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Identification and partial purification of a unique phenolic steroid sulphotransferase in rat liver cytosol

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Phenolic steroid sulphotransferase activity for both oestradiol and oestrone was identified in male rat liver cytosol in the 30000–40000-M_r fractions on gel filtration when activity was assayed at pH 5.5 (pH optimum 5.5–6.0). Activity for oestradiol but not oestrone was found in the 60000–70000-M_r range when assayed at pH 8.0 (pH optimum biphasic, 5.5–6.0 and 7.0–8.0). K_m for oestradiol (1.3 μM) was lower than published values for hydroxysteroid sulphotransferases (15–35 μM) and previously reported oestradiol sulphotransferases (71–85 μM). At above 2 μM-oestradiol phenolic sulphotransferase activity exhibited substrate inhibition. The phenolic steroid sulphotransferase activity was found to be distinct in chromatofocusing from organic-anion-binding and bile acid-binding proteins previously identified in this M_r range. Further purification on hydroxyapatite yielded a 44-fold enriched fraction that contained two monomeric bands, M_r 32500 and 29500.

Hydroxysteroid sulphotransferases I and II have been purified and found to have M_r 180000 and 290000 respectively (Marcus *et al.*, 1980; Lyon & Jakoby, 1980). These enzymes were characterized with dehydroepiandrosterone as substrate and were shown to sulphate the 17-position of oestradiol but did not sulphate the phenolic hydroxy group on oestradiol or oestrone. Singer and co-workers have identified an oestradiol phenolic sulphotransferase activity having M_r 54500 (Singer *et al.*, 1982; Green & Singer, 1983). We have examined the distribution of sulphotransferase activities on gel filtration of hepatic cytosol and identified a unique activity for phenolic steroids in the 30000–40000-M_r fractions. We have previously purified organic-anion-binding (Sugiyama *et al.*, 1982) and bile acid-binding proteins (Sugiyama *et al.*, 1983) from this molecular-mass fraction. However, the oestradiol sulphotransferase activity described in the present paper is distinct from these proteins.

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Materials and methods

Partial purification of sulphotransferase

All studies employed male Sprague–Dawley rats weighing approx. 250g. Cytosol was prepared from homogenates (33% w/v) of at least four rat livers as previously described (Sugiyama *et al.*, 1982, 1983) with 0.01M-sodium phosphate buffer, pH 7.4 (buffer A), containing 0.25M-sucrose as the homogenization buffer. Gel filtration was performed on Sephadex G-75 (superfine grade) with 30ml of cytosol on a 5cm × 101cm column. Fractions were examined for A_{280} and oestradiol sulphotransferase activity (see below). Fraction D from gel filtration (see below) was further fractionated by chromatofocusing as previously described (Sugiyama *et al.*, 1983) with a column (1.5cm × 25cm) of Polybuffer 94 exchanger (Pharmacia, Uppsala, Sweden) eluted with Polybuffer 74/HCl, pH 4.0. The individual column fractions were assayed for 8-anilinonaphthalene-1-sulphonate binding by fluorescence change, lithocholate binding with [^{14}C]carboxy-lithocholate (59mCi/nmol, New England Nuclear) by equilibrium dialysis as previously described (Sugiyama *et al.*, 1983), and oestradiol sulphotransferase activity. Fractions from chromatofocusing having oestradiol sulphotransferase activity were pooled,

adjusted to pH6.7 and applied to hydroxyapatite (0.9cm × 24cm column). The column was eluted with a linear gradient (100ml of 0.01M-potassium phosphate and 100ml of 0.17M-potassium phosphate, pH6.7). The fraction having peak sulphotransferase activity was checked by discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Sulphotransferase assays

Sulphotransferase activities in cytosol and in pooled and individual column fractions were assayed at 37°C as described by Sekura *et al.* (1979) with acetaminophen, 17β-oestradiol, oestrone, cholesterol and *p*-nitrophenol as substrates. The assay conditions for phenols and sterols differed: for phenols 0.2M-sodium phosphate buffer, pH6.5, with 500μM phenol substrate was used, and for sterols 0.2M-sodium acetate buffer, pH5.5, with 50μM sterol substrate was used unless otherwise stated. Other steroids examined included 17β-oestradiol 3-β-D-glucuronide, 17β-oestradiol 17-β-D-glucuronide, 17α-oestradiol, 17β-oestradiol 17-acetate, 17β-oestradiol 3-benzoate, oestrone 3-sulphate, oestrone β-D-glucuronide and dehydroepiandrosterone. Steroid sulphotransferase activity was assayed at pH5.5 (0.2M-sodium acetate buffer) as described by Sekura *et al.* (1979) or at pH8.0 (0.12M-Tris/HCl buffer) as described by Singer *et al.* (1982) and Green & Singer (1983), incubation of assay mixtures being for 60min. With the use of 0.12M-Tris/HCl or 0.1M-sodium phosphate buffer, the pH was varied between 5 and 9 to assess optima with oestradiol and oestrone as substrates. In addition, with the use of sodium phosphate buffer, ionic strength was varied to assess the effect on activity. Activities with taurolithocholate and lithocholate as substrates were assayed as described by Chen *et al.* (1977) and Barnes *et al.* (1979) respectively. All assays employed 3′-phosphoadenosine 5′-phospho[^{35}S]-sulphate (80μCi/μmol; New England Nuclear) with unlabelled carrier (final concentration 50μM). 3′-Phosphoadenosine 5′-phosphosulphate (labelled plus unlabelled) was purified on DEAE-cellulose with a 0.05–1M-triethylamine/carbonate buffer, pH7.6, gradient as described by Sekura *et al.* (1979). Under all conditions with various buffers, ionic strengths and pH values, the reaction rates were linear with time up to 60min and with protein concentration.

Kinetics of oestradiol sulphation

Kinetics of oestradiol sulphotransferase activity were determined with pooled column fractions from the 30000–40000-M_r range (protein 50μg/ml) as enzyme source, 50μM-3′-phosphoadenosine 5′-phospho[^{35}S]sulphate and a range of oestradiol concentrations with acetate buffer, pH5.5. Data were analysed by the procedure of Lineweaver & Burk (1934). The Michaelis constant (K_m) was calculated by the non-linear least-squares method with oestradiol in the concentration range 0.2–2μM.

Results

Sulphotransferase activities were assayed in pooled fractions from gel filtration of hepatic cytosol (Fig. 1). Although not shown in Fig. 1, albumin (M_r 70000) was co-eluted with fraction B, glutathione *S*-transferase activity (M_r 45000) was found in fraction C and fatty acid-binding protein (M_r 14000) was found in fraction E. For oestradiol and oestrone, sulphotransferase activity, assayed in sodium acetate buffer, pH5.5, as described by Sekura *et al.* (1979), was found exclusively in the 30000–40000-M_r fractions (Fig. 2). The specific enzyme activities of sulphotransferases for oestradiol (0.33–0.78nmol/min per mg) and oestrone (0.92–1.22nmol/min per mg) in fraction D were enriched 20–40-fold compared with cytosol (performed on three separate occasions). No sulphotransferase activities for *p*-nitrophenol, cholesterol or acetaminophen were found in the 30000–40000-M_r fractions (Fig. 2). Taurolithocholate sulphotransferase was identified in several fractions, including fraction D (Fig. 2).

Comparison of steroid sulphotransferase activity with a variety of substrates in cytosol with that in fraction D is listed in Table 1. Fraction D had phenolic steroid sulphotransferases activity, as evidenced by activity with oestrone and 17β-oestradiol 17-acetate. Since some activity was detected with 17β-oestradiol 3-benzoate, it is possible that the 17β-position may also be acted on by this activity. Neither the 3- nor the 17-glucuronide of 17β-oestradiol was a substrate for fraction D. Dehydroepiandrosterone, used previously as substrate to purify hydroxysteroid sulphotransferase (Marcus *et al.*, 1980; Lyon & Jakoby, 1980), was not a substrate for fraction D.

When oestradiol sulphotransferase activity was assayed at pH8.0, the activity was mainly associated with fraction B, with little activity in other fractions (Table 2). This finding agrees with previous reports (Singer *et al.*, 1982; Green & Singer, 1983). Oestrone sulphotransferase activity was absent from fraction B when assayed at pH8.0 and was mainly identified with fraction D. This predominance of activity for oestrone in fraction D was even more apparent when assays were performed at pH5.5.

The effects of varying the ionic strength on the

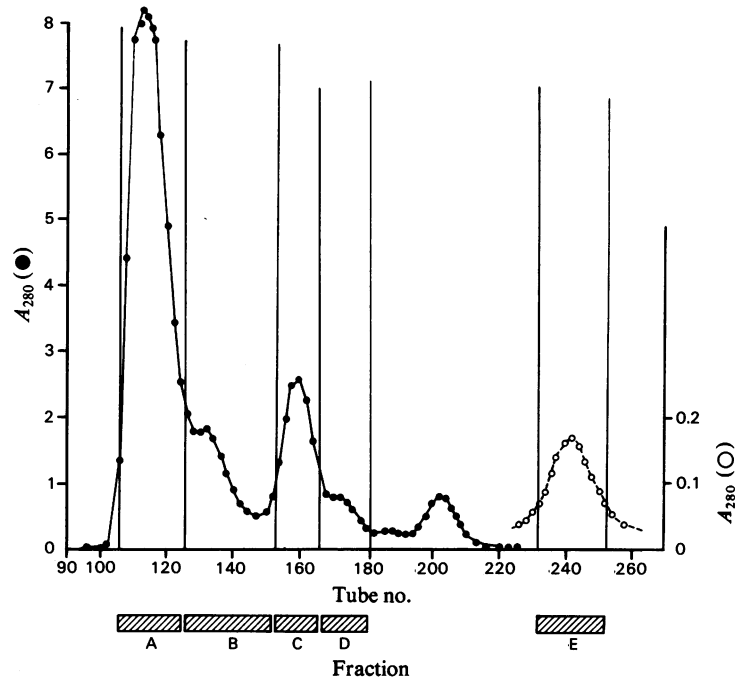

Fig. 1. *Gel filtration of rat liver cytosol*

Pooled protein fractions of different molecular-mass ranges were prepared by gel filtration of 30ml of hepatic cytosol on a Sephadex G-75 (superfine grade) column (5cm × 101cm). Individual fractions (5.3ml each) were pooled as follows: A, tubes 102–122; B, tubes 123–151; C, tubes 152–163; D, tubes 164–180; E, tubes 236–251. Fractions from tubes 181–235 were pooled but had no sulphotransferase activity for any substrate (results not shown).

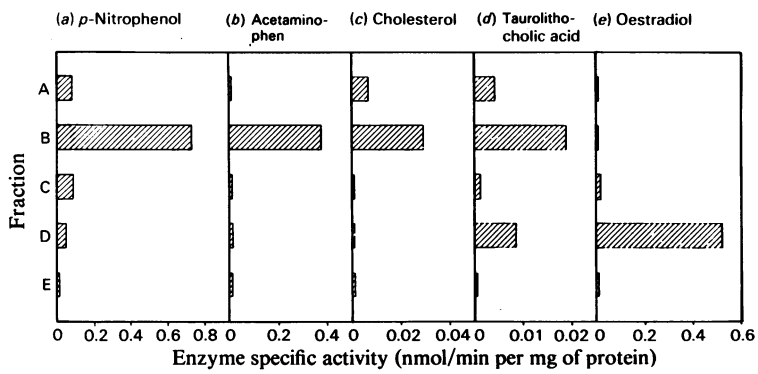

Fig. 2. *Distribution of sulphotransferase activities in pooled fractions of rat liver cytosol*

Sulphotransferase activity was assayed in the pooled fractions, as described in the Materials and methods section, by the method of Sekura *et al.* (1979).

activities of fractions B and E were assessed with the use of sodium phosphate buffer (0.01M to 0.2M). Activity of fraction B with oestradiol was constant at pH8.0 over the ionic-strength range investigated (conductivity 0.9 to 9.1mS) and identical with the results with 0.12M-Tris/HCl (3.2mS). Fraction D exhibited constant sulphotransferase activity (0.03nmol/h per mg) with sodium phosphate buffer at pH5.5 but showed no activity with 0.2M-sodium acetate (5.6mS). However, fraction D exhibited lower activity (approximately one-fifth) in 0.01M-sodium phosphate buffer but achieved maximum activity above 0.05M-sodium phosphate buffer at both pH5.5 and 8.0. At the higher ionic strengths the activities were comparable with the corresponding values

Table 1. *Oestradiol sulphotransferase activity in rat liver cytosol and pooled fractions from gel filtration in the 30000–40000-M_r region*

Fraction D was prepared as indicated in Fig. 1 and the Materials and methods section. Sulphotransferase activity was determined as described in the Materials and methods section. Assays employed 50μM steroid and sodium acetate buffer, pH5.5; a nearly identical ratio of activity of fraction D to that of cytosol was observed with 2μM steroid (not shown). Abbreviation: N.D., not detected.

Substrate	Enzyme specific activity (nmol/min per mg of protein)	
	Cytosol	Fraction D
17β-Oestradiol	0.016	0.78
17α-Oestradiol	0.006	0.64
17β-Oestradiol 3-benzoate	0.008	0.075
17β-Oestradiol 17-acetate	N.D.	0.39
17β-Oestradiol 3-β-D-glucuronide	0.011	N.D.
17β-Oestradiol 17-β-D-glucuronide	0.004	N.D.
Oestrone	0.034	1.22
Oestrone 3-sulphate	N.D.	N.D.
Dehydroepiandrosterone	0.038	N.D.

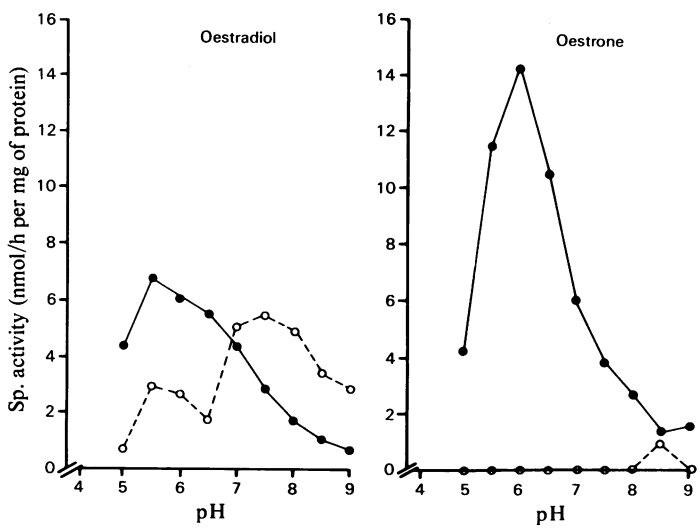

Fig. 3. *Optimum pH values for sulphotransferase activity with oestradiol and oestrone*

Assay conditions were as described in the Materials and methods section. pH was adjusted with HCl added to Tris base. Enzyme source was either fraction B (M_r 50000–70000, ○) or fraction D (M_r 30000–40000, ●).

Table 2. *Effect of pH on sulphotransferase activity*

Activity was determined with 50μM steroid and sodium acetate buffer, pH5.5, or Tris/HCl buffer, pH8.0, as indicated in the Materials and methods section. Abbreviation: N.D., not detected.

Fraction	Enzyme specific activity (nmol/min per mg of protein)			
	Oestradiol		Oestrone	
	pH5.5	pH8.0	pH5.5	pH8.0
A	N.D.	0.022	N.D.	0.018
B	N.D.	0.40	N.D.	N.D.
C	N.D.	0.013	N.D.	N.D.
D	0.39	0.051	0.92	0.071
E	N.D.	N.D.	N.D.	N.D.
Cytosol	0.014	0.049	0.030	N.D.

with sodium acetate and Tris/HCl buffers noted above.

The comparision of the pH optima of sulphotransferase activity in fractions B and D is shown in Fig. 3 with the use of Tris/HCl buffer. Nearly identical results were observed with 0.2M-sodium phosphate buffer (not shown). With oestrone an optimum at pH6.0 for fraction D was found, with little activity in fraction B. With oestradiol as substrate fraction D again showed a pH5.5–6.0 optimum, whereas fraction B demonstrated a biphasic pH optimum (5.5–6.0 and 7–8). Fraction B did not exhibit activity at pH5.5 with oestradiol when sodium acetate buffer was used, but did so when Tris/HCl or sodium phosphate buffer was used.

The kinetics of oestradiol sulphotransferase activity were studied over a concentration range of oestradiol with pooled gel-filtration fraction D as enzyme source; the K_m for oestradiol was found to be 1.3 μM (Fig. 4). Above 2 μM (up to 100 μM)-oestradiol, marked substrate inhibition was observed.

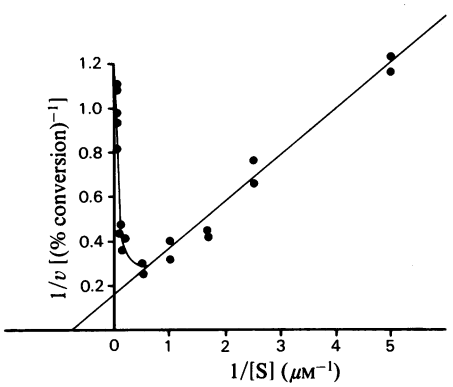

Fig. 4. *Lineweaver–Burk plot of oestradiol sulphotransferase activity*
Pooled fraction D from Fig. 1 was used as enzyme source. The reaction mixture contained 50 μg of protein/ml and 50 μM-3′-phosphoadenosine 5′-phosphosulphate. Incubations were carried out for 10 min with a range of oestradiol concentrations. The assay conditions were as described by Sekura *et al.* (1979).

The pooled fractions in the 30000–40000-M_r range from gel filtration, containing oestradiol sulphotransferase activity, were subsequently chromatofocused. As shown in Fig. 5, the oestradiol sulphotransferase activity was eluted at pH 5.1, whereas the previously identified 8-anilinonaphthalene-1-sulphonate-binding and lithocholic acid-binding activities were eluted at distinctly higher pH values. Taurolithocholate sulphotransferase activity was not detected in any of the fractions from chromatofocusing.

The pooled activities from chromatofocusing were chromatographed on hydroxyapatite. The peak activity, which was eluted at a conductivity of 2 mS, was enriched less than 2-fold over fraction D (Table 3), and, as shown in Fig. 6, was represented by two bands of roughly equal staining on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (M_r 32500 and 29500). It remains uncertain whether one or both of these proteins have enzyme activity. Further purification was hampered by marked instability of the enzyme activity. Storage of samples and purification in the presence of 1 mM-2-mercaptoethanol did not improve these results.

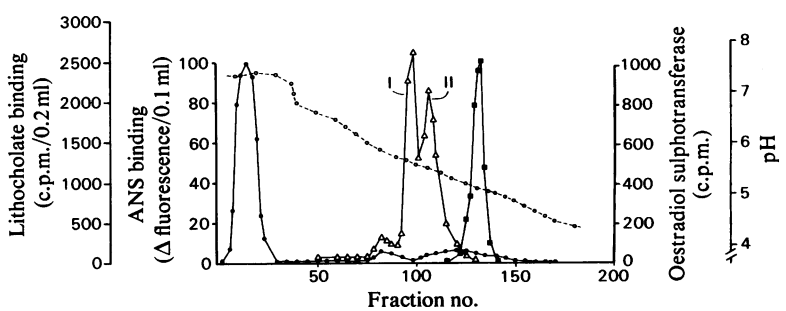

Fig. 5. *Chromatofocusing of the 30000–40000-M_r fractions from gel filtration*
Individual fractions were assayed for pH (●), 8-anilinonaphthalene-1-sulphonate (ANS) binding (○), lithocholate binding (△) and oestradiol sulphotransferase activity (■) assayed by the method of Sekura *et al.* (1979). ------, pH.

Table 3. *Purification of oestradiol sulphotransferase*
For experimental details see the Materials and methods section.

Step	Protein (mg)	Specific activity (nmol/min per mg of protein)	Purification (fold)
Cytosol		0.016	1
Sephadex G-75 (fraction D)	175	0.33*	20
Chromatofocusing	9	0.26	16
Hydroxyapatite	0.75	0.71	44

* N.B.: variability was found in enrichment of fraction D over cytosol from run to run.

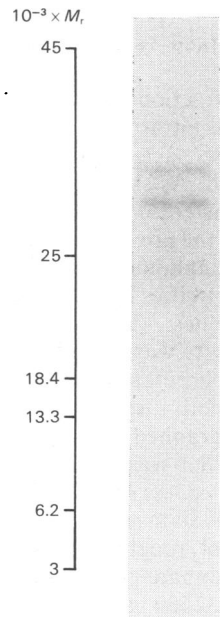

Fig. 6. *Discontinuous sodium dodecyl sulphate/12.5%-polyacrylamide-slab-gel electrophoresis of phenolic steroid sulphotransferases after hydroxyapatite chromatography*
Approx. 5 μg of fraction with peak enzyme activity was applied (lane on the right). Calibration proteins included ovalbumin (M_r 45000), chymotrypsinogen (M_r 25000), β-lactoglobulin (M_r 18400), lysozyme/cytochrome *c* (M_r 13300), bovine trypsin inhibitor (M_r 6200) and insulin (M_r 3000) and their positions are shown from top to bottom on the left.

Discussion

We examined the distribution of oestradiol sulphotransferase activities in gel filtration of rat liver cytosol in comparison with other substances known to undergo sulphation. This approach differs from that of others, who have initially separated the various sulphotransferases by ion-exchange chromatography. The molecular forms of bile acid sulphotransferases that we observed are in general agreement with findings obtained by Barnes & Spenney (1982) but not with those reported by Chen *et al.* (1977). Our identification of oestradiol sulphotransferase activity in the 30000–40000-M_r fractions, however, has not been previously reported. The purified hydroxysteroid sulphotransferases I and II are known to have activity for oestradiol (K_m 15–35 μM) but not for oestrone (Marcus *et al.*, 1980; Lyon & Jakoby, 1980). The sulphotransferase activity in 30000–40000-M_r fractions was relatively specific for phenolic steroids, including oestrone, and exhibited no activity with dehydroepiandrosterone. It had an affinity for oestradiol that was an order of magnitude greater (K_m 1.3 μM) than that reported for the higher-M_r hydroxysteroid sulphotransferases.

In the work of Singer *et al.* (1982), an oestrone sulphotransferase activity was separated from the major rat liver oestradiol sulphotransferase on DEAE-Sephadex, but the former comprised only a small fraction of the rat hepatic oestradiol sulphotransferase activity. In addition, Singer *et al.* (1982) showed that antiserum to rat glucocorticoid sulphotransferase III immunoprecipitated oestrone sulphotransferase activity. Sulphotransferase III has been reported to have M_r 61500 (Singer *et al.*, 1978). More recently, Green & Singer (1983) have demonstrated that the bulk of 17β-oestradiol sulphotransferase activity was associated with a 54500-M_r protein that could be separated from the hydroxysteroid sulphotransferases and glucocorticoid sulphotransferases in ion-exchange chromatography. However, they observed very little activity for this enzyme with oestrone, and reported discrepant results on the relation between activity for oestradiol and oestrone, first resolving these into two separate peaks on DEAE-Sephadex (Singer *et al.*, 1982) and then showing co-elution (Green & Singer, 1983). They demonstrated a pH optimum for this enzyme of 7.4–8.0. The explanation for our discovering a lower M_r (30000) and high activity with oestrone seems to be due to the utilization of different assay conditions, i.e. pH 5.5 in the method of Sekura *et al.* (1979). Comparing the assay conditions used by Green & Singer (1983) with those used by Sekura *et al.* (1979) clarifies the apparent discrepancy with our results. We also observe oestradiol sulphotransferase activity in the 60000–70000-M_r range for oestradiol assayed at pH 8.0 with the use of Tris/HCl buffer. However, even at this higher pH the bulk of activity with oestrone is in the 30000–40000-M_r fractions (Table 2). Thus we have identified a unique phenolic steroid sulphotransferase activity that has the bulk of activity with oestrone.

We have observed marked substrate inhibition of the oestradiol sulphotransferase at above 2 μM-oestradiol. Only slight substrate inhibition was noted by Singer *et al.* (1982), and only above 120 μM-oestradiol. Recently, more-marked substrate inhibition has been observed in mouse placenta (Hobkirk *et al.*, 1983), similar to our findings in rat liver.

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