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Purification from Rat Liver of an Enzyme that Catalyses the Sulphurylation of Phenols

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1. An enzyme (EC 2.8.2.1) that catalyses the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to phenols was purified approx. 2000-fold from male rat livers. 2. The purified preparation did not catalyse the sulphurylation of dehydroepiandrosterone, butan-1-ol, L-tyrosine methyl ester, 1-naphthylamine or serotonin. 3. At pH 8.0 and 37°C the K_m values of the enzyme for *p*-nitrophenol and adenosine 3′-phosphate 5′-sulphatophosphate are 51 and 14 μM respectively. The K_m value for either substrate is independent of the concentration of the other. 4. The sulphurylation of phenol is inhibited by thiol compounds and glutathione at a concentration of 3 mM caused an approx. 50% decrease in enzyme activity. 5. The K_m of the enzyme for adenosine 3′-phosphate 5′-sulphatophosphate is unaffected by the presence of added glutathione but at a concentration of 5 mM-glutathione the K_m of the enzyme for its phenolic substrate is decreased.

The sulphotransferases of mammalian tissue catalyse the transfer of the activated sulphate moiety from adenosine 3′-phosphate 5′-sulphatophosphate to a variety of acceptors (Spencer, 1960). None of these enzymes has been obtained in a pure state, but Banerjee & Roy (1966) described the partial separation from guinea-pig liver of a number of sulphotransferases. Phenol sulphotransferase (adenosine 3′-phosphate 5′-sulphatophosphate–phenol sulphotransferase, EC 2.8.2.1) was purified 17-fold over the crude extract and this enzyme preparation did not catalyse the sulphurylation of steroids but was active towards simple phenols and tyrosine methyl ester. This enzyme required added thiol for full activity and showed no requirement for magnesium ions. Mattock & Jones (1970) purified from female rat livers an enzyme that catalysed the sulphurylation of L-tyrosine methyl ester but this 70-fold purified preparation was contaminated with phenol sulphotransferase. The enzyme from rabbit liver which catalysed the sulphurylation of 5-hydroxytryptamine was purified 67-fold and this partially purified enzyme also catalysed the synthesis of *p*-nitrophenol sulphate (Hidaka, Nagatsu & Yagi, 1969).

The present work describes the purification from male rat liver of an enzyme that catalyses the sulphurylation of simple phenols and which has no activity towards steroids, aliphatic alcohols, aromatic amines and L-tyrosine methyl ester.

MATERIALS AND METHODS

Paper chromatography and electrophoresis. Samples (20 μl) were applied to Whatman no. 1 paper and subjected to descending chromatography for 16 h at room temperature in isobutyric acid–0.5 M-NH_3 (5:3, v/v) or butan-1-ol–acetic acid–water (50:12:25, by vol.). Electrophoresis was carried out on Whatman no. 1, paper for 2 h in 0.1 M-ammonium acetate buffer, pH 5.2, and a potential gradient of 10 V/cm.

Electrophoresis on polyacrylamide gel. Both preparative and analytical gel electrophoresis were performed by the method of Ornstein & Davis (1964) with some modification. The cathode chamber buffer was 0.04 M-diethylbarbituric acid adjusted to pH 7.2 with triethanolamine and the anode compartment buffer consisted of 0.04 M-diethylbarbituric acid containing 0.01 M-citric acid and adjusted to pH 8.4 with triethanolamine. The small-pore gel was prepared by mixing equal volumes of a 7% (w/v) solution of acrylamide in 0.05 M-phosphate–0.01 M-imidazole buffered to pH 8.4 with triethanolamine and an aqueous 0.35% solution of potassium persulphate. The large-pore gel was polymerized by the addition of riboflavin (1 mg %) to a 2.5% (w/v) acrylamide solution in 0.05 M-phosphate–0.01 M-imidazole buffered to pH 6.3 with triethanolamine. Preparative electrophoresis was carried out for 5 h at 400 V in the Shandon (London) apparatus.

Detection and measurement of radioactivity. Radioactive spots were located on dried chromatograms by radioautography with Kodak (Blue Brand BB-54) X-ray film. The areas of the chromatograms containing the ester sulphates were cut out and the radioactivity was counted in a Packard (model 3375) scintillation counter with a toluene phosphor scintillant.

Experimental animals. Male Wistar rats, 3–6 months old, were used throughout.

Preparation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. The liver from a male rat was homogenized with 4 vol. of 0.15 M-KCl by using a Potter–Elvehjem homogenizer and centrifuged at 100 000 *g* for 1 h. A 25 ml sample of the clear supernatant was incubated for 2 h at 37°C with 15 ml of a solution at pH 7.2 and containing

560 μmol of ATP [Sigma (London) Chemical Co., London S.W.6, U.K.], 300 μmol of K_2SO_4, 200 μmol of $MgSO_4$, 1 mmol of KH_2PO_4 and 20 mCi of $Na_2{}^{35}SO_4$ (carrier-free, code SJSI) (The Radiochemical Centre, Amersham, Bucks., U.K.). The reaction was terminated by immersion in boiling water for 1 min and the denatured protein was removed by centrifugation. The adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was isolated and purified by chromatography on a column of ECTEOLA cellulose (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) according to the method of Balasubramanian, Spolter, Rice, Sharon & Marx (1967). The yield of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was 15–20 μmol and the specific radioactivity was approx. 20000 c.p.m./nmol.

Assay of sulphotransferases. The final concentration of dehydroepiandrosterone in the assay mixtures was 50 μM and the final concentration of the other acceptor substrates used in this study was 2.5 mM. With the exceptions of 1-naphthylamine and butan-1-ol (British Drug Houses, Poole, Dorset, U.K.) and L-tyrosine methyl ester (Aldrich Chemical Co., Wis., U.S.A.) all acceptor substrates were purchased from Sigma (London) Chemical Co. Ltd. 1-Naphthylamine and dehydroepiandrosterone were added as solutions in hexane to the reaction tubes and the hexane was removed *in vacuo* before addition of the remaining components of the reaction mixture. The total volume of the assay mixture was 40 μl and contained in addition to the acceptor substrate: 1.6 nmol of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate, 200 μmol of tris–HCl buffer, pH 8, and 10 μl of rat liver enzyme solution. Incubation was for 5 min at 37°C and the reaction was terminated by immersion in boiling water for 1 min. Samples (20 μl) of the protein-free reaction mixtures were chromatographed on Whatman no. 1 paper and the yields of radioactive ester sulphates were determined.

One enzyme unit is defined as the amount of enzyme required to synthesize 1 nmol of ester sulphate in 5 min.

Determination of protein. This was by the method of Lowry, Rosebrough, Farr & Randall (1951) except in the case of eluates from polyacrylamide gels. The protein concentration of fractions obtained from polyacrylamide-gel electrophoresis was determined by a method based on that of Kihara & Kuno (1967). The procedure involved the adsorption of 0–60 μg amounts of protein in 0.2 M-$MgCl_2$ on to a Millipore filter (type HA) under slightly reduced pressure. The filter was washed with 2.5 ml of 0.1 M-$MgCl_2$ and this was followed by 3 ml of 0.1% Amido Black in acetic acid–methanol–water (1:5:4, by vol.). Excess of dye was removed by washing with 2 ml of 1% acetic acid and the Amido Black–protein complex was eluted from the filter in a test-tube with 2.5 ml of 0.1 M-NaOH. The extinction at 620 nm was determined and compared with the standard curve obtained with bovine serum albumin.

EXPERIMENTAL AND RESULTS

Isolation of the enzyme

All extraction and preparative steps were carried out at 0–4°C.

Stage 1. Rats were killed by a blow on the head and the livers were removed, blotted, chilled and homogenized in 4 vol. of 0.15 M-potassium chloride by using a Potter–Elvehjem homogenizer. The suspension was centrifuged at 100000 *g* for 60 min in an MSE Superspeed 75 ultracentrifuge and the supernatant was retained.

Stage 2. The supernatant from stage 1 was diluted with 0.15 M-potassium chloride to a protein concentration of 20 mg/ml and 2-mercaptoethanol was added to a final concentration of 10 mM. Finely powdered ammonium sulphate (108.7 g) was added with stirring and after 30 min the suspension was centrifuged and the precipitate was discarded. The supernatant was retained and to it was added 73.8 g of ammonium sulphate. After stirring for 30 min the suspension was centrifuged and the precipitated protein was retained. This precipitate was dissolved in 60 ml of 5 mM-tris–HCl buffer, pH 8, and dialysed for 72 h against large volumes of the same buffer. During the dialysis, inactive protein precipitated and this was removed by centrifugation and discarded.

Stage 3. Alumina C_γ gel (Sigma) was added to the stage 2 supernatant (1 mg dry wt. of gel/3 mg of protein) and the mixture was stirred at 2°C for 20 min before centrifugation at 30000 *g* for 10 min. The supernatant was discarded and the gel was extracted successively with 20 ml of 0.15 M-EDTA, pH 8, and then with 3 × 20 ml quantities of 0.3 M-EDTA, pH 8. Each extraction was for 20 min at 0°C and the extracts were collected by centrifugation and combined. The combined gel extracts were dialysed for 48 h against large volumes of 5 mM-phosphate buffer, pH 6.5, containing 1 mM-EDTA.

Stage 4. The solution from stage 3 was applied to a column (2 cm × 30 cm) of DEAE-cellulose previously equilibrated with 5 mM-phosphate buffer, pH 6.5, containing mM-EDTA. The column was then washed with 150 ml of the same buffer followed by a linear salt gradient formed from 200 ml of the equilibrating buffer and 200 ml of the same buffer but containing 0.1 M-potassium chloride. Fractions (5 ml) were collected and these were assayed for phenol sulphotransferase activity. The active fractions (fractions 35–49 from initiation of the gradient) were pooled and concentrated by ultrafiltration through a UM-2 membrane (Amicon Corp., The Netherlands). The concentrated enzyme solution was made 10 mM with respect to EDTA.

Stage 5. The stage 4 enzyme preparation (5 ml) was applied to a Sephadex G-200 column (2.5 cm × 40 cm) that had been equilibrated with 0.05 M-tris–HCl buffer, pH 8, containing 10 mM-EDTA. The column was developed with the same buffer at a flow rate of 20 ml/h and the eluate was collected in 3 ml fractions. The fractions were assayed for phenol sulphotransferase activity and those fractions containing the enzyme (fractions 33–39) were combined and concentrated by ultrafiltration. The ionic strength of the enzyme solution was

decreased by repeated washing on the ultrafilter with small quantities of 5mM-tris–HCl buffer, pH 8.

Stage 6. The stage 5 preparation was subjected to electrophoresis on polyacrylamide gel for 6h at 400V after which time the gel was cut transversely into 2mm slices and each slice was extracted with 2×3ml portions of 0.05M-tris–HCl buffer, pH 8. High phenol sulphotransferase activity was found in two gel fractions and these were pooled and concentrated by ultrafiltration.

Stability of the enzyme. The purified phenol sulphotransferase was unstable. The enzyme was, however, stabilized by the presence of EDTA and on storage at 2°C and pH 8 in solution of 10mM-EDTA there was a 20% loss in sulphotransferase activity after 4 days.

In a typical experiment (Table 1) an approx. 2000-fold purification of the enzyme was achieved by the procedure, 26% of the original activity being retained.

A sample (50μg) of the stage 6 enzyme fraction was subjected to electrophoresis on polyacrylamide gel (0.5cm×8cm) for 4h at 300V and protein was detected by staining with Amido Black (0.1%, w/v) in acetic acid–methanol–water (1:5:4, by vol.). One heavily stained and two weakly stained protein bands were detected.

Samples from each stage of the purification procedure were assayed for sulphotransferase activity towards other acceptors. The results, expressed as sulphotransferase activity towards dehydroepiandrosterone and various other substrates relative to the sulphotransferase activity towards *p*-nitrophenol are given in Table 2. The enzyme fraction recovered from stage 6 of the purification procedure had no detectable activity towards the compounds listed in Table 2. The addition of reduced glutathione or Mg^{2+} at concentrations ranging from 0 to 5mM did not restore sulphurylating activity towards these acceptor compounds. Further, it was shown that the following compounds were not substrates for this purified sulphotransferase: 5-hydroxyindole acetic acid, tyramine, oestrone, tri-iodothyronine and L-epinephrine. The purified enzyme had weak activity towards hydroxylated γ-pyrones and also towards 5-hydroxyindole. This catabolite of serotonin was sulphurylated at a rate of 4% of that of *p*-nitrophenol. The Michaelis constant (K_m) and the maximum velocity ($V_{max.}$) of the purified

Table 1. *Purification of adenosine 3′-phosphate 5′-sulphatophosphate–phenol sulphotransferase from rat liver*

Stage	Volume (ml)	Total protein (mg)	10^{-3} × Total activity (units)	Recovery (%)	Sp. activity (units/mg of protein)	Purification
(1) High-speed supernatant	520	10400	15.39	100	1.48	1
(2) $(NH_4)_2SO_4$ precipitation (35–55% saturation)	60	2400	16.39	106	6.83	4.6
(3) Alumina C_γ adsorption	80	360	9.07	59	25.19	17.0
(4) DEAE-cellulose (fractions 35–49)	70	42	6.81	44	162.11	109.5
(5) Sephadex G-200 (fractions 33–39)	21	7.4	5.22	34	705.58	476.7
(6) Polyacrylamide-gel electrophoresis	6	1.24	4.00	26	3224.5	2178.7

Table 2. *Sulphotransferase activity towards dehydroepiandrosterone,* L-*tyrosine methyl ester, butan-*1*-ol,* 1*-naphthylamine and* 5*-hydroxytryptamine relative to the activity towards* p*-nitrophenol at various stages of the purification procedure*

For experimental details see the text.

Stage	Substrate: Dehydroepiandrosterone	Tyrosine methyl ester	Butan-1-ol	1-Naphthylamine	5-Hydroxytryptamine
(1) High-speed supernatant	0.36	0.46	0.14	0.17	1.03
(2) $(NH_4)_2SO_4$ precipitation (35–55% saturation)	0.11	0.07	0.07	0.11	0.80
(3) Alumina C_γ adsorption	0.07	0.05	0.04	0.06	0.15
(4) DEAE-cellulose	0.06	0.04	0.02	0.04	0.09
(5) Sephadex G-200	0.03	0.02	0.02	0.01	0.04
(6) Polyacrylamide gel electrophoresis	0	0	0	0	0

enzyme towards some simple phenols is shown in Table 3.

Properties of the purified phenol sulphotransferase

Effect of metal ion. The effect of added Mg^{2+}, Mn^{2+} and EDTA on the activity of phenol sulphotransferase was investigated. A 0.1ml sample of the purified enzyme was separated from low-molecular-weight substances by gel filtration on a small column (15cm×0.7cm) of Sephadex G-25 (fine grade) equilibrated with 0.05M-tris–HCl buffer, pH8. The enzyme was preincubated for 10min at 37°C in the presence of added Mg^{2+}, Mn^{2+} or EDTA at concentrations in the range 0–10mM and then assayed over 5min with *p*-nitrophenol as acceptor substrate. Neither added metal ion nor EDTA had any effect on the activity of the enzyme.

Effect of pH *on the sulphurylation of* p*-nitrophenol.* A 0.1ml portion of the purified enzyme sample was subjected to gel filtration on a Sephadex G-25 column (15cm×0.7cm) previously equilibrated with water. The enzyme was collected in the excluded volume and its activity determined at a range of pH values by using 0.05M-tris–maleate buffer. The results (Fig. 1) show that the enzyme was active over a broad range of pH but the enzyme was inactivated rapidly at a pH below 6.

Michaelis constant of phenol sulphotransferase for adenosine 3′*-phosphate* 5′[^{35}S]*-sulphatophosphate and* p*-nitrophenol.* The rates of sulphurylation of *p*-nitrophenol were determined over a 5min assay at each of five concentrations of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate between 1 and 20μM and at three different concentrations of *p*-nitrophenol. The effect of the concentration of *p*-nitrophenol on the activity of the sulphotransferase was also determined at three different concentrations of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. The results are expressed as double-reciprocal plots (Lineweaver & Burk, 1934) and the Michaelis constant for each substrate was determined by extrapolating the lines to cut the abscissa. Fig. 2 shows that the reciprocal plots were linear and the K_m for one substrate was independent of the concentration of the second substrate. The K_m values for *p*-nitrophenol and adenosine 3′-phosphate 5′-sulphotransferase were 51 and 14μM respectively.

Table 3. *Comparison of the Michaelis constant* (K_m) *and the maximum velocity* ($V_{max.}$) *relative to that towards phenol of the purified adenosine* 3′*-phosphate* 5′*-sulphatophosphate–phenol sulphotransferase towards some phenolic compounds*

Substrate	K_m (mM)	Relative $V_{max.}$
Phenol	2.8	1.00
m-Aminophenol	8.7	0.66
p-Chlorophenol	0.73	1.28

Effect of thiol-blocking reagents on the biosynthesis of p*-nitrophenyl sulphate.* Samples (10μl) of the purified phenol sulphotransferase were incubated at 37°C for 10min with 10μl quantities of the reagents (dissolved in 0.05M-tris–HCl buffer, pH8) listed in Table 4; sulphotransferase activity towards *p*-nitrophenol was then determined. The final concentration of thiol-blocking reagent in the assay mixture was 0.1mM and the results are expressed relative to the sulphotransferase activity in the absence of thiol-blocking reagents. Under these conditions iodosobenzoate, iodoacetamide and *N*-ethylmaleimide caused slight activation but *p*-chloromercuribenzoate and mercuric chloride were strongly inhibitory.

Effect of glutathione on phenol sulphotransferase activity. The effect of glutathione on sulphotransferase activity was investigated by pre-incubating the purified enzyme with various concentrations of glutathione in 0.05M-tris–HCl buffer, pH8, for 10min at 37°C after which time the sulphotransferase was assayed over 5min by using *p*-nitrophenol as acceptor substrate. The results are expressed relative to the sulphotransferase activity without added glutathione (Fig. 3). Glutathione at a concentration of 3mM caused an approx. 50% decrease in sulphotransferase activity and no further decrease in enzymic activity was observed when the concentration of glutathione was increased to 10mM.

Added glutathione had no effect on the affinity of the enzyme for the nucleotide substrate. The effect of the concentration of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate on the rate of sulphurylation of *p*-nitrophenol in the absence and presence

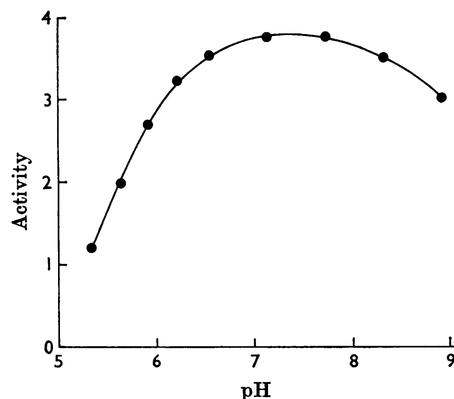
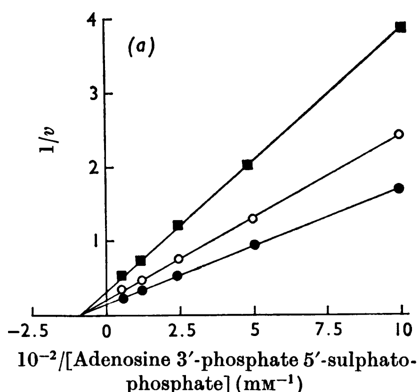


Fig. 1. Effect of pH on the synthesis of *p*-nitrophenyl sulphate by phenol sulphotransferase. See the text for details.



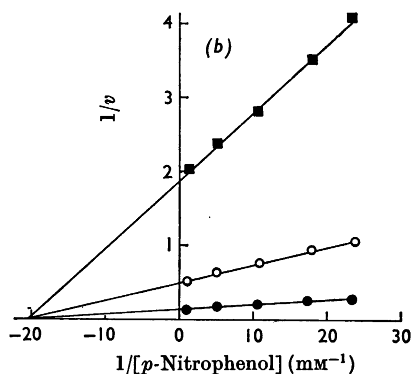


Fig. 2. Effect of varying the concentration of the second substrate on the reciprocal plots for adenosine 3′-phosphate 5′-sulphatophosphate and *p*-nitrophenol at pH 8. (*a*) For adenosine 3′-phosphate 5′-sulphatophosphate at different concentrations of *p*-nitrophenol: ●, 1.0 mM; ○, 0.1 mM; ■, 0.41 mM. (*b*) For *p*-nitrophenol at different concentrations of adenosine 3′-phosphate 5′-sulphatophosphate: ●, 20 μM; ○, 5 μM; ■, 1.25 μM. v = nmol of $^{35}SO_4$ transferred in 5 min.

Table 4. *Effect of various thiol-blocking reagents on the sulphotransferase activity towards* p-*nitrophenol*

The enzyme was incubated with each reagent (0.2 mM and pH 8) for 10 min at 37°C before assay of sulphotransferase activity. The results are expressed relative to the control without added thiol-blocking reagent.

Addition	Relative activity (%)
None	100
o-Iodosobenzoate	102
Iodoacetamide	116
N-Ethylmaleimide	109
p-Chloromercuribenzoate	43
Mercuric chloride	23

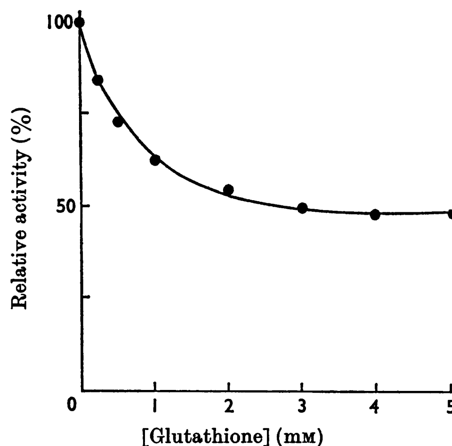


Fig. 3. Effect of the concentration of added glutathione on the synthesis of *p*-nitrophenyl sulphate by phenol sulphotransferase.

of 5 mM-glutathione was investigated. The results are shown as double-reciprocal plots (Fig. 4). The presence of glutathione had no effect on the K_m of the sulphotransferase for adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate but the $V_{max.}$ was lowered. However, glutathione at a concentration of 5 mM did alter both the K_m and $V_{max.}$ of the enzyme for its phenolic substrate. The effect of the concentration of *p*-nitrophenol on the rate of its sulphurylation in the absence and presence of glutathione is shown graphically as a double-reciprocal plot in Fig. 5. The affinity of phenol sulphotransferase for *p*-nitrophenol was increased greatly in the presence of glutathione as shown by a change in K_m from 51 to 21 μM. The K_m of the enzyme for phenol was affected similarly by glutathione and decreased from 2.8 mM to 0.63 mM.

DISCUSSION

The preparative procedures described in this paper permit the isolation of a sulphotransferase in a higher state of purity and more defined specificity than previously achieved. The specificity of the enzyme for its acceptor substrate has not been investigated thoroughly, but it is established that that purified enzyme cannot catalyse the sulphurylation of dehydroepiandrosterone, oestrone, aliphatic alcohols, L-tyrosine methyl ester and 5-hydroxytryptamine. The inability to isolate any other sulphotransferase free from phenol-sulphurylating activity might suggest that there exists in the liver more than one sulphotransferase with activity towards simple phenols. The enzyme purified in this present work may be a specific phenol sulphotransferase, whereas other sulphotransferases may

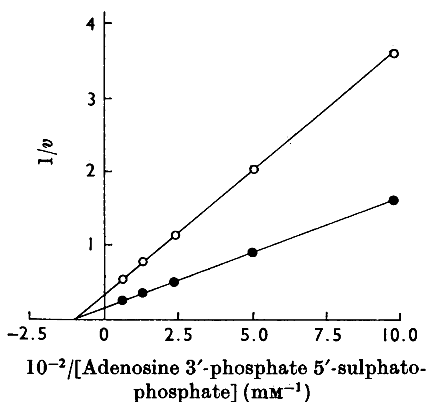


Fig. 4. Effect of added glutathione on the affinity of phenol sulphotransferase for adenosine 3′-phosphate 5′-sulphatophosphate at pH 8. Lineweaver & Burk (1934) plots showing the effect of the concentration of adenosine 3′-phosphate 5′-sulphatophosphate on the rate of synthesis of *p*-nitrophenyl sulphate measured in the absence (●) and presence (○) of 5mM-glutathione.

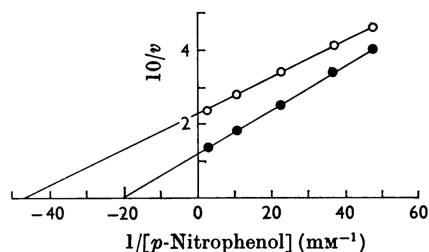


Fig. 5. Effect of added glutathione on the affinity of phenol sulphotransferase for *p*-nitrophenol at pH 8.0. Lineweaver & Burk (1934) plots showing the effect of the concentration of *p*-nitrophenol on the rate of its sulphurylation in the absence (●) and presence (○) of 5mM-glutathione.

have broader specificities which include weak activity towards phenols. This view is supported by the fact that the sulphurylation of phenols by unfractionated rat liver supernatant shows non-linear kinetics for the acceptor substrate: as purification of the phenol sulphotransferase progresses the deviation from linear kinetics disappears.

Glutathione is a potent inhibitor of the purified phenol sulphotransferase but glutathione has no effect on the activity of freshly prepared liver supernatant. The activity of these latter preparations is stimulated by added thiol-binding reagents (Carroll, 1969). This suggests that, in the cell, phenol sulphotransferase exists in a reduced state and becomes oxidized during its purification. The oxidized form of the enzyme is the more active but the reduced enzyme has the greater affinity for its phenolic substrate. The enzyme isolated in this present work has no activity towards the metabolically active phenols tested, which suggests that this enzyme may simply have a detoxifying function *in vivo*. The high affinity of the reduced enzyme for its acceptor substrate would be in accordance with such a function.

Examples of the inhibition of an enzyme by thiols and the activation of the enzyme by thiol-binding reagents are reported in the literature. Fructose 1,6-diphosphatase is activated by thiol-binding reagents and the activation is due to modification of thiol groups at the binding site of AMP, the allosteric inhibitor of the enzyme (Pontremoli, Tramello, Enser, Shapiro & Horecker, 1967; Little, Sanner & Pihl, 1969). Wolfenden, Tomozawa & Bamman (1968) have shown that the GTP binding to AMP aminohydrolase from rabbit muscle is relieved by the addition of mercurials. The inhibition of phenol sulphotransferase does not appear to be due to this type of mechanism and is not connected with inhibition by the products of the reaction (F. A. McEvoy, unpublished work).

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