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Biosynthesis of L-Tyrosine *O*-Sulphate from the Methyl and Ethyl Esters of L-Tyrosine

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1. Rat-liver supernatant preparations are capable of achieving the biological sulphation of L-tyrosine methyl ester, the reaction proceeding maximally at a substrate concentration of 30 mM and at pH 7·0. 2. Two sulphated products are formed, one of which has been identified as L-tyrosine *O*-sulphate. On the basis of indirect evidence the other product can be assumed to be L-tyrosine *O*-sulphate methyl ester. 3. An enzyme present in rat-liver supernatant preparations is capable of converting L-tyrosine *O*-sulphate methyl ester into L-tyrosine *O*-sulphate. This enzyme is inhibited by L-tyrosine methyl ester. 4. L-Tyrosine ethyl ester also yields two sulphated products when used as an acceptor in the liver sulphating system. One of these has been identified chromatographically as L-tyrosine *O*-sulphate and the other may be presumed to be L-tyrosine *O*-sulphate ethyl ester.

Enzyme preparations from mammalian livers and certain other tissues are able to accomplish the biological sulphation of many aromatic and aliphatic hydroxyl and amino groupings (see Gregory, 1961). In those cases that have been examined in detail adenosine 3′-phosphate 5′-sulphatophosphate (Robbins & Lipmann, 1957) has been identified as the sulphate donor. However, all attempts to transfer sulphate from this donor to free L-tyrosine have failed (Dodgson, Rose & Tudball, 1959; Grimes, 1959; Nose & Lipmann, 1958; Segal & Mologne, 1959; Suzuki & Strominger, 1960; Vestermark & Boström, 1959), and this finding is of particular interest in view of the fact that L-tyrosine *O*-sulphate has been identified as a normal constituent of human urine (Tallan, Bella, Stein & Moore, 1955) and a number of mammalian fibrinogens (Bettelheim, 1954; Blombäck, 1960). Evidence suggests that a carboxyl-substituted derivative of L-tyrosine with a free amino group may be the naturally occurring sulphate acceptor since L-tyrosine methyl ester and L-tyrosine ethyl ester will accept sulphate from adenosine 3′-phosphate 5′-sulphatophosphate in an enzyme system with *p*-nitrophenyl sulphate and adenosine 3′,5′-diphosphate as source of sulphate and coenzyme respectively (Segal & Mologne, 1959). However, an attempt to sulphate L-tyrosine methyl ester with inorganic sulphate, Mg^{2+} ions, ATP and rat-liver enzymes has failed (Grimes, 1959).

The present work directly confirms that the methyl and ethyl esters of L-tyrosine will accept sulphate from adenosine 3′-phosphate 5′-sulphatophosphate in the presence of rat-liver supernatant preparations and establishes that a product of this sulphation is free L-tyrosine *O*-sulphate. A preliminary account of this work has been given (Jones & Dodgson, 1964).

MATERIALS AND METHODS

Sulphate esters. L-Tyrosine *O*-sulphate was prepared according to the method described by Dodgson *et al.* (1959), and the ^{35}S-labelled ester was prepared as described by Dodgson, Rose & Tudball (1961).

Preparation of enzymes. Male M.R.C. hooded rats (approx. 200 g.) were killed by a blow on the head and the livers removed, blotted, weighed and immediately chilled to 0°. Suspensions (20%) of the tissue were then prepared in 0·15 M-KCl with the aid of a glass homogenizer and cellular particles were removed by centrifuging at 100000 $g_{av.}$ for 90 min. in a Spinco model L preparative ultracentrifuge. The supernatant (designated 'whole supernatant') was removed with a syringe and stored in 1·0 ml. portions at −10°.

In other experiments, rat livers were homogenized in 0·05 M-KCl (7 ml./5 g. of liver) and the 'pH 5 enzymes' precipitated by the method described by Hoagland, Keller & Zamecnik (1956). The precipitated material was redissolved in 0·1 M-tris–HCl buffer, pH 7·6 (1·0 ml./g. of liver), undissolved solid was removed by centrifuging and the supernatant (designated 'pH 5 supernatant') was stored in 1·0 ml. portions at −10°.

Incubation mixture. The standard reaction mixture contained 100 μl. of enzyme preparation, 4 μmoles of ATP (disodium salt) (Sigma Chemical Co., St Louis, Mo., U.S.A.), 2 μmoles of $MgCl_2$, 1 μmole of cysteine hydrochloride, 10 μc of $Na_2{}^{35}SO_4$ (code SJSI; The Radiochemical Centre, Amersham, Bucks.) and 0·5 μmole of acceptor dissolved in

10 μl. of 0·5 M-KH_2PO_4. All components were adjusted to pH 7·2 with 2 N-NaOH and the final volume of the reaction mixture was 160 μl. In later experiments it was found that the cysteine hydrochloride could be omitted from the incubation mixture without loss in sulphating activity. After incubation for 1 hr. at 38° the reaction tubes were immersed in boiling water for 2 min. and the precipitated protein was removed by centrifuging.

Paper chromatography and electrophoresis. Samples (5–10 μl.) of the deproteinized incubation mixtures were applied to Whatman no. 1 chromatography paper and subjected to descending chromatography for 16 hr. at room temperature with butan-1-ol–acetic acid–water (50:12:25, by vol.; solvent system A) or isobutyric acid–0·3 N-NH_3 (5:3, v/v; solvent system B). Samples were also subjected to electrophoresis on Whatman no. 1 paper for 2 hr. in 0·1 M-sodium acetate–acetic acid buffer, pH 4·5, and a potential gradient of 11 v/cm.

Ion-exchange chromatography. Samples (0·5–2·0 ml.) of deproteinized incubation mixtures were applied to a column (13 cm. × 2·5 cm.) of Dowex 50 ion-exchange resin (200–400 mesh; H^+ form) and the column was then washed with 100 ml. of water followed by N-NH_3 until the eluate (collected in 1·0 ml. fractions) was alkaline.

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 1–10 days. For quantitative assessment of the radioactive areas the papers were scanned with the C.100 Actigraph automatic chromatogram scanner (Nuclear–Chicago Corp., Ill., U.S.A.) at a speed of 12 in./hr., with a slit width of 0·25 in., an integration time of 40 sec. and a scale setting of 1000 counts/min. The relative radioactivity of each spot was then estimated by tracing the corresponding peak on the recording chart on to Abermill–Bond typing paper, cutting out the area under the peak and weighing. Each peak was traced in duplicate and the mean of the two weights recorded. The radioactivity of column eluates was estimated in a similar manner or, alternatively, by evaporating samples (0·25 ml.) to dryness on aluminium planchets and counting in automatic scaling equipment (Nuclear–Chicago Corp.) with a D.37A thin mica end-window Geiger–Müller tube.

EXPERIMENTAL AND RESULTS

In the absence of added acceptor, both enzyme preparations were capable of synthesizing adenosine 3′-phosphate 5′-sulphatophosphate, which was characterized by chromatography in solvent systems A and B and by paper electrophoresis. Radioautograms also showed that endogenous sulphate acceptors were present in the whole-supernatant preparation (see Spencer, 1960).

Both enzyme preparations were capable of using *p*-nitrophenol as sulphate acceptor but neither could use free L-tyrosine for this purpose. However, incubation of the pH 5 supernatant with either the methyl or ethyl ester of L-tyrosine gave rise to two new radioactive spots on chromatograms and electrophoresis strips. The mobilities of these metabolites in solvent systems A and B are compared with those of authentic L-tyrosine *O*[^{35}S]-sulphate in Table 1. Both acceptors gave rise to a radioactive metabolite that had chromatographic mobilities identical with those of L-tyrosine *O*[^{35}S]-sulphate. Similar agreement was obtained between the electrophoretic mobility of this metabolite and L-tyrosine *O*[^{35}S]-sulphate. The remaining metabolite in both cases exhibited a weak negative charge at pH 4·5 and was presumed to be the *O*-sulphate ester of the original acceptor molecule.

When whole supernatant was used as enzyme source with either the methyl or ethyl ester of L-tyrosine as acceptor, one new spot only (mobilities identical with those of L-tyrosine *O*[^{35}S]-sulphate) appeared on chromatograms or on electrophoresis strips.

Identification of L-*tyrosine* O[^{35}S]-*sulphate in incubation mixtures when* L-*tyrosine methyl ester was used as acceptor and whole supernatant as source of enzyme*

In these experiments the quantities of the standard reaction mixture were increased fivefold and L-tyrosine methyl ester was used as sulphate acceptor. After the usual incubation time the reaction mixtures were deproteinized and subjected to ion-exchange chromatography on Dowex 50 resin as described above. Two radioactive peaks only were located in the eluate: the first (mainly inorganic [^{35}S]sulphate) in the initial 4 ml. and the

Table 1. *Chromatographic mobilities of the radioactive metabolites found in standard reaction mixtures with* L-*tyrosine methyl ester or* L-*tyrosine ethyl ester as sulphate acceptor*

The R_F values for authentic L-tyrosine *O*[^{35}S]-sulphate were 0·20 and 0·24 in solvent systems A and B respectively.

	R_F values			
	Solvent system A		Solvent system B	
Sulphate acceptor	First metabolite	Second metabolite	First metabolite	Second metabolite
L-Tyrosine methyl ester	0·20	0·43	0·24	0·45
L-Tyrosine ethyl ester	0·20	0·50	0·24	0·53

other between 6 and 8 ml. The fractions corresponding to the second peak were pooled, neutralized with ammonia and freeze-dried. Electrophoresis of a portion of the freeze-dried material gave one radioactive compound only, which moved at the same rate as authentic L-tyrosine *O*[^{35}S]-sulphate. The remainder of the freeze-dried material was then mixed with 90 mg. of unlabelled L-tyrosine *O*-sulphate and recrystallized six times from a minimum volume of water by the dropwise addition of ether–ethanol (4:3, v/v) mixture. A small sample from each recrystallization was retained and analysed for [^{35}S]sulphate as described by Dodgson *et al.* (1961). Material of constant specific activity (60 counts/min./mg.) was obtained after the first recrystallization. The final precipitate was examined by electrophoresis, giving one ultraviolet-absorbing spot that was also radioactive and moved as expected for L-tyrosine *O*-sulphate.

Studies on the second radioactive metabolite formed when L-*tyrosine methyl ester is used as acceptor in the* pH 5 *supernatant system*

Considerable difficulty was encountered in identifying the second radioactive metabolite as the *O*-sulphate ester of L-tyrosine methyl ester. Several attempts to prepare adequate yields of this ester by chemical synthesis have all failed. Thus treatment of L-tyrosine methyl ester with concentrated sulphuric acid at $-8°$ yielded L-tyrosine *O*-sulphate as the sole end product. Treatment of solid L-tyrosine *O*-sulphate with diazomethane in anhydrous ether at room temperature for 2–3 hr. yielded an ether-soluble material that did not react with ninhydrin, indicating that *N*-methylation had occurred. When this methylation procedure was carried out at 0° for 15 min. a small portion only of the starting material dissolved in the ethereal solution of diazomethane. Undissolved material was separated, dissolved in water and examined by paper chromatography in solvent system A and by paper electrophoresis, when two ninhydrin-positive spots were located, the major one being unchanged L-tyrosine *O*-sulphate. The second compound had R_F (0·43) identical with that of the unknown metabolite and possessed a weak negative charge at pH 4·5. This minor component could be separated from unchanged starting material on a column of Dowex 50, when it appeared at approx. 50 ml. of the water eluate. The yield was extremely low, however, and could not be improved by varying conditions of temperature, time or concentration of reactants. Finally attempts were made to methylate *N*-carbobenzoxy-L-tyrosine *O*-sulphate (prepared by treatment of *N*-carbobenzoxy-L-tyrosine with chlorosulphonic acid; see Dodgson & Tudball, 1960), the product being treated with hydrogen at 0° in the presence of a palladium catalyst to remove the *N*-carbobenzoxy group. The final product was shown to be L-tyrosine *O*-sulphate, although a faint ninhydrin-positive spot with R_F 0·43 in solvent system A could be detected on chromatograms.

It was thought that further support for the assumed identity of this second radioactive metabolite might emerge from a more detailed study of the biological sulphation of L-tyrosine methyl ester by whole supernatant under various conditions of pH and at different concentrations of acceptor. The reaction mixtures contained 500 μl. of whole supernatant, 50 μl. of an aqueous solution containing magnesium chloride (10 μmoles) and $Na_2{}^{35}SO_4$ (10 μc), 100 μl. of an aqueous solution of ATP (20 μmoles) and 300 μl. of a solution of L-tyrosine methyl ester hydrochloride in 0·5 M-potassium dihydrogen phosphate. The concentrations of the ester were varied so as to give final concentrations in the reaction mixtures varying between 0 and 30 mM. All components were adjusted to pH 7·2 with 2 N-sodium hydroxide and suitable control determinations were made in which water was substituted for the ATP solution. After incubation at 38° for 1 hr. the mixtures were deproteinized and 10 μl. samples were examined by paper chromatography in solvent system A. Radioactive spots were located and their relative intensities assayed as described above. The results (Fig. 1) show that, at approx. 5 mM concentrations of L-tyrosine methyl ester, L-tyrosine *O*[^{35}S]-sulphate is the sole product, whereas, at higher concentrations of the acceptor, considerable

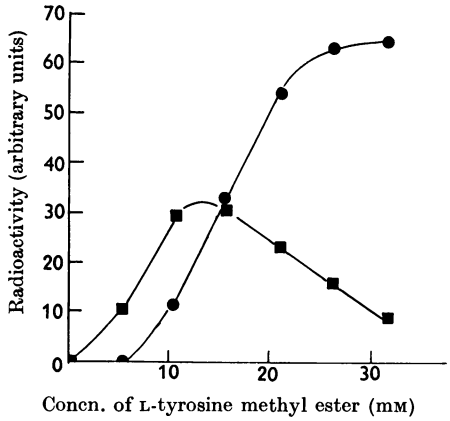

Fig. 1. Substrate concentration–activity curves for the sulphation of L-tyrosine methyl ester by rat-liver whole supernatant. ■, Curve following the production of L-tyrosine *O*[^{35}S]-sulphate; ●, curve following the production of the second radioactive metabolite. Details are given in the text.

Table 2. *Effect of* pH *on the activation of* [^{35}S]*sulphate and its subsequent transfer to* L-*tyrosine methyl ester by rat-liver whole supernatant*

The relative amount of ^{35}S associated with each metabolite was estimated as described in the text.

	Relative amount of ^{35}S incorporated (arbitrary units)		
pH	Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate	L-Tyrosine *O*[^{35}S]-sulphate	Second radioactive metabolite
6·2	12	0	0
7·0	98	25	22
7·9	46	10	8

quantities of the second radioactive metabolite are produced. Further, increasing concentrations of L-tyrosine methyl ester produce a progressive inhibition of the production of L-tyrosine *O*[^{35}S]-sulphate.

In studying the effect of varying the pH of the medium on the sulphation of L-tyrosine methyl ester, the reaction mixture described above was used with 20 mM-acceptor and at pH 6·2, 7·0 and 7·9. Products were estimated in the usual way, but in these experiments the amount of adenosine 3′-phosphate 5′-sulphatophosphate produced during the 1 hr. incubation period at 38° was also measured by submitting a 10 μl. portion of the deproteinized mixtures to paper electrophoresis and estimating the radioactivity of the appropriate area of the paper. The results (Table 2) indicate that the activation of sulphate and its transfer to L-tyrosine methyl ester proceeds maximally at pH 7·0.

Having established optimum conditions for the formation of the second metabolite (30 mM at pH 7·0), we used these conditions to prepare it on a larger scale. Components and volumes of the incubation mixture were increased fivefold and, after incubation and deproteinization, 1·0 ml. portions of the mixture were fractionated on columns of Dowex 50 ion-exchange resin as described above. Three radioactive peaks were detected in the water eluate (Fig. 2). The first peak contained predominantly inorganic [^{35}S]-sulphate and was not examined further. The identity of the component of the second peak was confirmed to be L-tyrosine *O*[^{35}S]-sulphate by paper chromatography in solvent system A. Fractions corresponding to the third radioactive peak were pooled and freeze-dried, and the residue was reconstituted in 1·0 ml. of water. Paper chromatography of this solution in solvent system A confirmed the presence of the second radioactive metabolite (R_F 0·43), although small amounts of L-tyrosine *O*[^{35}S]-sulphate were also present, probably as a result of the loss of methyl group during the manipulative procedure.

Conversion of the metabolite into L-*tyrosine* O[^{35}S]-*sulphate*. Four tubes were prepared each containing

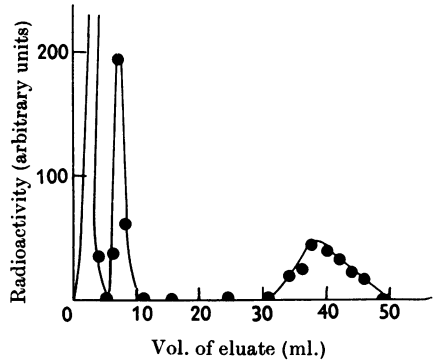

Fig. 2. Behaviour, on Dowex 50 (H^+ form) ion-exchange columns, of deproteinized whole-supernatant reaction mixtures in which L-tyrosine methyl ester (approx. 30 mM) was used as acceptor for sulphate. Details are given in the text.

the following solutions: 5 μl. of whole supernatant, 5 μl. of 0·5 M-potassium dihydrogen phosphate, 20 μl. of a solution of the second radioactive metabolite in water. The pH of all components was adjusted to 7·0 with 2 N-sodium hydroxide. The tubes were incubated at 38° for 10, 20, 30 and 40 min. respectively. Protein was then removed by centrifuging after heating for 30 sec. at 100°; samples (10 μl.) were subjected to paper chromatography in solvent system A and radioactive spots were located and their relative intensities estimated. The results (Fig. 3) show that a decrease in the concentration of the second radioactive metabolite is accompanied by a corresponding increase in the concentration of L-tyrosine *O*[^{35}S]-sulphate, the slopes of the curves being identical but opposite in sign. No other radioactive spots were located on chromatograms. In control experiments in which water was substituted for whole supernatant there was no measurable decrease in the concentration of the second radioactive metabolite during the incubation period. L-Tyrosine methyl ester (incorporated in the reaction mixture at 16 mM con-

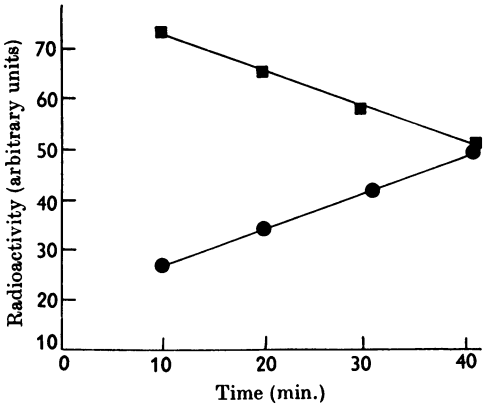

Fig. 3. Progress of enzymic conversion of the second radioactive metabolite into L-tyrosine O[^{35}S]-sulphate. ■, Second radioactive metabolite; ●, L-tyrosine O[^{35}S]-sulphate. Details are given in the text.

centration) completely inhibited the conversion of the metabolite into L-tyrosine O[^{35}S]-sulphate.

Metabolism of L-tyrosine methyl ester by whole supernatant. L-Tyrosine methyl ester (100 μl. of a 0·1 M solution in 0·5 M-potassium dihydrogen phosphate at pH 7·0) was incubated for 30 min. at 38° with 100 μl. of whole supernatant. Protein was removed in the usual way and samples (5 μl.) of the clear supernatant were subjected to paper chromatography in solvent system A. Authentic samples of L-tyrosine methyl ester and free L-tyrosine were used as markers. The dried chromatograms were sprayed with a 0·2% solution of ninhydrin in acetone and heated at 100° for 10 min. Two ninhydrin-positive spots were located on chromatograms, one spot (R_F 0·62) corresponding to that of authentic L-tyrosine methyl ester and the second spot (R_F 0·44) to that of free L-tyrosine. The intensity of the spot corresponding to free L-tyrosine was considerably less in control experiments where water was substituted for whole supernatant in the incubation mixture.

DISCUSSION

The present work, in agreement with that of Segal & Mologne (1959), shows that L-tyrosine methyl ester and L-tyrosine ethyl ester are capable of acting as acceptors for sulphate in the 3′-phosphoadenylylsulphate–phenol-sulphotransferase (EC 2.8.2.1) system of rat liver. Further, it has now been established that the optimum concentration of L-tyrosine methyl ester for this reaction sequence is approx. 30 mM, with little or no sulphating activity apparent at concentrations of acceptor below 2 mM. This could explain the negative results reported for an enzyme system similar to the one described above with L-tyrosine methyl ester at approx. 1 mM concentration (Grimes, 1959). The optimum pH of 7·0 reported in the present paper probably represents the optimum conditions for the synthesis of the adenosine 3′-phosphate 5′-sulphatophosphate and should not be compared with the higher value for the transfer of sulphate from *p*-nitrophenyl sulphate via adenosine 3′,5′-diphosphate to L-tyrosine methyl ester (Segal & Mologne, 1959).

The final product of the incubation of L-tyrosine methyl ester with whole supernatant has been identified as L-tyrosine O[^{35}S]-sulphate. In the presence of the pH 5 supernatant a second radioactive metabolite is produced, the same compound being produced by the whole-supernatant system when higher concentrations of acceptor were employed. This second compound is metabolized, by an enzyme present in whole supernatant, to yield L-tyrosine O[^{35}S]-sulphate, and this reaction is inhibited by L-tyrosine methyl ester. The behaviour of the second metabolite on paper electrophoresis, paper chromatography and ion-exchange chromatography is identical with that of a compound detected in small amounts during attempts to synthesize the O-sulphate ester of L-tyrosine methyl ester by methylation of L-tyrosine O-sulphate.

The collective results lead to the conclusion that the methyl ester of L-tyrosine will accept sulphate from adenosine 3′-phosphate 5′-sulphatophosphate and the initial product then loses its methyl group to produce L-tyrosine O-sulphate. The enzyme catalysing the second reaction is present in whole supernatant and in limited amounts in the pH 5 supernatant. The same enzyme may also be responsible for the removal of the methyl group from L-tyrosine methyl ester, thus explaining the inhibitory effect of this latter compound on the production of L-tyrosine O[^{35}S]-sulphate by whole-supernatant systems.

These findings support the view that a carboxyl-substituted derivative of L-tyrosine with a free amino group may act as sulphate acceptor *in vivo*. Small peptide fragments containing an L-tyrosine residue in the N-terminal position may well act in this capacity. Such peptides, having been sulphated, may in turn yield free L-tyrosine O-sulphate as a result of proteolysis *in vivo*. In this connexion it has already been shown (Jones, Dodgson, Powell & Rose, 1963) that rabbit fibrinopeptide B, which contains one residue of L-tyrosine O-sulphate, is metabolized *in vivo* to yield free L-tyrosine O-sulphate, which can then be recovered in the urine.

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