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Partial Purification and Properties of an Enzyme from Rat Liver that Catalyses the Sulphation of L-Tyrosyl Derivatives

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1. An enzyme that catalyses the transfer of sulphate from adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate to L-tyrosine methyl ester and tyramine was purified approx. 70-fold from female rat livers. 2. The partially purified preparation is still contaminated with adenosine 3′-phosphate 5′-sulphatophosphate–phenol sulphotransferase (EC 2.8.2.1), but a partial separation of the two enzymes can be achieved by chromatography on columns of Sephadex G-200 and DEAE-Sephadex. 3. The enzyme responsible for the sulphation of L-tyrosine methyl ester and tyramine is activated by dithiothreitol, 2-mercaptoethanol and GSH, the degree of activation being more marked with preparations previously stored at 0 or −10°C. In contrast, the enzymic sulphation of *p*-nitrophenol is inhibited by all three thiols. Again, there is a quantitative difference in the degree of inhibition of the two enzymes by *o*-iodosobenzoate, *p*-chloromercuribenzoate, *N*-ethylmaleimide and iodoacetate. 4. Mixed-substrate experiments support the hypothesis that the enzyme responsible for the sulphation of L-tyrosine methyl ester and tyramine is separate from that responsible for the sulphation of *p*-nitrophenol. However, *p*-nitrophenol is a potent inhibitor of the sulphation of both tyrosyl derivatives whereas these latter compounds have no effect on the sulphation of *p*-nitrophenol.

The biogenesis of L-tyrosine *O*-sulphate is of interest partly in view of its presence in mammalian urines (see John, Rose, Wusteman & Dodgson, 1966) and particularly as it is a constituent of many mammalian fibrinogens (see Doolittle & Blömback, 1964; Blömback, Blömback & Grondahl, 1965) and a number of mammalian gastrins (see Agarwal *et al.* 1968). Sulphated tyrosine residues are also present in some physiologically active peptides found in certain amphibian skins. It is found in phyllokinin from *Phyllomedusa rohdei* (Anastasi, Bertaccini & Erspamer, 1966), caerulein from *Hyla caerula* (Anastasi, Erspamer & Endean, 1968*b*) and phyllocaerulein from *Phyllomedusa sauvagi* (Bernardi, Bosisio, De Castiglione & Goffredo, 1969). The sulphate ester group is essential for full physiological activity of phyllokinin and caerulein (Anastasi *et al.* 1968*a*,*b*). Finally, the sulphated amino acid has been identified as a constituent of porcine cholecystokinin–pancreozymin (Mutt & Jorpes, 1968).

Evidence suggests that the biosynthesis of L-tyrosine *O*-sulphate involves the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to carboxyl-substituted derivatives of L-tyrosine but not to the free amino acid. The enzymic sulphation of dipeptides containing L-tyrosine in the *N*-terminal position, tyramine, L-tyrosine amide and the methyl and ethyl esters of L-tyrosine has been demonstrated with a partially purified enzyme preparation from rat liver (Segal & Mologne, 1959). With L-tyrosylglycine, L-tyrosyl-L-alanine and the methyl and ethyl esters of L-tyrosine, the product of this reaction has been identified as the corresponding *O*-sulphate ester (Jones & Dodgson, 1965; Jones, Scotland & Dodgson, 1966). Segal & Mologne (1959) suggested that the sulphotransferase catalysing these reactions could not be identified with adenosine 3′-phosphate 5′-sulphatophosphate–phenol sulphotransferase (EC 2.8.2.1) in view of a quantitative difference in their requirement for the cofactor, adenosine 3′,5′-diphosphate, in an enzyme system employing *p*-nitrophenyl sulphate as donor of sulphate. However, Roy (1960) questions this conclusion on the grounds that the adenosine 3′-phosphate 5′-sulphatophosphate produced in such a system is attached to the phenol sulphotransferase and is not readily available for sulphate transfer involving a second enzyme. In support of this argument, the author quotes the failure to transfer sulphate from *p*-nitrophenyl sulphate to dehydroepiandrosterone via adenosine

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3′,5′-diphosphate even in the presence of added steroid sulphotransferase (Gregory & Lipmann, 1957). Banerjee & Roy (1968) have shown that tyrosine methyl ester is a competitive inhibitor of the formation of *p*-nitrophenyl sulphate by the phenol sulphotransferase of guinea-pig liver, suggesting that this enzyme might be capable of using tyrosine derivatives as substrates. In unpublished experiments we have confirmed that the enzyme preparation used by Banerjee & Roy (1968) can transfer sulphate to tyrosine methyl ester.

The present work describes a partial purification from rat liver of the enzyme that catalyses the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to tyrosine methyl ester and its properties are contrasted with those of phenol sulphotransferase.

MATERIALS AND METHODS

Paper chromatography and electrophoresis. Samples (5μl) were applied to Whatman no. 1 paper and subjected to descending chromatography for 16h at room temperature with butan-1-ol–acetic acid–water (50:12:25, by vol.). Samples (5μl) were also subjected to electrophoresis on Whatman no. 1 paper for 2h in 0.1M-sodium acetate–acetic acid buffer, pH4.5; a potential gradient of 11V/cm was applied.

Detection and measurement of radioactivity. Radioactive spots were located and measured on dried chromatograms and electrophoretograms by the method described by Jones & Dodgson (1965).

The total ^{35}S content of solutions was measured by applying samples (100μl) to discs (2cm^2) of Whatman no. 3 paper supported in polythene planchets (Bettix Ltd., New Malden, Surrey, U.K.) and dried at 60°C. The radioactivity on the dried discs was then measured by counting in an IDL type 6050 automatic scaling equipment (Isotope Development Ltd., Beenham, Berks., U.K.) with a thin mica end-window Geiger–Müller tube. All counts were corrected for coincidence and background and sufficient counts were recorded to give a standard error of less than 2%.

Experimental animals. Female M.R.C. hooded rats, 3–6 months old, were used throughout.

Preparation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. Rat liver whole supernatant was prepared as described by Jones & Dodgson (1965) and portions (1.0ml) were incubated in a total volume of 1.8ml containing 20μmol of $MgCl_2$, 10μmol of KH_2PO_4, 2μmol of K_2SO_4, 72μmol of ATP (disodium salt) [Sigma (London) Chemical Co., London S.W.6, U.K.] and 100μCi of $Na_2{}^{35}SO_4$ (carrier-free, code SJS1) (The Radiochemical Centre, Amersham, Bucks., U.K.). All components were adjusted to pH7.5 with 1M-NaOH. After incubation for 1h at 37°C the reaction was stopped by immersing the incubation tubes in a boiling-water bath for 30s and the denatured protein was removed by centrifuging. Isolation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate from the deproteinized incubation mixture was then achieved by the method of Banerjee & Roy (1966). The final preparation was filtered through a Millipore disc (50nm pore size; Millipore Filter Corp., Bedford, Mass., U.S.A.) to remove remaining traces of charcoal.

Determination of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. Solutions containing the radioactive nucleotide were assayed by one of three different methods. First, the method described by Spencer (1960) was used, which measures the quantitative enzymic transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to *p*-nitrophenol. Secondly, the nucleotide was determined by measuring the extinction of its solution at 260nm with a Hilger–Uvispek model H700.308 spectrophotometer (ϵ_{260} 14500; Cherniak & Davidson, 1964). Finally, the total ^{35}S content of solutions of the nucleotide was determined as described above. From the known specific radioactivity of the ^{35}S (10^7 c.p.m./μmol when measured under the conditions described here) and assuming 1mol of ^{35}S/mol of nucleotide, the amount of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was calculated. The three methods of assay, which measure transferable sulphate, nucleotide and ^{35}S content respectively, agreed within 5%. The usual yield of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was approx. 1μmol/g of liver with a specific radioactivity of 10^7 c.p.m./μmol. The preparation was stored as an aqueous 0.2mM solution at −10°C.

Assay of sulphotransferases. The standard assay mixtures for the determination of the activities of sulphotransferases contained 100μl of 0.1M-tris–HCl buffer, 0.48μmol of dithiothreitol (Calbiochem Ltd., Basingstoke, Hants., U.K.), 50μl of an aqueous solution of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate (0.01μmol), 10μl of enzyme preparation and the appropriate acceptor (the dithiothreitol and the acceptor were dissolved in the buffer). The amount of each acceptor and the pH of the final incubation mixture for each acceptor were: *p*-nitrophenol (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; recrystallized twice from water), 0.32μmol, pH7.5; L-tyrosine methyl ester (Ralph N. Emanuel Ltd., London S.E.1, U.K.), 1.0μmol, pH7.5; tyramine [Sigma (London) Chemical Co. Ltd.], 0.6μmol, pH9.0. The inclusion of dithiothreitol in the incubation mixtures produced little stimulation of L-tyrosine methyl ester or tyramine sulphotransferase activity in fresh enzyme preparations. However, the degree of stimulation became more pronounced with preparations that had been stored at 0 or −10°C; the longer the period of storage, the more marked was the degree of stimulation. Maximum activity was attained with 3mM-dithiothreitol or with 20mM-2-mercaptoethanol. In contrast, the activity of phenol sulphotransferase did not decline on storage and the enzyme was inhibited by dithiothreitol and 2-mercaptoethanol. However, for practical convenience the assay mixtures for all substrates contained 3mM-dithiothreitol unless otherwise stated.

All assay mixtures were incubated for 15min at 37°C, the reaction was stopped and the amount of ^{35}S-labelled sulphate ester produced was determined by one of two methods. In the first method, the reaction was stopped by immersing the tubes in a boiling-water bath for 30s and the ^{35}S-labelled sulphate esters were then separated from unchanged nucleotide by paper electrophoresis (when the acceptor was tyramine or L-tyrosine methyl ester) or by paper chromatography (when the acceptor was *p*-nitro-

phenol). The area corresponding to the sulphate ester was located and its radioactivity measured as described above. The second method was essentially that described by Wengle (1964); the reaction was stopped by the addition of 0.4ml of 0.05M-H_2SO_4, and unchanged ^{35}S-labelled nucleotide was precipitated by the addition of 0.4ml of 0.05M-$Ba(OH)_2$ [containing 5ml of aq. ammonia (sp.gr. 0.88) and 10ml of 0.1% (w/v) phenolphthalein in ethanol/l]. Excess of Ba^{2+} was precipitated by bubbling CO_2 through the solution until the phenolphthalein was decolourized. The mixture was then centrifuged at 500*g* for 10min and the ^{35}S-labelled esters in the supernatant were determined by measuring the radioactivity of samples on paper disks as described above. For both methods of assay the amount of sulphate ester was calculated from the measured specific radioactivity of the original adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. Control values were obtained by using identical procedures except that substrate was added immediately before the reaction was stopped. Both methods of assay were compared for all three acceptors and found to agree within 5%. Under these conditions of assay, the rate of production of sulphate ester was linear for 30min and was directly proportional to the amount of enzyme added. With each acceptor, the identity of the corresponding *O*-sulphate ester was presumed from the electrophoretic and chromatographic mobilities of the single radioactive product detected in each case. The R_F values of the ^{35}S-labelled esters produced from L-tyrosine methyl ester, tyramine and *p*-nitrophenol were 0.43, 0.32 and 0.60 respectively (see Jones & Dodgson, 1965; Dodgson, Rose & Tudball, 1959). The ^{35}S-labelled product produced from either L-tyrosine methyl ester or tyramine showed no significant electrophoretic mobility under the conditions described here, whereas that derived from *p*-nitrophenol moved approx. 6.0cm.

One enzyme unit is defined as the amount of enzyme necessary to produce 1μmol of product/h under the conditions described.

Determination of protein. This was by the method of Lowry, Rosebrough, Farr & Randall (1951) or (for later stages of the purification procedure) the extinction at 280nm in quartz cells of 1cm path length was used as a measure of protein concentration.

Assay of other enzymes. The degradation, by various enzyme preparations, of L-tyrosine methyl ester and its *O*-sulphate ester and the enzymic synthesis of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate were measured as described by Jones & Dodgson (1965). The enzymic degradation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was measured by using the standard reaction mixture, described above for the assay of sulphotransferase activity, except that the acceptor was omitted. Radioactive breakdown products of the nucleotide were then detected by paper electrophoresis as described above.

EXPERIMENTAL AND RESULTS

Isolation of the enzyme

All extraction and preparative steps were carried out at 0–4°C. All solutions, employed in the isolation of the enzyme, contained 3mM-dithiothreitol.

Stage 1. Rats were killed by a blow on the head, and the livers were removed, blotted, chilled and then passed through a fine sieve (mesh 1.0mm^2 approx.). Suspensions (20%, w/v) of sieved liver were prepared in 0.25M-sucrose with the aid of a Teflon–glass homogenizer, clarified by centrifuging at 81000$g_{av.}$ for 45min in a Spinco model L preparative ultracentrifuge and the supernatant was retained.

Stage 2. Alumina C_γ gel [Sigma (London) Chemical Co. Ltd.] was added to the stage 1 supernatant (0.4g dry wt. of the gel/g of protein) and the mixture stirred at 0°C for 10min before centrifuging at 800*g* for 10min. The gel was resuspended in 50ml of 0.8M-ammonium sulphate and maintained in suspension by stirring for 10min at 0°C before centrifugation at 800*g* for 10min and the supernatant was retained.

Stage 3. To the supernatant from stage 2 (50ml) was added 9.25g of finely powdered ammonium sulphate with gentle stirring. After 10min at 0°C, the precipitate was collected by centrifugation at 8000*g* for 10min and dissolved in 20ml of 0.05M-hydrochloric acid buffer, pH7.5, containing 0.01M-EDTA and 3mM-dithiothreitol.

Stage 4. The solution from stage 3 was adjusted to pH4.8 by the cautious addition of M-acetic acid. After standing for 10min at 0°C the mixture was centrifuged at 8000*g* for 10min, the precipitate discarded and the supernatant adjusted to pH7.5 with M-sodium hydroxide.

Stage 5. The stage 4 supernatant (approx. 20ml) was stirred with 4g of dry Sephadex G-25 (coarse grade) for 10min at 0°C. The slurry was then transferred to a sintered-glass filter funnel supported in a straight-walled centrifuge tube and the whole assembly centrifuged at 500*g* for 5min at 0°C. The concentrated protein solution was recovered from the bottom of the centrifuge tube and the procedure repeated with a further 2g of dry Sephadex. The final filtrate (approx. 5ml) was applied to a column (2.4cm × 67cm) of Sephadex G-200 equilibrated with 0.05M-tris–hydrochloric acid buffer, pH7.5, containing 0.01M-EDTA and 3mM-dithiothreitol. The column was developed with the same buffer at a flow rate of 30ml/h and the eluate collected in 5ml fractions. The fractions were assayed for protein and for tyrosine methyl ester sulphotransferase activity, and those containing the bulk (80–90%) of the enzyme (usually in the range of fractions 34–48) were combined and retained.

Stage 6. The stage 5 preparation was applied to a column (1.5cm × 15cm) of DEAE-Sephadex A-50 (medium grade) that had been equilibrated with 0.05M-tris–hydrochloric acid buffer, pH7.5, containing 0.01M-EDTA and 3mM-dithiothreitol. The column was then washed with 50ml of the same buffer followed by a linear salt gradient formed from 100ml of the equilibrating buffer and 100ml of the same buffer but containing 0.3M-sodium chloride.

Table 1. *Partial purification of adenosine 3′-phosphate sulphatophosphate–L-tyrosine methyl ester sulphotransferase from rat liver*

Stage	Volume (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Sp. activity (units/mg of protein)	Purification
(1) High-speed supernatant	100	1340	125	100	0.09	1
(2) Alumina C_γ gel adsorption	50	480	96	76.8	0.20	2.2
(3) $(NH_4)_2SO_4$ precipitation (20–50% saturation)	20	180	59	47.2	0.33	3.7
(4) pH4.8 supernatant	20	146	65	52	0.45	5.0
(5) Sephadex G-200 (fractions 34–48)	71	74	57	45.6	0.77	8.6
(6) DEAE-Sephadex (fractions 20–25)	30	4	24	19.2	6.0	66.7

From the initiation of the salt gradient, the eluate was collected in 5ml fractions and assayed for protein and for enzyme activity. The fractions (usually in the range of fractions 20–25) containing material of highest specific activity were pooled and retained. At this stage of purification the enzyme was found to be unstable, at 0°C having a half-life of 1–2 days. Its stability was not significantly improved by increasing the concentration of protein and dithiothreitol to 1mg/ml and 9mM respectively.

In a typical experiment (Table 1) an approx. 70-fold purification of the enzyme was achieved by this procedure, 20% of the original activity being retained. No enzymic degradation of adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate, L-tyrosine methyl ester or L-tyrosine *O*-sulphate methyl ester, nor enzymic synthesis of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate, could be detected with enzyme preparations from stage 4 or subsequent stages.

Samples from each stage of the preparative procedure were also assayed for sulphotransferase activity towards tyramine and *p*-nitrophenol. The results, expressed as sulphotransferase activity towards each substrate relative to the sulphotransferase activity towards L-tyrosine methyl ester, are given in Table 2. It can be inferred that the enzyme catalysing the transfer of sulphate to *p*-nitrophenol is not identical with the enzyme catalysing the sulphation of L-tyrosine derivatives, and a partial separation of the two activities is apparent at stages 5 and 6 of the preparative procedure.

Table 2. *Sulphotransferase activity towards tyramine and p-nitrophenol relative to the sulphotransferase activity towards L-tyrosine methyl ester at various stages of the preparative procedure*

Stage	Sulphotransferase activity relative to that towards L-tyrosine methyl ester: Tyramine as substrate	*p*-Nitrophenol as substrate
(1) High-speed supernatant	0.9	0.6
(2) Alumina C_γ gel adsorption	0.8	0.6
(3) $(NH_4)_2SO_4$ precipitation (20–50% saturation)	0.9	0.7
(4) pH4.8 supernatant	0.7	0.6
(5) Sephadex G-200	0.8	0.4
(6) DEAE-Sephadex	1.0	0.2

Comparison of the properties of phenol sulphotransferase and L-tyrosine methyl ester sulphotransferase

Effect of glutathione. An enzyme preparation was obtained by employing stages 1–4 of the preparative procedure except that dithiothreitol was omitted from all solutions. Samples (10μl) of freshly prepared enzyme were then incubated at 37°C with 50μl of a solution containing glutathione at a concentration of 38mM (expressed as GSH; dissolved in 0.1M-tris–hydrochloric acid buffer, pH7.5) but with GSSG/GSH ratios ranging from zero to infinity. After 30min, 50μl of 0.2mM-adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate was added to each tube followed by 50μl of an appropriate solution of one of the acceptors (L-tyrosine methyl ester, tyramine or *p*-nitrophenol) dissolved in the usual buffer. The concentration of each acceptor, the incubation time and the temperature and the method of assay for sulphotransferase activity were identical with those described above except that dithiothreitol was omitted. The results (Fig. 1) show that the sulphotransferase activity towards L-tyrosine methyl ester and tyramine is maximal in the presence of GSH and minimal in the presence of GSSG. Conversely, the activity of phenol sulphotransferase is maximal in the presence of GSSG and minimal in the presence of GSH. However, the activity of phenol sulphotransferase in the presence of GSSG was approx. 50% of its activity when assayed in the absence of any added glutathione.

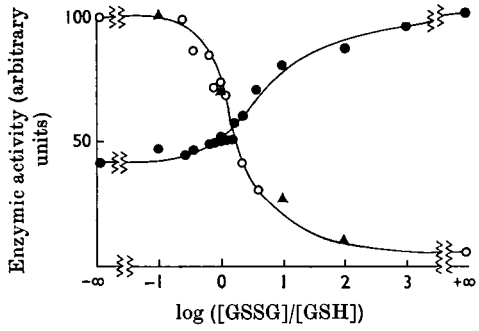

Fig. 1. Rate of synthesis of the *O*-sulphate esters of L-tyrosine methyl ester, tyramine and *p*-nitrophenol by a partially purified enzyme preparation in the presence of different ratios of GSH to GSSG. The final concentration of glutathione (expressed as GSH) in each tube was 12mM. The acceptors used for sulphotransferase assay were: ○, L-tyrosine methyl ester; ▲, tyramine; ●, *p*-nitrophenol. See the text for details.

In the absence of glutathione, the sulphotransferase activity towards L-tyrosine methyl ester and tyramine in this freshly prepared enzyme was approx. 85% of the activity when measured in the presence of GSH.

Effect of thiol-blocking reagents. Again an enzyme preparation was obtained as described in the preceding experiment and samples (10μl) were pre-incubated at 37°C for 30min with various reagents (dissolved in 50μl of the tris–hydrochloric acid buffer, pH7.5) known to react with thiol groups in proteins. Sulphotransferase activity towards L-tyrosine methyl ester and *p*-nitrophenol was then determined as described in the previous experiment. The results (Table 3) show that the two activities are affected to different extents by all the reagents tested.

Mixed-substrate experiment. Enzyme from stage 4 of the preparative procedure was used together with the appropriate standard incubation mixture for the assay of sulphotransferase activity. The K_m values obtained for L-tyrosine methyl ester, tyramine and *p*-nitrophenol at pH7.5 (obtained by the double-reciprocal plot of Lineweaver & Burk, 1934) were 0.3mM, 2mM and 0.06mM respectively. The rate of sulphation of each substrate, alone and in the presence of one of the other substrates, was then measured at pH7.5 in the usual way, the sulphate esters being determined independently after separation by paper chromatography or electrophoresis. In all experiments the relative concentration (defined as the ratio of the substrate concentration to the K_m) of each substrate was 5.0. The experimental results were then compared with theoretical values calculated on the assumption either that one enzyme (adenosine 3′-phosphate 5′-sulphatophosphate phenol sulphotransferase, EC 2.8.2.1) catalyses the transfer of sulphate to all three substrates or that a second enzyme is responsible for the sulphation of the tyrosine derivatives (see Table 4). It can be seen that, as predicted, the sulphation of tyramine and L-tyrosine methyl ester is probably catalysed by the same enzyme. However, the presence of either of these substrates has no significant effect on the rate of sulphation of *p*-nitrophenol. By contrast, *p*-nitrophenol prevents the transfer of sulphate to either tyramine or L-tyrosine methyl ester.

Table 3. *Effect of various thiol-blocking reagents on the sulphotransferase activity towards* L-*tyrosine methyl ester and* p-*nitrophenol*

The enzyme was incubated with each inhibitor (6mM) for 30min at pH7.5 and 37°C before assay of sulphotransferase activity towards each substrate.

Inhibitor	Sulphotransferase activity remaining after 30min (arbitrary units): L-Tyrosine methyl ester as substrate	Sulphotransferase activity remaining after 30min (arbitrary units): *p*-Nitrophenol as substrate
None	100	100
o-Iodosobenzoate	3	13
p-Chloromercuribenzoate	<1	34
N-Ethylmaleimide	3	85
Iodoacetate	24	98

DISCUSSION

The preparative procedures described in this paper provide some evidence for the separate identity of the enzymes responsible for the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to *p*-nitrophenol and to derivatives of L-tyrosine. Chromatography on columns of DEAE-Sephadex and Sephadex G-200 shows partial but definite separation of the two enzymes.

The separate identity of the two enzymes discussed here is again suggested by the results of the mixed-substrate experiment (Table 4). In the case of one enzyme acting on two substrates, the rate of sulphation of either substrate alone would be approximately halved in the presence of the same high relative concentration of the other substrate (see Dixon & Webb, 1964). This situation is apparent during the simultaneous sulphation of tyramine and L-tyrosine methyl ester (Table 4). However, it is not the case when *p*-nitrophenol is used as substrate simultaneously with either of the two tyrosine derivatives, and the situation seems more complex. The simplest interpretation of the

Table 4. *Sulphotransferase activity of stage 4 enzyme preparation in the presence of mixtures of any two of* L-*tyrosine methyl ester*, p-*nitrophenol and tyramine*

The theoretical mixed-substrate velocity (V_M) for each substrate in a mixture, assuming one enzyme, was calculated from the observed velocity (V_A) for each substrate (when assayed alone) from the following expression:

$$V_M = V_A\left(\frac{1+O^-}{2+O^-}\right)$$

where O^- = the relative concn. of the substrate (which is defined as substrate concn./K_m). V_A values (expressed as nmol of ^{35}S/30min) for L-tyrosine methyl ester, tyramine and *p*-nitrophenol were 1.5, 2.3 and 0.68 respectively.

	Amount of sulphate transferred to each acceptor (nmol/of ^{35}S/30min)		
	Experimental value	Theoretical value	
Substrate mixtures		(1) Only one enzyme	(2) Two enzymes
(1) L-Tyrosine methyl ester	0	0.82	1.5
+	+	+	+
p-nitrophenol	0.72	0.37	0.68
(2) L-Tyrosine methyl ester	0.60	0.82	1.5
+	+	+	+
tyramine	1.08	1.25	2.3
(3) Tyramine	0	1.25	2.3
+	+	+	+
p-nitrophenol	0.67	0.37	0.68

results is that *p*-nitrophenol is an inhibitor of L-tyrosine methyl ester sulphotransferase with a K_i value considerably less than 0.06mM (the K_m value for *p*-nitrophenol in the sulphation reaction). Conversely, neither of the two tyrosine derivatives has any effect on the rate of sulphation of *p*-nitrophenol. Banerjee & Roy (1968) have shown that L-tyrosine methyl ester is a competitive inhibitor of phenol sulphotransferase from guinea-pig liver at pH 5.6, but the K_i value quoted for the inhibition was 20mM. This degree of inhibition would not be apparent under the conditions employed here as the concentration of L-tyrosine methyl ester was 2mM and that of *p*-nitrophenol was five times its K_m value.

There is a quantitative difference in the response of the two enzymes to treatment with various reagents that would be expected to interact with thiol groups in proteins. In particular, *N*-ethylmaleimide and iodoacetic acid have a marked effect on the activity of L-tyrosine methyl ester sulphotransferase but very little effect on the activity of *p*-nitrophenol sulphotransferase. Again there is a striking difference in the response of the two enzymes to treatment with different ratios of GSH to GSSG. L-Tyrosine methyl ester sulphotransferase shows maximum activity in the presence of GSH alone and is least active in the presence of GSSG alone. In contrast, *p*-nitrophenol sulphotransferase shows maximum activity in the presence of GSSG alone and the activity is decreased to approx. 40% of this value in the presence of GSH alone. However, even the presence of GSSG, under the conditions described here, decreases the activity of this enzyme to 50% of its activity in the native form. It is not yet clear whether this is an effect on the thiol groups of the protein or a relatively non-specific binding of GSSG by the protein resulting in decreased enzyme activity.

The presence of sulphated tyrosine residues in molecules such as fibrinogen raises the important question of the mode of their incorporation into proteins. By analogy with other 'modified amino acids' it could be argued that the sulphate group is probably introduced when the tyrosine is already part of the completed protein or a polypeptide precursor (see Peterson, 1967). This argument would be supported to some extent by the failure, in this laboratory, to activate L-tyrosine *O*-sulphate with a rat liver enzyme preparation that was capable of catalysing the ATP-dependent activation of L-tyrosine (W. H. B. Denner & J. G. Jones, unpublished work). Again, L-tyrosyl-tRNA synthetase from *Escherichia coli* cannot utilize L-tyrosine *O*-sulphate as substrate, nor is the ester an inhibitor of the enzyme (Calendar & Berg, 1966). However, it seems possible that the sulphate ester group could conceivably be introduced either at the level of the aminoacyl-tRNA or after incorporation of the L-tyrosine residues into a peptide chain. Whichever answer emerges as the true one it seems

likely that a sulphotransferase will participate in the process. Whether the enzyme described here as adenosine 3′-phosphate 5′-sulphatophosphate–L-tyrosine methyl ester sulphotransferase fulfils this function is still obscure, and a detailed investigation of the properties of this enzyme may throw light on the nature of its physiological substrate and role.

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REFERENCES

Agarwal, K. L., Beacham, J., Bentley, P. H., Gregory, R. A., Kenner, G. W., Sheppard, R. C. & Tracey, H. J. (1968). *Nature, Lond.*, **219**, 614.

Anastasi, A., Bernardi, L., Bertaccini, G., Bosisio, G., De Castiglione, R., Erspamer, V., Goffredo, O. & Impicciatore, M. (1968*a*). *Experientia*, **24**, 771.

Anastasi, A., Bertaccini, G. & Erspamer, V. (1966). *Br. J. Pharmac. Chemother.* **27**, 479.

Anastasi, A., Erspamer, V. & Endean, R. (1968*b*). *Archs Biochem. Biophys.* **125**, 57.

Banerjee, R. K. & Roy, A. B. (1966). *Molec. Pharmac.* **2**, 56.

Banerjee, R. K. & Roy, A. B. (1968). *Biochim. biophys. Acta*, **151**, 573.

Bernardi, L., Bosisio, G., De Castiglione, R. & Goffredo, O. (1969). *Experientia*, **25**, 7.

Blömback, B., Blömback, M. & Grondahl, N. (1965). *Acta chem. scand.* **19**, 1789.

Calendar, R. & Berg, P. (1966). *Biochemistry, Easton*, **5**, 1681.

Cherniak, R. & Davidson, E. A. (1964). *J. biol. Chem.* **239**, 2986.

Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 87. London: Longman, Green and Co. Ltd.

Dodgson, K. S., Rose, F. A. & Tudball, N. (1959). *Biochem. J.* **71**, 10.

Doolittle, R. F. & Blömback, B. (1964). *Nature, Lond.*, **202**, 147.

Gregory, J. D. & Lipmann, F. (1957). *J. biol. Chem.* **229**, 1081.

John, R. A., Rose, F. A., Wusteman, F. S. & Dodgson, K. S. (1966). *Biochem. J.* **100**, 278.

Jones, J. G. & Dodgson, K. S. (1965). *Biochem. J.* **94**, 331.

Jones, J. G., Scotland, S. M. & Dodgson, K. S. (1966). *Biochem. J.* **98**, 138.

Lineweaver, H. & Burke, D. (1934). *J. Am. chem. Soc.* **56**, 658.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Mutt, V. & Jorpes, J. E. (1968). *Eur. J. Biochem.* **6**, 156.

Peterson, P. J. (1967). *Biol. Rev.* **42**, 552.

Roy, A. B. (1960). *Adv. Enzymol.* **22**, 205.

Segal, H. L. & Mologne, L. A. (1959). *J. biol. Chem.* **234**, 909.

Spencer, B. (1960). *Biochem. J.* **77**, 294.

Wengle, B. (1964). *Acta Soc. Med. upsal.* **69**, 105.