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PURIFICATION AND IMMUNOCHEMICAL CHARACTERIZATION OF A RAT LIVER SULPHOTRANSFERASE CONJUGATING PARACETAMOL

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Abstract—Paracetamol sulphotransferase (ST) was purified 250-fold from male rat liver, and the pure enzyme used to elicit antibodies in rabbit. The enzyme was active towards paracetamol at pH 9.0, as well as towards several commonly used drugs, and formed sulphates at both O- and N-atoms. Comparison of the substrate specificity of paracetamol ST with that of aryl sulphotransferases isolated by other workers suggested that we have purified a previously unknown isoenzyme of rat liver ST, although the difficulties of characterization of STs based on their substrate specificities is noted. The antibody preparation recognized only one polypeptide (M_r = 35,000) on immunoblot analysis of rabbit liver cytosol, corresponding to purified paracetamol ST. Analysis of the tissue distribution of this protein demonstrated that its expression was restricted to the liver, as was the enzyme activity. The observed sex difference in paracetamol ST (males > females) was determined by immunoblot analysis to be the result of reduced enzyme protein levels in females. In human liver cytosol, the antibody recognized two polypeptides, probably corresponding to M- and P-phenol STs, suggesting significant sequence similarity between rat and human phenol sulphotransferases.

Conjugation with sulphate represents a major route of inactivation and excretion of a wide range of xenobiotics, as well as endogenous compounds such as catecholamines, steroid hormones and bile salts. These reactions are catalysed by the sulphotransferases (ST†)—a family of isoenzymes found principally in the cytoplasm of most body tissues [1–3]. In most cases, the addition of the sulphate group (from the donor molecule adenosine 3′-phosphate 5′-phosphosulphate, PAPS) significantly increases the polarity of the compound, thereby facilitating excretion in the urine or bile. Biological activity of xenobiotics is usually decreased as a result of sulphate conjugation, however there are some examples of increased toxicity or carcinogenicity following sulphation—particularly in the case of the *N*-hydroxyarylamines [2]. Although much work has been done on the purification and characterization of different STs at the level of substrate specificity, there is little information as to the molecular origins of the heterogeneity of this enzyme family, and our overall understanding of the organization of this complex group of enzymes is confused. This is particularly true for the sub-family of STs involved in the sulphation of small planar phenolic molecules, the aryl (or phenol) sulphotransferases (ASTs or PSTs). In rats this group of STs has been subdivided to accommodate at least four isoenzymes—ASTs I–IV [2, 4]. Although these enzyme activities can be resolved chromatographically [2, 4, 5] they have very similar substrate specificities, and it is difficult to distinguish between members of the group without purification of the enzyme activities, and even then classification is awkward. It is widely accepted that these four isoforms fall into two sub-groups—ASTs I and II constitute one group, characterized by a pH optimum for 2-naphthol sulphation of 6.6, and the more acidic ASTs III and IV forming another group with maximal activity towards 2-naphthol at pH 5.5 [1, 4]. These two sub-groups are immunologically distinct, but the two members of each group have extremely similar properties, including substrate specificity. The availability of molecular probes for the different members of this family will further our understanding of this important group of proteins and assist in correlating enzyme activities with individual isoenzymes.

Paracetamol is an extremely widely used analgesic drug, and following therapeutic doses is eliminated from the body principally as the sulphate conjugate and the glucuronide [6, 7]. However, even in moderate overdose it can result in severe (often fatal) hepatic failure [8–10]. In normal human volunteers, the ratio of glucuronide to sulphate conjugates formed from therapeutic doses of paracetamol exhibits a large degree of variability (>5-fold), with the most frequent ratio being approximately 1 [11], and recent evidence of a genetic polymorphism for human platelet thermostable phenol ST suggests a molecular basis for such variation exists [12]. In rats, paracetamol is sulphated by the aryl sulphotransferase (EC 2.8.2.1) sub-family of the STs [2], although which AST isoenzyme(s) is primarily responsible for the reaction has not been conclusively demonstrated [2, 13].

In order to investigate more fully the metabolism

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† Abbreviations: ST, sulphotransferase; PST, phenol sulphotransferase; AST, aryl sulphotransferase; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; DHEA, dehydroepiandrosterone; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; ADP, adenosine-2′,5′-diphosphate.

and toxicity of this important drug, we have isolated and characterized a form of AST from rat liver with a high specific activity for paracetamol sulphation, which is distinct both chromatographically and immunologically from hydroxysteroid ST, and also from the ASTs classified I and II by the nomenclature of Jakoby and co-workers [1, 4, 5]. The purified preparation was also shown to be capable of sulphating other commonly used drugs, such as ritodrine and metoclopramide, suggesting an important role for this protein in protection against toxic side effects of drugs. Immunological characterization of the paracetamol ST, using a polyclonal antibody raised in rabbit against the purified enzyme, showed that the liver is the principal site of expression of this protein in rats, with virtually no appearance of the enzyme in the extrahepatic tissues examined, and that the protein was more abundant in males than in females. In addition, immunological cross reaction with two polypeptides in human liver cytosol was observed.

MATERIALS AND METHODS

Materials. 1-[1-^{14}C]Naphthol (58 mCi/mmol) was purchased from Amersham (Aylesbury, U.K.) and [1,2,6,7-^{3}H]dehydroepiandrosterone (100 Ci/mmol), [^{3}H(G)]4-hydroxyacetanilide (paracetamol) (1.2 Ci/mmol) and [^{35}S]3′-phosphoadenosine-5′-phosphosulphate (1.5 Ci/mmol) were obtained from New England Nuclear (Stevenage, U.K.). Paracetamol, PAPS, DHEA, dopamine, metoclopramide and pargyline were obtained from the Sigma Chemical Co. (Poole, U.K.). 1-Naphthalene methanol was from Aldrich (Gillingham, U.K.), and 1-naphthol, 3-nitrophenol, 4-nitrophenol, 2-aminophenol and 4-chlorophenol were obtained from BDH (Glasgow, U.K.). Ritodrine HCl was from the hospital pharmacy. DEAE-Sepharose Fast Flow, Q-Sepharose Fast Flow, Sephacryl S-200HR and adenosine 2′,5′-disphosphate-agarose were from Pharmacia (Milton Keynes, U.K.). Hydroxylapatite was purchased from Bio-Rad. Electrophoresis reagents were from BDH. Alkaline phosphatase-conjugated anti-rabbit IgG (raised in donkey) was purchased from Sigma, as were the alkaline phosphatase substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt). Other reagents were obtained from commonly used local suppliers, and were of analytical grade whenever possible. All chromatography instrumentation was purchased from Pharmacia, and electrophoresis apparatus was from Hoeffer Scientific Instruments.

Preparation of cytosolic fractions. Adult Wistar rats (approx. 300 g) were used throughout, and were from the colony maintained in the Medical School Animal Unit. Animals were killed by a sharp blow to the head followed by cervical dislocation, and tissues removed into ice-cold homogenization buffer (10 mM triethanolamine, 0.25 M sucrose, 3 mM 2-mercaptoethanol, pH 7.4). Following homogenization in 3 volumes of the above buffer using a Teflon/glass homogenizer, homogenates were centrifuged at 10,000 *g* for 12 min and the resulting supernatant filtered through glass wool and further centrifuged for 50 min at 105,000 *g*. The supernatant was removed with a pasteur pipette, taking care to avoid the layer of lipid at the very surface of the tubes. These cytosolic fractions were then aliquoted and stored at −70° until use (within 3 months). Human liver cytosol (from a 60-year-old male who died from a brain hemorrhage) was prepared in an identical manner.

Enzyme assays. Incubation times and amounts of protein resulting in linear reaction rates were determined and employed throughout.

Paracetamol sulphotransferase activity was measured by the method described by Pacifici *et al.* [14] using 500 μM paracetamol (0.1 μCi) and 320 μM PAPS. The standard buffer employed was 0.5 M Tris–HCl, 5 mM 2-mercaptoethanol, pH 9.0, and incubations were generally carried out for 1 hr. Sulphation of 1-naphthol was measured radiometrically using modifications of a method described by Hjelle *et al.* [15], which is based on the method for assaying 1-naphthol UDP-glucuronosyltransferase first described by Otani *et al.* [16]. Briefly, aliquots (50 μL) of appropriately diluted cytosol (or purification fractions) were incubated with 12 μM [^{14}C]1-naphthol (0.05 μCi) and 6.5 μM PAPS in buffer containing 30 mM potassium phosphate, 1.6 mg/mL bovine serum albumin and 24 mM dithiothreitol, pH 6.6 (or 5.5) in a total volume of 200 μL. Reactions were terminated by the addition of 200 μl of 0.4 M trichloroacetic acid/0.6 M glycine, pH 2.7, and unreacted 1-naphthol was removed by a single extraction with 3 mL chloroform. Following brief centrifugation to separate the phases, an aliquot of the aqueous phase (200 μL) was subjected to liquid scintillation counting in 3 mL Scintran Cocktail EX (BDH).

Sulphation of DHEA was also measured radiometrically by modifications of the method described by Okuda *et al.* [17]. The concentrations of DHEA and PAPS in the incubations were 75 and 200 μM, respectively, and incubations were generally performed for 20 min at 37°. Unreacted DHEA was removed by two extractions with 3 mL diethyl ether, and an aliquot of the aqueous layer was subjected to liquid scintillation counting as described above.

Sulphotransferase activity towards all other substrates were assayed by the barium precipitation method, using [^{35}S]PAPS, originally described by Foldes and Meek [18]. Reactions generally contained enzyme solution, buffer containing 0.25 M Tris–HCl pH 7.15 (pH 8.5 for metoclopramide and ritodrine), 8 mM 2-mercaptoethanol, substrate at 250 μM and 0.05 μCi (200 μM) PAPS in a final volume of 100 μL. When dopamine was used as acceptor substrate, pargyline (an inhibitor of monamine oxidase) was included in the incubation mixture at a concentration of 1.2 mM.

Purification of rat liver paracetamol sulphotransferase. All operations were performed at 4°. Cytosol was prepared in buffer A (10 mM triethanolamine, 0.25 M sucrose, 3 mM 2-mercaptoethanol pH 7.4) from approximately 175 g of adult male Wistar rat liver as described above. Approximately 500 mL of cytosol was applied directly to a column of DEAE-Sepharose Fast Flow (85 × 2.6 cm) in buffer A at a flow rate of 180 mL/hr, and after elution of unbound material, ST activity was

eluted using a linear gradient of 0–0.4 M NaCl in a total volume of 1300 mL buffer A at a flow rate of 60 mL/hr. Fractions (10 mL) enriched in paracetamol ST activity were pooled, concentrated to approximately 40 mL in an ultrafiltration apparatus with a PM30 membrane (Amicon), and dialysed against three changes of 80 volumes of buffer A. The pool obtained was applied to a column of Q-Sepharose Fast Flow (60 × 2.6 cm) at a flow rate of 60 mL/hr and after unbound material had passed through the column, paracetamol ST activity was eluted using a linear gradient of 0–0.25 M NaCl in a total volume of 600 mL buffer A. Fractions of 6 mL were collected and assayed for ST activity, and the paracetamol ST-positive fractions were pooled, concentrated to about 15 mL as above and applied to a column of Sephacryl S-200 HR (100 × 2.6 cm) at a flow rate of 20 mL/hr in buffer A. Fractions (4 mL) were collected and assayed for ST activity, and those containing paracetamol ST activity were pooled, concentrated to about 10 mL and dialysed against buffer B (10 mM potassium phosphate, 0.25 M sucrose, 3 mM 2-mercaptoethanol pH 6.8) and subjected to chromatography on hydroxylapatite (30 × 1.6 cm) at a flow rate of 20 mL/hr in buffer B. Bound material was eluted with a linear gradient of 0.01–0.3 M potassium phosphate in 350 mL buffer B. Fractions containing paracetamol ST activity were pooled, concentrated to approx. 4 mL and dialysed against buffer A prior to affinity chromatography on adenosine 2′,5′-diphosphate-agarose (ADP-Agarose). Briefly, 0.5 mL aliquots of the pooled material were applied to a column (5 × 1.6 cm) of ADP-Agarose equilibrated in buffer A at a flow rate of 40 mL/hr. Once unbound material had been eluted, non-specifically bound protein was removed by washing with two column volumes of 50 mM NaCl in buffer A. Paracetamol ST activity was eluted using a linear gradient of 0–100 μM PAPS in a total volume of 20 ml buffer A containing 50 mM NaCl. A final wash with 0.5 M NaCl in buffer A removed any tightly bound material.

In order to remove a few minor contaminants, the fractions eluted from the affinity column containing paracetamol ST activity were pooled, and the buffer exchanged with FPLC buffer (20 mM triethanolamine, 0.25 M sucrose, 3 mM 2-mercaptoethanol pH 7.4) using a short prepacked column of Sephadex G-25 (PD-10, Pharmacia). Samples (2 mL) were applied to a MonoQ anion exchange column attached to a FPLC apparatus (Pharmacia) at a flow rate of 60 mL/hr. Gradient elution of paracetamol ST was performed using a linear gradient of 0–0.2 M NaCl in FPLC buffer over a period of 30 min. Fractions of 1 mL were collected and analysed by assay for paracetamol ST activity and by SDS–polyacrylamide gel electrophoresis. Fractions containing only one polypeptide species which were positive for paracetamol ST activity were pooled and stored at 4°.

Preparation of anti-paracetamol ST antibody. Electrophoretically pure paracetamol ST was used to elicit antiserum in an adult female New Zealand White rabbit. Initially, 100 μg of purified antigen was mixed with Freund's complete adjuvant, and administered by several intramuscular injections. This was followed by two subsequent booster immunizations 2 weeks apart each of 50 μg purified antigen (from the same preparation) in Freund's incomplete adjuvant. Blood was collected at weekly intervals until antibody production was evident and thereafter every other day until peak titre was reached (about 7 weeks after the first immunization) at which point the rabbit was bled under general anaesthesia to yield about 65 mL antiserum. IgG was enriched from the antiserum by ammonium sulphate precipitation (at 50% saturation) followed by extensive dialysis against 0.1 M phosphate buffer, pH 7.4. Production of specific antiserum was monitored by dot-blot and immunoblot analysis (see below) using both purified paracetamol ST and rat liver cytosol.

Gel electrophoresis and immunoblot analysis. Samples were resolved on 11% gels in the presence of 0.1% SDS, by the method of Laemmli [19], and stained with Coomassie Blue. For immunoblot analysis, proteins resolved by SDS–PAGE were electrophoretically transferred to nitrocellulose (Schleicher and Schuell, BA 85) as described originally by Towbin *et al.* [20], and chromogenic detection of immunoreactive polypeptides was performed using the alkaline phosphatase-linked double antibody method, with the substrates BCIP and NBT as described by Leary *et al.* [21], and modified by Coughtrie *et al.* [22]. The only change made to the above modifications [22] was that all blocking steps, incubations with antibodies and washing steps were performed at pH 9.0, a modification which resulted in a decrease in non-specific background immunostaining.

Protein determination. Protein content was estimated by the method of Lowry *et al.* [23], with bovine serum albumin (Fraction V) as standard.

RESULTS

Purification of rat liver paracetamol sulphotransferase

The method reported here represents considerable modification of published methods for the purification of rat liver PSTs [1, 4, 5]. In our hands, these procedures failed to produce paracetamol ST or sufficient purity for the purpose of raising antibodies, and therefore we introduced extra chromatographic steps in order to remove unwanted impurities.

The purification of rat liver paracetamol ST activity is summarized in Table 1. Chromatography on DEAE-Sepharose Fast Flow resulted in one principal peak of paracetamol ST activity, eluting at 180 mM NaCl (Fig. 1). This peak was separated from the activity towards DHEA ST activity (eluting at approx. 150 mM NaCl). The sulphation of the planar phenol 1-naphthol at two different pH values (6.6 and 5.5) was used to follow the purification of the two groups of PST activity proposed by Jakoby's group [1, 4] [i.e. those corresponding to forms I and II (pH 6.6) and to forms III and IV (pH 5.5)]. The 1-naphthol ST activity at pH 5.5 followed the sulphation of paracetamol on the DEAE-Sepharose column (and indeed throughout the purification procedure), suggesting that paracetamol ST is chromatographically similar on DEAE-cellulose to either the PST III or PST IV of Jakoby's nomenclature. The fractions containing paracetamol ST activity were

Table 1. Purification of sulphotransferase activity towards paracetamol from male Wistar rat liver

Purification step	Specific activity (nmol/min/mg)	Activity (nmol/min)	Yield (%)	Purification (fold)
Cytosol	0.159	1015	100	1
DEAE-cellulose	0.341	503	50	2.2
Q-Sepharose	0.411	171	17	2.6
Sephacryl S-200	0.633	134	13	4.0
Hydroxylapatite	3.12	109	11	20
ADP-agarose	17.5	41	4	110
FPLC/Mono Q	39.6	21	2	250

Enzyme activity was determined with paracetamol as substrate, at pH 9.0 as described in Materials and Methods.

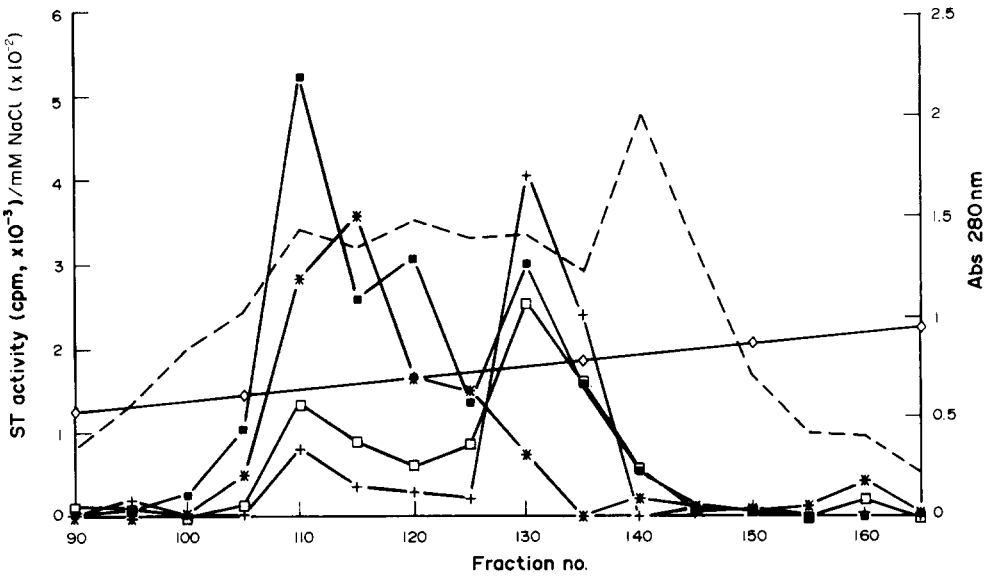

Fig. 1. DEAE-cellulose chromatography of rat liver sulphotransferases. Chromatographic conditions were as described in Materials and Methods. Fractions (10 mL) were collected and assayed for sulphotransferase activity towards paracetamol (□), 1-naphthol at pH 6.6 (□, 1-naphthol at pH 5.5 (+), dehydroepiandrosterone (*). Protein was monitored by absorbance at 280 nm (----). The course of the NaCl gradient is indicated by (◇).

pooled and subjected to chromatography on Q-Sepharose Fast Flow. Although this step did not result in a significant purification of the paracetamol ST activity, it served to refine the separation obtained on the DEAE-Sepharose column, and did result in an improvement in the specific activity of the final preparation compared with purifications when this step was not included (data not shown). Chromatography on hydroxylapatite removed any residual sulphotransferase activity towards DHEA, thereby ensuring that the preparation applied to the ADP-Agarose affinity column contained principally the paracetamol ST isoenzyme. No second peak of 1-naphthol ST activity (at pH 5.5) was observed on hydroxylapatite chromatography, which is contrary to the results of Jakoby's group [5], who claimed that AST III eluted as a trailing peak after AST IV (not shown). Aliquots (approx. 0.5 mL) of the concentrated hydroxylapatite column eluate exhibiting high activity for paracetamol and 1-naphthol (at pH 5.5) were applied to the ADP-Agarose column, and followed elution of non-specifically adsorbed material with 50 mM NaCl, paracetamol ST activity was washed from the column with a gradient of 0–100 μM PAPS, with the enzyme eluting at about 50 μM PAPS (not shown).

The material obtained from the affinity column contained several contaminating polypeptides upon electrophoretic analysis, so the fractions containing paracetamol ST activity were further resolved by FPLC/MonoQ. This yielded protein which was considered of sufficient purity, as determined by SDS–polyacrylamide gel electrophoresis (Fig. 2), for use in raising antibodies. The final preparation was purified 250-fold with respect to the cytosolic activity. Analysis of the purified preparations on SDS–PAGE demonstrated a single polypeptide of M_r = 35,000 as determined by comparison with commercial mol-

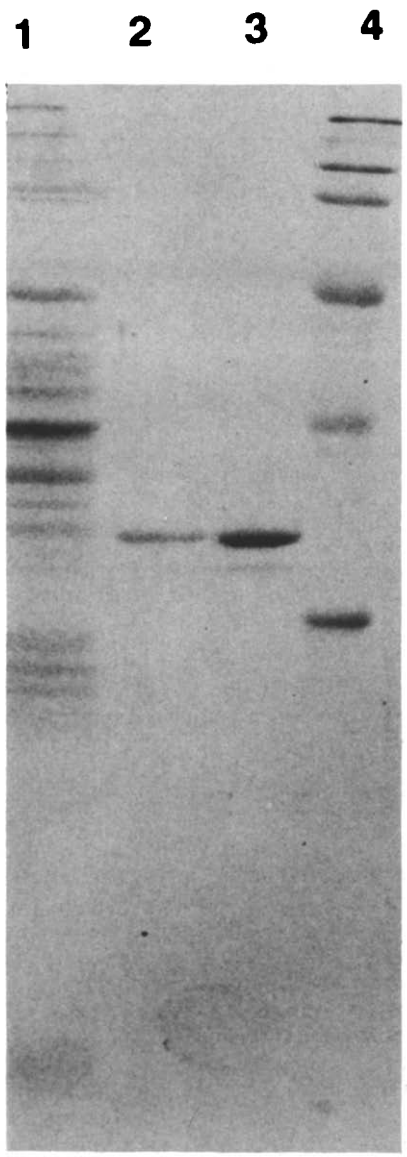

Fig. 2. SDS–polyacrylamide gel electrophoresis of purified rat liver paracetamol sulphotransferase. Samples were subjected to electrophoresis on 11% gels, and proteins were visualized by staining with Coomassie Brilliant Blue. Lane 1, rat liver cytosol (30 μg); Lanes 2 and 3, purified paracetamol ST (2 and 7.5 μg, respectively); Lane 4, protein molecular weight standards (2.5 μg of each): myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa.

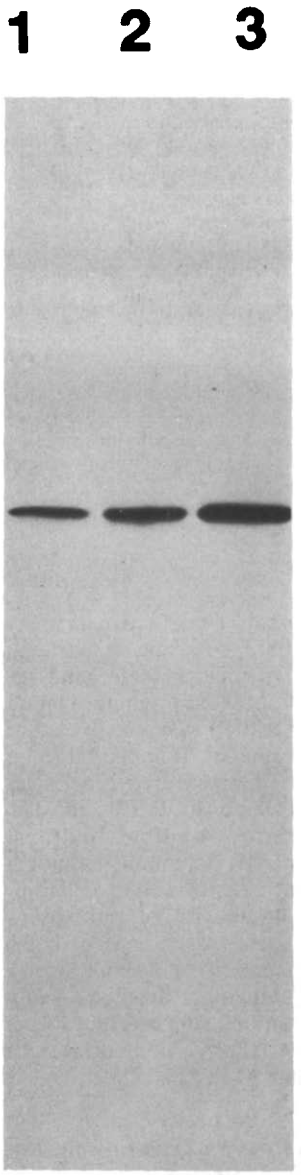

Fig. 3. Immunoblot analysis of purifed rat liver paracetamol sulphotransferase. Samples of purified paracetamol ST (lanes 1, 2 and 3, 0.05, 0.1, 0.2 μg, respectively) were subjected to electrophoresis on an 11% polyacrylamide gel in the presence of 0.1% SDS followed by electrophoretic transfer to nitrocellulose. The immobilized protein was exposed to anti-rat liver paracetamol ST antibody and immunoreactive protein was visualized using an alkaline phosphatase-conjugated 2° antibody and the chromogenic substrates BCIP and NBT.

ecular weight standards (Fig. 2). When larger amounts of purified enzyme were examined by this method, one additional minor band was observed (Fig. 2, lane 3). The identity of this band is not known, but it is possible that it represents a breakdown fragment of paracetamol ST generated during the purification procedure or during storage. The antibody preparation raised against the purified preparation does not recognize this protein on immunoblot analysis of purified paracetamol ST (Fig. 3).

Substrate specificity of purified paracetamol sulphotransferase

The ability of purified preparations of rat liver paracetamol ST to conjugate a range of xenobiotics was determined, and compared with the cytosolic fraction from which it was prepared (Table 2). It was demonstrated that, in addition to paracetamol, the

Table 2. Substrate specificity of purified rat liver paracetamol sulphotransferase

Substrate	Specific activity in rat liver cytosol (nmol/min/mg)	Specific activity of purified paracetamol ST (nmol/min/mg)
Paracetamol	0.171	39.7
1-Naphthol (pH 5.5)	0.555	20.2
Phenol	0.434	5.29
Ritodrine	0.010	3.95
Metoclopramide	0.0*	4.75
1-Naphthalene methanol	0.0*	3.52
4-Chlorophenol	0.0004	0.007
4-Nitrophenol	0.0002	0.008
3-Nitrophenol	0.0002	0.009
2-Aminophenol	0.0007	0.012
Dopamine	0.004	0.084

Enzyme activities were determined on purified male rat liver paracetamol ST and the cytosol fraction from which it was prepared by the procedures outlined in Materials and Methods.

* Below the limit of detection of the assay method [18].

purified preparation was active against several commonly used therapeutic agents, particularly the antiemetic drug metoclopramide, and also ritodrine, widely used to prevent premature labour. While the purified preparation was capable of the extensive sulphation of phenol, it appeared to be sensitive to the presence of substituted groups on the phenol molecule, as demonstrated by the lack of activity towards 4-chloro-, 4-nitro-, 3-nitro and 2-aminophenols. Similarly, dopamine was not conjugated to any significant extent by the purified preparation.

The lack of activity of purified rat liver paracetamol ST towards a range of benzylic alcohols (with the exception of 1-naphthalene methanol), along with the inability to sulphate substituted phenols and dopamine, suggests that this enzyme is not the PST IV of Jakoby's nomenclature since the PST IV has been shown to be active towards these compounds [24].

Production of specific antiserum against rat liver paracetamol ST and immunological characterization of paracetamol sulphotransferase

Purified paracetamol ST (200 μg in total) was used to immunize a female New Zealand White rabbit, and the resulting antiserum was characterized by immunoblot analysis of purified enzyme and cytosolic protein. Figure 3 shows the interaction of IgG purified from the antiserum with purified rat liver paracetamol ST. The antibody preparation appeared specific for this isoenzyme of ST, since it did not cross react with polypeptides upon immunoblot analysis in fractions obtained from other regions of the DEAE-Sepharose column which exhibited ST activity towards 1-naphthol at pH 6.6 or towards DHEA (not shown).

The antibody preparation raised during the course of the current work was used to probe Western blots of total cytosolic protein from male and female rat liver cytosols (Fig. 4). The antibodies recognized a polypeptide of identical electrophoretic mobility (corresponding to a molecular weight of 35,000 Daltons) from both sources. However the amount of immunoreactive protein was decreased in female rat liver cytosol, an observation which agreed with the approx. 50% reduction in paracetamol sulphotransferase activity measured in female rat liver cytosol (Table 3). A similar reduction in 1-naphthol ST activity was also observed, but in agreement with previously published work (e.g. Ref. 25) activity towards DHEA was significantly higher (approx. 9-fold) in female rat liver cytosol, confirming that the antibody preparation does not recognize DHEA ST. Paracetamol sulphotransferase activity was not detectable in rat brain, lung or kidney, and this was confirmed by the fact that the antibodies were not able to detect immunoreactive protein in the cytosolic fractions prepared from these tissues (Fig. 5).

The anti-paracetamol ST antibodies also recognized two polypeptides on immunoblot analysis of human liver cytosol, but the strength of interaction was drastically reduced compared to rat liver. The human liver cytosol used for this experiment exhibited a paracetamol ST activity of 440 pmol/min/mg.

DISCUSSION

We report here the purification of a form of rat liver sulphotransferase conjugating paracetamol to high specific activity. The purified paracetamol ST was also highly active towards phenol, 1-naphthol and certain other drugs, in particular ritodrine and metoclopramide (Table 2)—therefore the enzyme is capable of forming both O-sulphates and N-sulphates. The purified enzyme was used to elicit antibodies by immunization of a rabbit, and the resultant antibodies were used to investigate the tissue- and sex-specific distribution of paracetamol ST by immunoblot analysis.

More than a decade after their first isolation the classification of rat aryl STs is still unclear, and the principal isoenzyme responsible for paracetamol sulphation has until now not been identified. Jakoby's group determined that both AST I and II were capable of sulphating this compound, and had

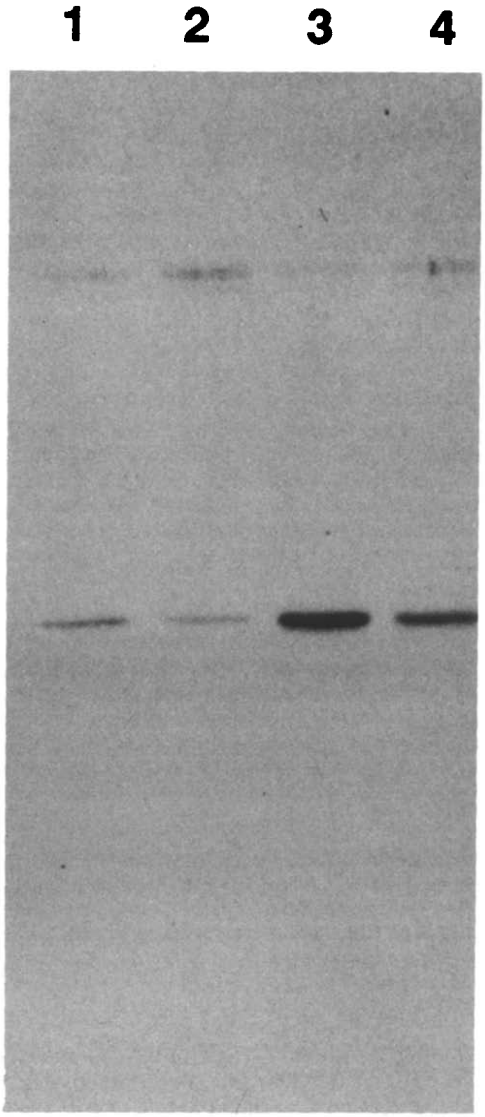

Fig. 4. Sex differences in the expression of paracetamol ST in rat liver. Cytosol fractions prepared from male (lanes 1 [2 μg] and 3 [7 μg]) or female (lanes [2 μg] and 4 [7 μg]) rat liver were electrophoresed on an 11% SDS–polyacrylamide gel and transferred to nitrocellulose. Following incubation with anti-rat liver paracetamol ST antibody, immunoreactive polypeptides were visualized using alkaline phosphatase-conjugated anti-rabbit IgG.

Table 3. Comparison of cytosolic sulphotransferase activities in male and female rat liver

	Paracetamol	1-Naphthol*	DHEA
Male	202 ± 17	491 ± 21	22.3 ± 2.3
Female	113 ± 7	218 ± 11	209 ± 14
Female/male	0.56	0.44	9.4

Enzyme activities were measured as described in Materials and Methods and are expressed as mean pmol/min/mg ± SD of determinations on cytosol fractions prepared from three different age-matched animals.

* Measured at pH 6.6.

almost identical kinetic parameters for the drug. However, these measurements were made at pH 5.5 or 6.6, not at pH 9.0 as used in the current work. At this high pH, the less acidic AST isoenzymes I and II have virtually no activity towards paracetamol (Fig. 1), therefore we suggest that rat liver paracetamol ST activity (whether in cytosol or purified preparations) should be measured at pH 9.0 to ensure that only the contribution of the paracetamol ST isoenzyme is being determined. The activity of paracetamol ST in rat liver cytosol at various pH values was determined to be: 109 (pH 5.5), 164 (pH 6.6), 159 (pH 7.15) and 139 (pH 9.0) pmol/min/mg (means of determinations on three different rat liver cytosol samples), whereas at pH 9.0 1-naphthol ST activity is only approx. 20% of the value at either pH 5.5 or 6.6 (not shown). The paracetamol ST eluted from the DEAE-cellulose column after the point where the AST I and II isoenzymes eluted (i.e. 1-naphthol ST activity at pH 6.6 (Fig. 1), suggesting it to be similar to the AST III/IV sub-group. However it differed from the reported substrate specificity of AST IV in that it was unable to sulphate either a range of benzylic alcohols, except 1-naphthalene methanol, or substituted phenols (Table 2). AST IV has been previously shown to sulphate both of these groups of compounds [1, 24, 26]. AST III has not been fully characterized in respect of substrate specificity, so it is not possible to say unequivocally whether the paracetamol ST reported here corresponds to this isoenzyme, however it is possible that we have isolated a previously unknown form of aryl sulphotransferase. These results illustrate the difficulty encountered in determining the substrate specificities of even the purified enzymes, and the necessity for a molecular approach to the problem of identifying the different isoenzymes of AST.

The anti-paracetamol ST antibody preparation was used to investigate the sex differences in the expression of paracetamol ST in rat liver. Paracetamol was sulphated at approximately twice the rate in male rat liver cytosol than in female liver cytosol (Table 2). A similar difference was observed for 1-naphthol ST activity (measured at pH 6.6), but female liver cytosol was able to sulphate dehydroepiandrosterone almost 10 times as fast as male liver cytosol. When total cytosolic protein from male and female rat liver was subjected to immunoblot analysis with the antibody, the degree of immunostaining was indeed reduced by approximately 50% in female liver compared to male liver, confirming that the reduced enzyme activity in females is the result of a decreased amount of paracetamol ST enzyme protein (Fig. 4). A similar method has been used recently to investigate the molecular basis of the female-specific expression of DHEA ST in rats [27], which was also shown to be directly related to the amount of immunoreactive protein detectable on immunoblot analysis, and to a decreased amount of DHEA ST mRNA.

Investigation of the expression of paracetamol ST in different rat tissues revealed that the enzyme protein is not present to any significant extent in the extrahepatic tissues, corresponding to the absence of paracetamol ST enzyme activity (Fig. 5). AST activity is known to be present in most rat tissues,

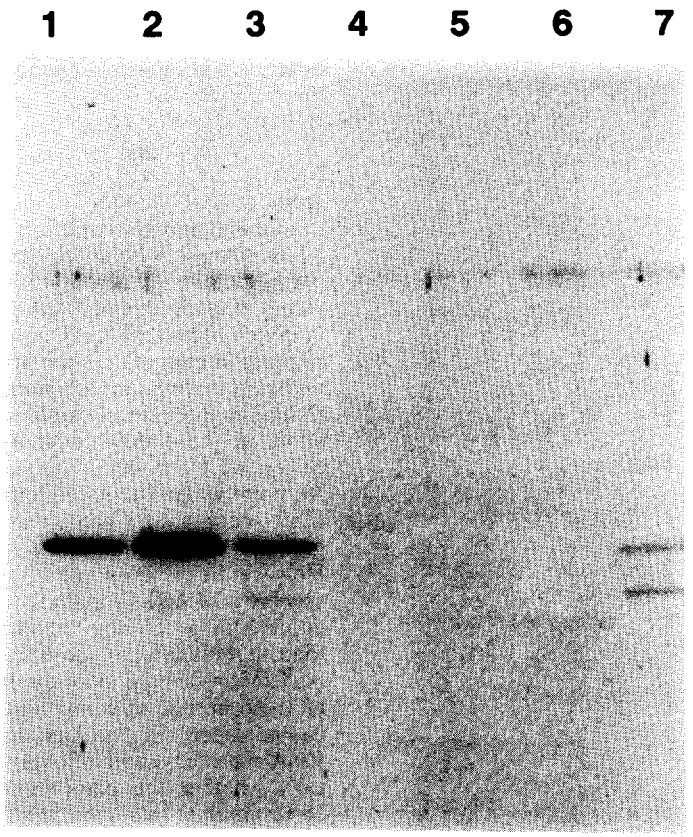

Fig. 5. Immunochemical investigation of the distribution of paracetamol ST in rat tissues and in human liver. Immunoblot analysis was performed exactly as described in the legend to Fig. 4. Lanes 1 and 2, purified rat liver paracetamol ST (0.2 and 0.5 μg, respectively); lane 3, male rat liver cytosol (4 μg); lane 4, rat kidney cytosol (10 μg), lane 5, rat lung cytosol (10 μg); lane 6, rat brain cytosol (10 μg); lane 7 human liver cytosol (40 μg).

as determined by measurements of enzyme activity towards a variety of phenolic substrates [28–30], however it has not been determined which of the different isoforms of AST are responsible for these reactions in the extrahepatic tissues. This report contains the first demonstration of the hepatic-specific expression of a rat AST isoenzyme by immunochemical methods. The restriction of an enzyme such as this to the liver has important implications, as any decrease in the complement of detoxifying enzymes present in an organ is likely to lead to increased risk of toxicity following exposure to harmful compounds or their metabolites which are excreted in the urine.

Human liver cytosol has previously been shown to sulphate paracetamol [14], and we have demonstrated here that the anti-rat liver paracetamol ST antibody recognized two major immunoreactive polypeptides on immunoblot analysis of a sample of human liver cytosol with a high ST enzyme activity towards paracetamol as substrate (Fig. 5). This finding is particularly interesting in the light of recent immunological characterization of human liver phenol sulphotransferases by Heroux *et al.* [31] using an antibody raised against purified human platelet M_{II}phenol sulphotransferase (M_{II}-PST). These investigators found that their antibody reacted on immunoblots with two polypeptides, of molecular weight 32 and 34 kDa, and determined that the 32 kDa protein corresponded to P-PST (the phenol-metabolizing PST) and the 34 kDa protein corresponded to the M-PST. Our antiserum also reacted with 32 and 34 kDa proteins in human liver cytosol, although the amount of protein required to elicit a response was >10-fold higher than for rat liver cytosol, suggesting that (i) our antiserum is recognizing the same two proteins as that of Heroux *et al.* [31], and (ii) the different forms of phenol sulphotransferase in human liver share more sequence similarity than their rat liver counterparts.

In conclusion, we have demonstrated that there is a form of rat liver AST with high specificity towards paracetamol which is also capable of sulphating at N-atoms, and that this enzyme is unlikely to be any of the previously reported rat ASTs. Using an immunochemical method, we have demonstrated that biological variation in paracetamol ST activity in rats correlates with alterations in the amount of enzyme protein present. The necessity for the application of the techniques of molecular biology to the classification of sulphotransferases has been demonstrated, and this work is currently underway in our laboratory.

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