Thiol-Dependent Changes in the Properties of Rat Liver Sulphotransferases

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1. Two enzymes (A and B) which catalyse the sulphation of *p*-nitrophenol and L-tyrosine methyl ester have been isolated from female rat livers. One of these enzymes (A) also catalyses the sulphation of dehydroepiandrosterone. 2. The K_m values for the sulphation of *p*-nitrophenol and L-tyrosine methyl ester by enzyme B at pH 7.5 are $1.5 \mu\text{M}$ and 2.9 mM respectively. 3. Enzyme B is oxidized on keeping at 0°C when the K_m and V_{max} values for the sulphation of *p*-nitrophenol are increased approx. 200-fold and fourfold respectively. This oxidized preparation of enzyme B fails to catalyse the sulphation of L-tyrosine methyl ester. 4. When the oxidized form of enzyme B is kept at 0°C and low ionic strength then further forms of *p*-nitrophenol sulphotransferase are produced having even lower affinities for the sulphate acceptor. 5. The K_m value for adenosine 3'-phosphate 5' [^{35}S]-sulphatophosphate is not affected during storage of the enzyme under these conditions. 6. Prolonged storage of enzyme B at low ionic strength leads to a considerable degree of polymerization of *p*-nitrophenol sulphotransferase and L-tyrosine methyl ester sulphotransferase. 7. The changes in the kinetic properties and molecular size of enzyme B during storage are reversed by dithiothreitol.

There is conflicting evidence about the effect of thiols on the catalytic activity and molecular size of mammalian sulphotransferases. GSH and cysteine have been found necessary for the maximum activity of phenol sulphotransferase (adenosine 3'-phosphate 5'-sulphatophosphate-phenol sulphotransferase, EC 2.8.2.1) from rabbit and rat livers respectively (Gregory & Lipmann, 1957; Subba Rao, Seshadri Sastry & Ganguly, 1963). Conversely GSH, 2-mercaptoethanol and dithiothreitol have been shown to inhibit the sulphation of *p*-nitrophenol but to enhance the sulphation of derivatives of L-tyrosine by rat liver enzymes (Mattock & Jones, 1970). The molecular weights of phenol sulphotransferases from guinea-pig and rat livers have been estimated as 60 000 and about 200 000 respectively (Banerjee & Roy, 1966; Carroll, 1969). The enzyme from rat livers could be partially dissociated by treatment with 2-mercaptoethanol. The complex kinetics of the sulphation of *p*-nitrophenol by this enzyme preparation were attributed to the presumed different kinetic properties of the molecules of various size which probably co-existed under the experimental conditions that were used (Carroll, 1969). A report by Carroll & McEvoy (1970) claims that the molecular weight of freshly prepared rat liver phenol sulphotransferase is about 60 000, and

that aggregates of larger molecular size are formed on storing in the cold. These aggregates are enzymically active but are dissociated and inhibited by added thiols.

The present paper describes changes in the kinetic properties and molecular size of phenol sulphotransferase from rat livers that occur when preparations are stored in the cold and in the absence of thiol-reducing agents. A preliminary account of this work has already been given (Barford & Jones, 1970).

MATERIALS AND METHODS

Preparation and determination of adenosine 3'-phosphate 5' [^{35}S]-sulphatophosphate. The ^{35}S -labelled nucleotide was prepared and measured as described by Mattock & Jones (1970).

Assay of sulphotransferases. The sulphotransferase activity of enzyme preparations was determined by a modification of the method described by Wengle (1964) as outlined by Mattock & Jones (1970). The assay mixture contained $10 \mu\text{l}$ of enzyme preparation, $0.015 \mu\text{mol}$ of adenosine 3'-phosphate 5' [^{35}S]-sulphatophosphate, $100 \mu\text{l}$ of 0.1 M -tris-HCl buffer, pH 7.5, $50 \mu\text{l}$ of water and *p*-nitrophenol (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; recrystallized twice from water), L-tyrosine methyl ester (Ralph Emanuel Ltd., London S.E.1, U.K.) or dehydroepiandrosterone (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) according to the plan of each experiment.

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Determination of protein. This was by the method of Lowry, Rosebrough, Farr & Randall (1951).

Ion-exchange chromatography. Samples (approx. 150 mg of protein) were applied at 4°C to a column (1.5 cm × 25 cm) of DEAE-cellulose (DE 32; Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, U.K.) that had been equilibrated with 0.01 M-tris-HCl buffer, pH 7.5, containing 0.2 mM-EDTA and 0.01 M-2-mercaptoethanol (buffer solution A). The column was washed with 50 ml of this buffer before applying a linear 0–0.4 M-NaCl concentration gradient in the same buffer. The column was developed at a flow rate of 8 ml/h, the eluate collected in 5 ml fractions and assayed for sulphotransferase activity towards *p*-nitrophenol (0.3 μmol), L-tyrosine methyl ester (1 μmol) and dehydroepiandrosterone (0.1 μmol).

Chromatography on Sephadex G-200. Samples (2 ml) were examined by chromatography at 4°C on a column (1.5 cm × 73 cm) of Sephadex G-200 that had been equilibrated with 0.01 M-tris-HCl buffer, pH 7.5, containing 0.2 mM-EDTA. The column was developed with the same buffer at a flow rate of 8 ml/h and the eluate collected in 2 ml fractions. The fractions were assayed for sulphotransferase activity towards *p*-nitrophenol and L-tyrosine methyl ester as described above.

Concentration of enzyme solutions. Solutions of enzymes containing about 40 mg of protein were dialysed at 4°C for 4 h against 500 vol. of buffer solution A and then applied to a column (0.5 cm × 2.0 cm) of DEAE-cellulose that had been equilibrated with the same buffer at 4°C. The column was developed with 1 M-NaCl in buffer solution A and the eluate run into 5 ml of 10% (w/v) trichloroacetic acid until the first sign of turbidity was noted. The next 2.0 ml of eluate contained the bulk of the enzyme activity (about 30 mg of protein) and was retained.

EXPERIMENTAL AND RESULTS

Preparation of the enzyme. Phenol sulphotransferase was isolated from the livers of female M. R. C. hooded rats (Mattock & Jones, 1970). Enzyme from stage 3 of that preparative procedure was reduced by treatment with 0.01 M-dithiothreitol at 37°C for 5 min. A sample (15 ml) was then subjected to ion-exchange chromatography as described above. Two peaks (A and B) of *p*-nitrophenol and L-tyrosine methyl ester sulphotransferase activity were obtained (Fig. 1). Enzyme A also contains sulphotransferase activity towards dehydroepiandrosterone. When the enzyme preparation was examined without prior reduction, the elution profile of sulphotransferase activity measured towards L-tyrosine methyl ester remained essentially the same although there was some loss of resolution of the two peaks. Tubes corresponding to enzyme A and enzyme B were pooled separately, dialysed for 4 h against 500 vol. of buffer solution A and kept at 0°C. After 1 week, enzymes A and B were examined separately by ion-exchange chromatography and in each case sulphotransferase activity towards *p*-nitrophenol and L-tyrosine methyl ester was found to be eluted from the column as a single homo-

Fig. 1. Chromatography of rat liver sulphotransferases on DEAE-cellulose at pH 7.5. The column was equilibrated with 0.01 M-tris-HCl buffer containing 0.2 mM-EDTA and 0.01 M-2-mercaptoethanol. The enzymes were eluted with a linear 0–0.4 M-NaCl concentration gradient in the same buffer. Sulphotransferase activities were measured with L-tyrosine methyl ester (○), *p*-nitrophenol (▲) and dehydroepiandrosterone (□). One unit is the amount of enzyme necessary to form 1 μmol of product/h under the conditions described in the text.

geneous peak in the same position as the original peak of enzyme A or B. Samples of enzyme B were pooled, concentrated as described earlier and used for subsequent experiments.

Kinetics of sulphation of L-tyrosine methyl ester and *p*-nitrophenol by enzyme B. The rates of transfer of [³⁵S]sulphate from adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate to L-tyrosine methyl ester (final concn. 0–6 mM) and *p*-nitrophenol (final concn. 0–20 μM) were measured separately by using the assay procedure outlined above. The results were expressed as double-reciprocal plots (Lineweaver & Burk, 1934) and the Michaelis constant for each substrate was determined by extrapolating lines to cut the abscissa (Fig. 2). The *K_m* values for L-tyrosine methyl ester and *p*-nitrophenol were 2.9 mM and 1.5 μM respectively.

Time-dependent changes in the catalytic properties of enzyme B. A sample of enzyme B was kept at 0°C and assayed periodically for sulphotransferase activity towards L-tyrosine methyl ester (final concn. 6 mM) and *p*-nitrophenol (final concn. 20 μM). Both enzyme activities decreased to zero in approx. 2 weeks but sulphotransferase activity towards *p*-nitrophenol could be detected at a higher concentration of this substrate. Fig. 3(b) shows the effect of varying the concentration of *p*-nitrophenol on the rate of its sulphation by this preparation of enzyme B that had been kept at 0°C for 3 weeks. The *V_{max}*.

Fig. 2. Effect of varying the concentration of (a) *p*-nitrophenol and (b) *L*-tyrosine methyl ester on the reciprocal plots for the transfer of ^{35}S sulphate from adenosine 3'-phosphate 5' [^{35}S] sulphatophosphate (0.1 mM) with enzyme B. $v = \mu\text{mol}$ of ^{35}S transferred/h per ml of enzyme.

Fig. 3. Effect of varying the concentration of *p*-nitrophenol on the rate of transfer of ^{35}S sulphate from adenosine 3'-phosphate 5' [^{35}S] sulphatophosphate (0.1 mM) with a preparation of enzyme B that had been stored at 0°C at I approx. 1 M for (a) 1 week and (b) 3 weeks. In (b) the points are experimental and the curve is theoretical, calculated from an assumed K_m value of 0.34 mM.

value for the sulphation of *p*-nitrophenol increased fourfold during this period and the K_m value increased to 0.34 mM. Shorter periods of storage merely decreased V_{max} for the sulphation of *L*-tyrosine methyl ester but appeared to complicate the kinetics of the sulphation of *p*-nitrophenol. The rates of sulphation of varying concentrations of *p*-nitrophenol by enzyme B that had been stored for 1 week at 0°C is shown in Fig. 3(a).

All of the original kinetic properties of enzyme B could be restored at any time by treatment with 0.025 M-dithiothreitol at 37°C for 5 min (Table 1). The changes that occur in the catalytic properties of enzyme B during storage may therefore be attributed to the oxidation of thiol groups in the protein. Fig. 4 compares the kinetics of the sulphation of *p*-nitrophenol by an oxidized preparation of enzyme B with the kinetics of the same reaction by the same enzyme preparation after reduction with dithiothreitol as described above.

The oxidation of enzyme B can be expedited by carrying out the usual concentrating procedure in the absence of 2-mercaptoethanol. After 1 day at 0°C this enzyme preparation failed to catalyse the sulphation of *L*-tyrosine methyl ester and the kinetics of the sulphation of *p*-nitrophenol were identical with those shown in Fig. 3(b) and were unaltered after a further 3 weeks at 0°C .

A sample of enzyme B that had been oxidized in 1 day was dialysed for 4 h against 500 vol. of 0.01 M-tris-HCl buffer, pH 7.5, containing 0.2 mM-EDTA and kept at 0°C . The preparation was assayed periodically for phenol sulphotransferase activity (final concentration of *p*-nitrophenol was

Table 1. *Kinetic constants for the sulphation of L-tyrosine methyl ester and p-nitrophenol using enzyme B*

The concentration of adenosine 3'-phosphate 5' [35 S]-sulphatophosphate was 0.1 mM in each experiment. $V_{\max}$ values are as μmol of [35 S]sulphate transferred/h per ml of enzyme.

Enzyme preparation	With <i>p</i> -nitrophenol as variable substrate		With L-tyrosine methyl ester as variable substrate	
	K_m (μM)	$V_{\max}$	K_m (mM)	$V_{\max}$
Freshly prepared enzyme B	1.5	0.61	2.9	0.39
Enzyme B stored for 2 weeks at 0°C and <i>I</i> approx. 1 M	340	2.4	—	—
Enzyme B stored for 2 weeks at 0°C and <i>I</i> approx. 1 M and then treated with 25 mM-dithiothreitol at 37°C for 5 min	1.7	0.60	3.0	0.37

Fig. 4. Effect of varying the concentration of *p*-nitrophenol on the rate of transfer of [35 S]sulphate from adenosine 3'-phosphate 5' [35 S]-sulphatophosphate (0.1 mM) with a preparation of enzyme B that had been stored for 2 weeks at 0°C and *I* approx. 1 M. The enzyme was assayed in the oxidized form (●) and after reduction with dithiothreitol (○).

10 mM) before and after treatment with 0.025 M-dithiothreitol at 37°C for 5 min. The results (Table 2) show that the activity of the oxidized enzyme decreases relative to the activity of the reduced enzyme during a period of 5 weeks. The effect of varying the concentration of *p*-nitrophenol on the rate of its sulphation by the oxidized enzyme after keeping for 3 weeks and 5 weeks is shown in Figs. 5(a) and 5(b) respectively. The results cannot be described by the normal rectangular hyperbola and are consistent with the accumulation, during storage, of one or more other forms of phenol sulphotransferase with a lower affinity for *p*-nitrophenol

than the newly oxidized sample of enzyme B. The kinetics of sulphation of *p*-nitrophenol and L-tyrosine methyl ester by this enzyme preparation after reduction by dithiothreitol remained normal during this period although the $V_{\max}$ value for both reactions decreased approx. threefold.

A dialysed sample of the oxidized enzyme B was kept for 3 weeks at 0°C and then assayed for phenol sulphotransferase activity by using 10 mM-*p*-nitrophenol and various concentrations (5 μM –100 μM) of adenosine 3'-phosphate 5' [35 S]-sulphatophosphate. The experiment was also performed with the reduced form of the same enzyme preparation and with 20 μM -*p*-nitrophenol in the assay mixture. The results (Fig. 6) show that the interaction between enzyme and nucleotide is independent of the state of oxidation of the enzyme.

Effect of dithiothreitol on the sulphotransferase activity of fresh rat liver supernatant. A suspension (20%, w/v) of livers from adult female rats was prepared in 0.25 M-sucrose with the aid of a Teflon-glass homogenizer, clarified by centrifuging at 81 000 g_{av} for 45 min in a Spinco model L preparative ultracentrifuge and the supernatant retained. Samples (20 μl) of the supernatant were assayed for sulphotransferase activity towards L-tyrosine methyl ester (6 mM) and *p*-nitrophenol (20 μM and 10 mM) by using the usual assay mixture both in the absence and presence of 50 mM-dithiothreitol. The results (Table 3) show that the thiol-reducing agent has no effect on these sulphotransferase activities in a freshly prepared liver supernatant.

Behaviour of enzyme B on Sephadex G-200. A freshly prepared sample of enzyme B was examined by chromatography on a column of Sephadex G-200 as described above. The results (Fig. 7a) show that sulphotransferase activity towards L-tyrosine methyl ester and *p*-nitrophenol emerge from the column as a single peak, and the elution volume (73 ml) was slightly larger than that measured for a pure sample (2 mg in 2 ml of buffer) of bovine serum

Table 2. *Time-dependent changes in the activity of the oxidized enzyme B*

The enzyme was kept at 0°C in 0.01M-tris-HCl buffer, pH 7.5, containing 0.2mM-EDTA. Enzyme activity was measured with 10mM-*p*-nitrophenol and with a sample of the enzyme before and after reduction with 0.025M-dithiothreitol at 37°C for 5 min.

Time (days)	Phenol sulphotransferase activity of oxidized enzyme B Phenol sulphotransferase activity of reduced enzyme B
0	3.68
1	2.63
7	1.63
14	1.43
21	1.16
35	0.53

Fig. 5. Effect of varying the concentration of *p*-nitrophenol on the rate of transfer of [^{35}S]sulphate from adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate (0.1 mM) with a preparation of enzyme B that had been stored at 0°C and *I* approx. 0.01 M for (a) 3 weeks and (b) 5 weeks.

albumin (70ml) and corresponds to the elution volume expected for a globular protein of molecular size about 60 000.

Fig. 6. Effect of varying the concentration of adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate on the reciprocal plots for the sulphation of *p*-nitrophenol by a preparation of enzyme B that had been stored for 3 weeks at 0°C and *I* approx. 0.01 M. The enzyme was assayed with 10mM-*p*-nitrophenol without prior reduction (●) and with 20 μM-*p*-nitrophenol after reduction of the protein with dithiothreitol (Δ). *v* = μmol of [^{35}S]sulphate transferred/h per ml of enzyme.

A second sample of enzyme B was concentrated in the absence of 2-mercaptoethanol and dialysed for 4h against 500 vol. of 0.01M-tris-HCl buffer, pH 7.5, containing 0.2mM-EDTA. This preparation was kept at 0°C and samples were examined by chromatography on Sephadex G-200 after 3 weeks and 5 weeks. The kinetics of the sulphation of *p*-nitrophenol by these two samples were identical with those shown in Figs. 5(a) and 5(b) respectively. The results of the gel-filtration experiments (Figs. 7b and 7c) show that changes in the molecular size of phenol sulphotransferase are apparent after 5 weeks at 0°C. Further, identical changes are apparent in the chromatographic distribution of L-tyrosine methyl ester sulphotransferase activity. This heterogeneous sample of the sulphotransferases

Table 3. *Effect of dithiothreitol on the sulphotransferase activity of rat liver high-speed supernatant*

Each enzyme activity was assayed as described in the text.

Sulphate acceptor	Concn. (mM)	Enzyme activity (μmol of [^{35}S]sulphate transferred/h per ml of enzyme)	
		Assayed with 25 mM-dithiothreitol	Assayed without dithiothreitol
L-Tyrosine methyl ester	6.0	0.64	0.65
<i>p</i> -Nitrophenol	10.0	0.24	0.24
<i>p</i> -Nitrophenol	0.02	0.13	0.12

was treated with 0.025M-dithiothreitol at 37°C for 5 min and again examined by chromatography on Sephadex G-200. The sulphotransferase activities now emerged from the column as a single component with an elution profile identical with that shown in Fig. 7(a) for the freshly prepared enzyme B.

DISCUSSION

Two enzymes (A and B), capable of catalysing the sulphation of *p*-nitrophenol and L-tyrosine methyl ester, have been isolated from female rat livers. Enzyme A also catalyses the sulphation of the non-phenolic steroid dehydroepiandrosterone. This situation is analogous to the one described for sulphotransferases from guinea-pig liver (Banerjee & Roy, 1966). In this latter case a relatively specific phenol sulphotransferase was separated by ion-exchange chromatography from a number of steroid sulphotransferases but all the enzymes were capable of catalysing the sulphation of *p*-nitrophenol (Banerjee & Roy, 1966) and L-tyrosine methyl ester (J. G. Jones & A. B. Roy, unpublished work).

From results quoted here it is clear that enzyme B can exist in a number of different forms. The freshly prepared enzyme preparation catalyses the sulphation of L-tyrosine methyl ester and *p*-nitrophenol. The K_m values for these sulphate acceptors are 1.5 μM and 2.9 mM for *p*-nitrophenol and L-tyrosine methyl ester respectively. The K_m value for *p*-nitrophenol is low compared with values quoted elsewhere for phenol sulphotransferase (Banerjee & Roy, 1966; Carroll, 1969; Mattock & Jones, 1970). On standing in the cold, enzyme B is converted into a different form with quite distinct kinetic properties. The new form of the enzyme catalyses the sulphation of *p*-nitrophenol with a K_m of 0.34 mM, and V_{max} for the reaction is fourfold greater than that with the original enzyme B. This enzyme fails to catalyse the sulphation of L-tyrosine methyl ester but the low solubility of the ester precludes experiments at much higher concentrations of sulphate acceptor. All of the original kinetic properties of enzyme B are restored by treatment with the thiol-reducing agent dithiothreitol. It is therefore con-

cluded that the interconversion of these two forms of enzyme B involves the reversible oxidation of thiol-groups in the protein.

A mixture of the oxidized and reduced forms of the enzyme will continue to exhibit normal kinetic properties when assayed with L-tyrosine methyl ester because only the reduced enzyme is active towards this substrate. However, with *p*-nitrophenol as substrate, a mixture of the two forms of the enzyme will exhibit complex kinetics as shown in Fig. 3(a).

When the oxidized form of enzyme B is kept at 0°C in a buffer of relatively low ionic strength (I approx. 10 mM) then further changes in its kinetic properties are observed. The results shown in Table 2 and Figs. 5(a) and 5(b) are consistent with the accumulation, during storage of the preparation, of another form or other forms of phenol sulphotransferase with a lower affinity for *p*-nitrophenol. The appearance of these new forms of phenol sulphotransferase does not appear to be directly related to changes in the molecular size of the enzyme, although aggregates of larger molecular size are formed on more prolonged storage. These aggregates are activated and dissociated by reduction with dithiothreitol, when they also show sulphotransferase activity towards L-tyrosine methyl ester.

The results quoted here show that thiol-reducing agents are likely to increase the rate of sulphation of L-tyrosine methyl ester by all enzyme B preparations other than those freshly prepared. The effect of thiols on the rate of sulphation of *p*-nitrophenol on the other hand will clearly depend upon the age of the enzyme preparation, the conditions of storage and the concentration of substrate used for the estimation. A newly oxidized sample of enzyme B is apparently inhibited by dithiothreitol if the enzyme is assayed by using concentrations of *p*-nitrophenol greater than the value at the intersection of the curves shown in Fig. 4 (0.1 mM). Conversely, the same enzyme preparation is apparently activated by dithiothreitol at concentrations of *p*-nitrophenol below this value. A preparation of enzyme B stored at low ionic strength is apparently unaffected by

Fig. 7. Chromatography of enzyme B on a column of Sephadex G-200. The enzyme preparation was examined in the reduced form immediately after isolation (a) and in the oxidized form after storing at 0°C and I approx. 0.01M for 3 weeks (b) and 5 weeks (c). Some fractions were assayed for sulphotransferase activity towards *p*-nitrophenol (10 mM) after reduction with dithiothreitol (○). In the case of the oxidized preparation of the enzyme the fractions were also assayed with *p*-nitrophenol (10 mM) without the addition of dithiothreitol (●). In (a) and (c) fractions were also assayed after reduction with dithiothreitol for sulphotransferase activity towards *L*-tyrosine methyl ester (6 mM; ▲). One unit is the amount of enzyme necessary to form 1 μ mol of product/h under the conditions described in the text.

dithiothreitol after keeping for 3 weeks at 0°C but is again activated on reduction after keeping for 5 weeks even when assayed with 10 mM *p*-nitrophenol (see Table 2). After this time the enzyme preparation also shows some degree of aggregation. This

observation contrasts with that of Carroll & McEvoy (1970) who report that an aggregated form of rat liver phenol sulphotransferase is inhibited by added thiols, although the concentration of *p*-nitrophenol used for the assay was not quoted. A similar result could arise if the aggregated form described here were assayed at a concentration of *p*-nitrophenol considerably greater than 10 mM.

The changes in the properties of enzyme B which have been shown to result from the oxidation of thiol groups is not reflected in the affinity of the enzyme for the sulphate donor adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate. This observation is consistent with the findings that sulphotransferase reactions in general seem to proceed via a rapid-equilibrium random Bi Bi mechanism (Banerjee & Roy, 1966, 1967, 1968; Mattock & Jones, 1970; Orsi & Spencer, 1964). Such a mechanism allows an independent variation in the properties of the two substrate-binding sites. Finally it should be pointed out that the sulphotransferase activity of freshly prepared rat liver supernatant towards *L*-tyrosine methyl ester and *p*-nitrophenol is unaffected by added dithiothreitol. It is therefore presumed that these enzymes exist in the cell in the reduced form and the different conformational states of the oxidized enzyme reported here probably have no significance *in vivo*. It seems unlikely that reversible oxidation and reduction of the enzyme by glutathione *in vivo* can serve as a control mechanism. Determinations of the glutathione content of various tissues, including liver, show that the proportion of the total peptide in the oxidized form is usually of the order of 0.2–3% (Battacharya, Robson & Stewart, 1955; Martin & McIlwain, 1959; Guntherberg & Rost, 1966; Wendell, 1970). Previous results (Mattock & Jones, 1970) show that the sulphotransferase activity of a rat liver preparation towards *L*-tyrosine methyl ester and *p*-nitrophenol was insensitive to changes in the GSSG/GSH ratio when the oxidized form constituted less than 30% of the total glutathione present.

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