. After 30 min. at 4° the crystals of monopotassium L-tyrosylglycine *O*-sulphate were separated, washed with 5 ml. of ethanol followed by 5 ml. of ether and dried *in vacuo* over P_2O_5 at 100° for 12 hr. The yield was 20 mg. (Found: ester SO_4^{2-} , 25%; K^+ , 10.3%. Monopotassium L-tyrosylglycine *O*-sulphate, $C_{11}H_{13}KN_2O_7S$, requires ester SO_4^{2-} , 27%; K^+ , 10.9%).

L-Tyrosyl-L-alanine (500mg.; Yeda Research and Development Co. Ltd.) was sulphated by the procedure described for L-tyrosylglycine. The yield was 40 mg. (Found: ester SO_4^{2-} , 25.8%; K^+ , 11.0%. Monopotassium L-tyrosyl-L-alanine *O*-sulphate, $C_{12}H_{15}KN_2O_7S$, requires ester SO_4^{2-} , 26.0%; K^+ , 10.5%).

The homogeneity of the preparations of the sulphate esters was checked by descending chromatography with Whatman no. 1 paper, the solvent (solvent system A) being butan-1-ol-acetic acid-water (50:12:25, by vol.). After 16 hr. the chromatograms were dried and developed by spraying with a 0.25% (w/v) solution of ninhydrin in acetone and drying in air at 100°. The R_F values of the parent compounds and their sulphate esters were: L-tyrosine, 0.45; L-tyrosine *O*-sulphate, 0.20; L-tyrosylglycine, 0.42; L-tyrosylglycine *O*-sulphate, 0.24; L-tyrosyl-L-alanine, 0.48; L-tyrosyl-L-alanine *O*-sulphate, 0.33. Hydrolysis of each sulphate ester in *N*-HCl at 100° for 4 min. yielded inorganic sulphate and the corresponding amino acid or peptide. The ultraviolet-absorption spectra of the parent peptides and their sulphate esters were identical with those of L-tyrosine and L-tyrosine *O*-sulphate respectively. This is in accord with the conclusion that the compounds described above are true *O*-sulphate esters (see Dodgson *et al.* 1959).

Preparation of enzymes. Rat-liver whole supernatant was prepared by the method described by Jones & Dodgson (1965) and stored in 1.0 ml. portions at -10°.

Incubation mixture. The standard incubation mixture contained 100 μ l. of enzyme preparation, 4 μ moles of ATP (disodium salt; Sigma Chemical Co., St Louis, Mo., U.S.A.), 2 μ moles of $MgCl_2$, 1 μ mole of cysteine hydrochloride, 10 μ C of $Na_2^{35}SO_4$ (carrier-free; code SJS1, The Radiochemical Centre, Amersham, Bucks.), 5 μ moles of KH_2PO_4 and sulphate acceptors according to the plan of the experiment (the acceptor compounds were either dissolved in the solution of KH_2PO_4 or, for amounts greater than 2 μ moles, they were added as solid to the incubation mixture). The pH of all components was adjusted to 7.0 with 2N-NaOH and the final mixture (volume 170 μ l.) was incubated for 1 hr. at 38°. After incubation the reaction tubes were immersed in boiling water for 2 min. and the precipitated protein was removed by centrifuging.

Paper chromatography and electrophoresis. Samples (6 μ l.) of deproteinized incubation mixtures were applied to Whatman no. 1 paper and subjected to descending chromatography for 16 hr. at room temperature with solvent system A or isobutyric acid-0.3N- NH_3 (5:3, v/v) (solvent system B). Samples (6 μ l.) were also subjected to electrophoresis on Whatman no. 1 paper for 2 hr. in 0.1M-sodium acetate-acetic acid buffer, pH 4.5, with a potential gradient of 11 v/cm. Under these conditions L-tyrosine O-sulphate moved 3.2 cm., L-tyrosylglycine O-sulphate moved 2.3 cm. and L-tyrosyl-L-alanine O-sulphate moved 2.0 cm.

Detection and estimation of radioactivity. Radioactive spots were located on dried chromatograms and electrophoresis strips by exposure to Ilford Industrial B X-ray film for 3 days or by scanning with the C-100 Actigraph automatic chromatogram scanner (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). The relative amount of radioactivity associated with each spot was estimated from the record of the scanner as described by Jones & Dodgson (1965).

EXPERIMENTAL AND RESULTS

In the absence of added acceptor the enzyme preparation synthesized ^{35}S -labelled 3'-phosphoadenylyl sulphate which was characterized by chromatography in solvent systems A and B and by paper electrophoresis.

The enzyme preparation was able to use *p*-nitrophenol, L-tyrosine methyl ester or L-tyrosine ethyl ester as sulphate acceptor, but could not use L-tyrosine or glycyl L-tyrosine.

Enzymic synthesis of L-tyrosylglycine O[^{35}S]-sulphate by the rat-liver sulphating system. L-Tyrosylglycine was incubated in the standard reaction mixture for 1 hr. at 38°. The amounts of the peptide were varied so as to give final concentrations in the reaction mixture varying from 0 to 60 mM. At the end of the incubation period, samples (6 μ l.) of deproteinized mixtures were examined by paper chromatography in solvent system A and by paper electrophoresis.

Radioautography of dried chromatograms and electrophoresis strips indicated that incubation mixtures, with L-tyrosylglycine as sulphate acceptor, contained two new radioactive compounds in addition to ^{35}S -labelled inorganic sulphate and

3'-phosphoadenylylsulphate. The chromatographic mobility of the major compound (metabolite I; R_F 0.24 in solvent system A) was identical with that of authentic L-tyrosylglycine O-sulphate. Similar agreement was obtained between the electrophoretic mobility of this metabolite and that of L-tyrosylglycine O-sulphate. The second radioactive metabolite detected on chromatograms (metabolite II) had R_F 0.28 in solvent system A. The second radioactive compound detected on electrophoretograms (assumed to be metabolite II) possessed no net charge when examined by paper electrophoresis at pH 4.5. L-Tyrosine O[^{35}S]-sulphate was not detected on chromatograms or electrophoretograms. The amount of radioactivity associated with both metabolites was assayed as described above by scanning the paper chromatograms. Owing to the close proximity of the spots, the combined area under the peaks on the recording chart was taken as a measure of the sulphation of L-tyrosylglycine. The results (Fig. 1) show that the transfer of [^{35}S]sulphate to L-tyrosylglycine proceeds maximally when the concentration of the acceptor is approx. 50 mM. A visual appraisal of radioautograms indicated that the relative amounts of both metabolites increased in parallel with increasing concentration of L-tyrosylglycine. Finally, chromatograms (developed with 0.25% ninhydrin in acetone) exhibited spots corresponding in mobilities to free L-tyrosine (R_F 0.45) and glycine (R_F 0.23).

Metabolite I was then separated from 0.5 ml. of pooled deproteinized reaction mixtures by paper chromatography (solvent system A) and the appropriate areas were eluted with water. To the

Fig. 1. Substrate concentration-activity curve for the sulphation of L-tyrosylglycine by rat-liver whole supernatant. Details are given in the text.

combined eluates (approx. 10ml.) was added 15mg. of authentic carrier L-tyrosylglycine *O*-sulphate and the whole was applied to a column (10cm. $\times$ 1cm.) of Dowex 50 resin (H^+ form; 20–50 mesh). This latter procedure was necessary to remove glycine, which travels with metabolite 1 on paper chromatography in solvent system *A*. The column of resin was then washed with 20ml. of water, and the eluate and washings were neutralized with *n*-potassium hydroxide, concentrated *in vacuo* at 40° to approx. 0.5ml. and added to 5ml. of ethanol. The white solid that was precipitated was collected by centrifuging, washed with 5ml. of ethanol followed by 5ml. of ether and finally dried *in vacuo* over phosphorus pentoxide at room temperature (the yield was approx. 10mg.). The product was then examined by descending paper chromatography in solvent systems *A* and *B*, by two-way paper chromatography with phenol–water (4:1, w/v) as first solvent and solvent system *A* as second solvent and by paper electrophoresis under the conditions described above. All methods showed a single spot, which was radioactive, gave a positive ninhydrin reaction, absorbed ultraviolet light and moved as expected for L-tyrosylglycine *O*-sulphate.

Finally, the product was dissolved in saturated sodium hydrogen carbonate solution (2mg./0.5ml.) and added to 0.5ml. of ethanol containing 10mg. of 1-fluoro-2,4-dinitrobenzene. The mixture was shaken at room temperature for 2hr. and then evaporated to dryness *in vacuo*. The residue was dissolved in 1ml. of water and examined by paper chromatography in solvent system *A*. Chromatograms showed the presence of a single DNP derivative, which was also radioactive.

Enzymic synthesis of L-tyrosyl-L-alanine $O[^{35}S]$ -sulphate by the rat-liver sulphating system. L-Tyrosyl-L-alanine was incubated in the standard reaction mixture for 1hr. at 38°. The final concentration of the peptide in the reaction mixture varied from 0 to 120mm. At the end of the incubation period the deproteinized mixtures were examined by paper chromatography and electrophoresis followed by radioautography and scanning in the usual way. Two new radioactive metabolites were detected in incubation mixtures when the concentration of L-tyrosyl-L-alanine was between 10mm and 30mm, whereas a third metabolite was detected when the concentration of the peptide was 60mm and 120mm. The chromatographic mobility of one of these metabolites (metabolite 1; R_F 0.33 in solvent system *A*) was identical with that of authentic L-tyrosyl-L-alanine *O*-sulphate. Similar agreement was obtained between the electrophoretic mobilities of these two compounds. No free L-tyrosine $O[^{35}S]$ -sulphate was detected on chromatograms or electrophoretograms, but the behaviour of the other two radioactive metabolites on paper

Fig. 2. Substrate concentration–activity curves for the sulphation of L-tyrosyl-L-alanine by rat-liver whole supernatant. ○, Production of L-tyrosyl-L-alanine $O[^{35}S]$ -sulphate (metabolite 1); ●, production of metabolite 2; ■, production of metabolite 3. Details are given in the text.

electrophoresis suggests that one of them (metabolite 2; R_F 0.22 in solvent system *A*) is less acidic and the other (metabolite 3; R_F 0.43 in solvent system *A*) is more acidic than L-tyrosyl-L-alanine *O*-sulphate. The relative amounts of each metabolite in incubation mixtures were estimated by scanning chromatograms in the usual way. The results (Fig. 2) show that the optimum concentration of L-tyrosyl-L-alanine for this sulphating system is approx. 60mm. Chromatograms (developed with 0.25% ninhydrin in acetone) showed the presence of L-tyrosine (R_F 0.45 in solvent system *A*) and L-alanine (R_F 0.31 in solvent system *A*).

Metabolite 1 was separated from 100 μ l. of pooled deproteinized incubation mixtures by paper chromatography followed by ion-exchange chromatography in the presence of 25mg. of authentic carrier L-tyrosyl-L-alanine *O*-sulphate, the whole procedure being carried out as described above for the isolation of L-tyrosylglycine *O*-sulphate. The final white solid (approx. 20mg.) was precipitated twice from 100 μ l. of water by the addition of 5ml. of ethanol. After each precipitation a small sample (approx. 1mg.) was retained for estimation of its specific activity as described by Dodgson *et al.* (1961), and for examination by paper electrophoresis. Both materials had identical specific activities (100 counts/min./mg.) and, when examined by electrophoresis, gave one spot, which was radioactive, gave a positive ninhydrin reaction and absorbed ultraviolet light.

DISCUSSION

The present work establishes that L-tyrosylglycine and L-tyrosyl-L-alanine can act as acceptors for sulphate in the 3'-phosphoadenylyl sulphate-phenol-sulphotransferase system (EC 2.8.2.1) of rat-liver at pH 7.0. In contrast, glycyl-L-tyrosine could not act as sulphate acceptor in this system.

The optimum concentration of acceptors for the rat-liver sulphating system was approx. 50mM and approx. 60mM for L-tyrosylglycine and L-tyrosyl-L-alanine respectively. However, these values undoubtedly reflect, to some extent, the presence of enzymes in the system that hydrolyse both peptides to their constituent amino acids. Incubation of L-tyrosylglycine or L-tyrosyl-L-alanine with the rat-liver sulphating system leads to the appearance of the corresponding ³⁵S-labelled O-sulphate ester among the products formed. These findings support the view that small peptide fragments with L-tyrosine in the N-terminal position may act as sulphate acceptors *in vivo*. Further, it is possible that these sulphated peptides may be hydrolysed *in vivo* to yield free L-tyrosine O-sulphate and hence account, in part, for the occurrence of this sulphated amino acid in mammalian urines. In this connexion it has been shown (Jones, Dodgson, Powell & Rose, 1963) that rabbit fibrinopeptide B, which contains one residue of L-tyrosine O-sulphate, is metabolized by the rabbit to yield free L-tyrosine O-sulphate, which can then be detected in the urine.

It is not yet clear whether the other radioactive compounds, detected in incubation mixtures when either L-tyrosylglycine or L-tyrosyl-L-alanine was

used as sulphate acceptor, represent breakdown products of the sulphated peptides or sulphated products of degraded fragments of the original peptides.

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