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PURIFICATION AND PROPERTIES OF RAT KIDNEY UDP-GLUCURONOSYLTRANSFERASE

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Abstract—Rat kidney microsomes catalysed the glucuronidation of 1-naphthol, 4-nitrophenol, bilirubin and β-estradiol. Unlike rat hepatic microsomes, UDP-glucuronosyltransferase activity towards morphine and testosterone was not detectable. Treatment of rats with β-naphthoflavone resulted in a 3-fold induction of renal UDPGT activity towards 1-naphthol, 4-nitrophenol and phenol, and a 2-fold induction of bilirubin and β-estradiol glucuronidation. No induction of renal UDPGT was observed after phenobarbital treatment, but renal bilirubin UDPGT activity was specifically induced after treatment of rats with clofibrate. UDPGT activity was purified from rat kidney by a combination of ion-exchange chromatography, gel filtration and affinity chromatography on UDP-hexanolamine Sepharose. One major protein-staining polypeptide was observed on silver-stained SDS–polyacrylamide gels, of molecular weight 55,000 Da, and a minor band of 54,000 Da was also present. Indeed, immunoblot analysis of purified renal UDPGTs with anti-rat liver UDPGT antibodies revealed two immuno-reactive polypeptides of molecular weight 55,000 and 54,0000 Da. The highly purified preparations catalysed the glucuronidation of 1-naphthol and bilirubin. Glucuronidation of bilirubin by purified renal UDPGT preparations required the presence of phospholipid, the activity being further enhanced by incubation with rat lung microsomes. The data presented indicate that two UDPGT isoenzymes have been co-purified.

Conjugation with UDP-glucuronic acid represents a major route of inactivation and elimination of xenobiotics and certain endogenous compounds, such as steroid hormones and bilirubin [1]. UDP-glucuronosyltransferases (EC 2.4.1.17) are a family of enzymes, located in the endoplasmic reticulum, catalysing these reactions, and are present in the major organs of most mammals, particularly the liver, small intestine and kidneys [1, 2]. The heterogeneity of hepatic UDPGT is well characterised by a number of methods: differential induction [3], developmental studies [4], genetic deficiency [5, 6] and purification [7–12].

The kidney had been shown to glucuronidate a variety of compounds in the rat, but activity towards certain substrates (notably steroids) appears to be absent [13, 14]. Differential induction has been observed, with phenobarbital having no effect on renal UDPGT activity, whereas β-naphthoflavone and *trans*-stilbene oxide induced activity towards 1-naphthol, 4-nitrophenol and 4-methylumbelliferone [14]. These data suggest a different distribution of UDPGT in the kidney compared with liver, and heterogeneity of renal UDPGT. UDPGT activity in the kidney has also been observed in man [1], although the function is not clear. However, glucuronidation of β-estradiol [15] and estriol [16] has been demonstrated, indicating a role for the human kidney in the inactivation of endogenous steroids. No bilirubin UDPGT was detectable in human kidney [17].

The purpose of the present study was to extend the characterization of renal UDPGT to include endogenous substrates, in particular bilirubin, and to purify UDPGT activities from Sprague–Dawley rat kidney. Data obtained from substrate specificity determinations and induction studies on renal UDPGT activities indicated that purification of UDPGTs from rat kidney would result in preparations of phenol UDPGT and/or bilirubin UDPGT not contaminated with other UDPGT isoenzymes. Minor contamination with other UDPGT isoenzymes has been observed with preparations of rat liver UDPGTs [5, and M. Coughtrie, unpublished observations]. We report the isolation of a very highly purified preparation containing only two rat kidney UDPGTs (as determined by SDS–polyacrylamide gel electrophoresis and silver-staining) possessing activity towards 1-naphthol and bilirubin, containing two molecular weight species of 55,000 and 54,000 Da, respectively. These preparations exhibited two immuno-reactive polypeptides (55,000 and 54,000 Da) on immunoblot analysis with anti-rat liver testosterone UDPGT antibody, presumably the result of the co-purification of two different isoenzymes.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley CD rats (200–250 g) were obtained from Charles River Laboratories, Cambridge, MA. For induction experiments, animals received either β-naphthoflavone (100 mg/kg in corn oil i.p. for 3 days), phenobarbital (2 g/l in drinking water for 7 days and a single i.p. dose of 100 mg/kg in 0.9% NaCl 48 hr prior to sacrifice) or clofibrate (200 mg/kg i.p. in corn oil twice

daily for 4 days). Control animals received the appropriate vehicle only.

Materials. 1-[1-^{14}C]Naphthol (58 mCi/mmol), 4-nitro[2,6-^{14}C]phenol (58 mCi/mmol), [*N-methyl*-^{14}C]morphine HCl (58 mCi/mmol), [4-^{14}C]testosterone (57 mCi/mmol) and [2,4,6,7-^{3}H]estradiol (50 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL, and were determined by the manufacturer to be of >97% radiochemical purity. 1-naphthol, 4-nitrophenol, testosterone β-estradiol, UDP-glucuronic acid, UDP-hexanolamine, Lubrol PX, β-naphthoflavone, CNBr-activated Sepharose 4B, L-α-dioleoyl phosphatidylcholine and diaminobenzidine were obtained from Sigma Chemical Co., St. Louis, MO. DEAE-cellulose (DE52) and CM-cellulose (CM52) were from Whatman, and Sephacryl S-200 was from Pharmacia. Immunochemical reagents were purchased from Cappel Laboratories.

Preparation of microsomes. Livers or kidneys were chopped in a blender and homogenised in 4 volumes of ice-cold 0.25 M sucrose, the homogenates centrifuged for 15 min at 10,000 *g* and the resulting supernatant was centrifuged at 105,000 *g* for 60 min. Pellets were resuspended in 0.25 M sucrose and centrifuged for a further 45 min at 105,000 *g*. Microsomal pellets were resuspended in 0.25 M sucrose to a protein content of 15 mg/ml, frozen in liquid nitrogen and stored at −80° until use (within 2 months).

Analytical procedures. Assays for UDPGT towards various aglycones were performed using previously described methods: bilirubin [18], 1-naphthol [19], 4-nitrophenol [20], phenol [21], testosterone [22], β-estradiol [23] and morphine [24]. Protein in microsomal samples was determined by the method of Lowry *et al.* [25] and in purification fractions as described by Bradford [26]. SDS–polyacrylamide gel electrophoresis (7.5%) was carried out by the method of Laemmli [27]. Gels were stained by a commercial silver-stain procedure (Bio-Rad Laboratories).

Proteins separated on SDS–polyacrylamide gels were electrophoretically transferred to nitrocellulose (Schleicher and Schuell) as described by Towbin *et al.* [28], and immunostaining with antibody raised in sheep against rat liver testosterone UDPGT [29] was performed as in ref. 30.

Purification of UDP-glucuronosyltransferase. UDPGT from rat kidney was purified by modification of a method previously described [31]. Kidneys (~ 150 g) from 50–60 rats were chopped in a blender and homogenised in 4 volumes of ice-cold 0.25 M sucrose. All further operations were performed at 0–4°. The homogenate was centrifuged at 10,000 *g* for 15 min. The resulting supernatant was centrifuged at 105,000 *g* for 60 min, and the microsomal pellets resuspended in a volume (in ml) of 1% Lubrol PX, 0.2 M potassium phosphate, pH 7.0 equal to twice the wet weight (in grams) of the kidneys. The suspension was stirred on ice for 30 min and then centrifuged at 105,000 *g* for 60 min, to remove insoluble material. The supernatant was subjected to 25–60% ammonium sulphate fractionation, and the 60% pellet resuspended in ~30 ml of 5 mM potassium phosphate buffer, pH 8.0 containing 20% glycerol, 0.05% Lubrol PX, 2 mM EDTA and 0.25 mM 2-mercaptoethanol (buffer A), and dialysed overnight against two changes of buffer A (200 volumes).

The dialysed fraction was applied to a column of DE52 (3.2 × 60 cm) previously equilibrated with buffer A. Unbound protein was eluted with buffer A, and fractions containing GT activity were pooled, concentrated to ~15 ml by ultrafiltration, and dialysed overnight against 200 volumes of 5 mM potassium phosphate, pH 6.5 containing 20% glycerol, 0.05% Lubrol PX, 2 mM EDTA and 0.25 mM 2-mercaptoethanol (buffer B).

The dialysed fraction was applied to a column of CM52 (2.2 × 30 cm) equilibrated in buffer B, and active fractions eluting in the flow-through were pooled and concentrated to 10 ml. After dialysis overnight against 200 volumes of 20 mM Tris–HCl pH 7.4 containing 20% glycerol and 0.05% Lubrol PX (buffer C), the CM52 eluate was applied to a column of Sephacryl S-200 (1.6 × 60 cm) equilibrated in buffer C. The highly active fractions eluting early in the protein peak were pooled, concentrated to 5 ml and made 5 mM in $MgCl_2$, by the addition of 12.5 μl of 2 M $MgCl_2 \cdot 6H_2O$. This fraction was then applied to a column containing 15 ml UDP-hexanolamine Sepharose 4B equilibrated with buffer C containing 5 mM $MgCl_2$ (buffer D). (UDP-hexanolamine was coupled to CNBr-activated Sepharose 4B as described in ref [31].) The flow was stopped for 1 hr to facilitate binding, and unbound material was eluted by extensive washing with buffer D. UDPGT was eluted with 3 ml of buffer C containing 1 mM UDP-glucuronic acid. UDP-glucuronic acid-eluted fractions exhibiting high specific activity were pooled and stored on ice at 0–4°.

RESULTS AND DISCUSSION

Substrate specificity of renal UDPGT

Renal and hepatic microsomes catalysed the glucuronidation of a variety of substrates (Table 1). Enzyme assays were performed in the presence of optimally activating concentrations of the non-ionic detergent Lubrol PX, to overcome the well-known latency of microsomal UDPGTs [1]. Renal microsomal UDPGT specific activity for the substrates 1-naphthol, 4-nitrophenol and bilirubin was 2- to 2.5-fold lower than hepatic activities, in the presence and absence of detergent. The lower rate of β-estradiol glucuronidation observed in the kidney (4-fold) could be the result of this substrate being conjugated by a different UDPGT isoenzyme in the kidney compared with the liver, as no other steroid substrates have been found to be glucuronidated by rat kidney microsomes [13, 32 and M. Coughtrie, unpublished results].

These data are in agreement with those from other sources (e.g. [13, 14]), and suggest that a different distribution of UDPGT isoenzymes exists in the liver and kidney of the rat. This is confirmed by immunoblot analysis of rat liver and kidney microsomes using anti-rat liver UDPGT antibody (Fig. 1), which shows the presence of at least four immuno-reactive polypeptides in rat liver microsomes (50,000–54,000 Da), whereas microsomes prepared from rat

Table 1. Substrate specificity of microsomal UDPGT in rat liver and kidney

Substrate	Specific activity (nmol/min/mg)	
	Liver	Kidney
1-Naphthol	40 ± 4	19 ± 1
4-Nitrophenol	37 ± 6	20 ± 3
Phenol	10 ± 3	4.9 ± 0.7
Bilirubin	0.52 ± 0.07	0.2 ± 0.07
β-Estradiol	0.95 ± 0.36	0.25 ± 0.09
Testosterone	1.3 ± 0.2	N.D.*
Morphine	1.6 ± 0.4	N.D.†

UDPGT enzyme activities were measured in the presence of optimally-activating concentrations of Lubrol PX, determined for each tissue and substrate.

Values represent mean ± SD for microsomes prepared from at least three pools of tissue.

N.D., not detectable.

* Limit of detection >0.1 nmol/min/mg.

† Limit of detection >0.3 nmol/min/mg.

kidney appear to possess only one major immunostaining polypeptide (54,000 Da). It has previously been demonstrated for rat liver microsomes that the 54,000 immunostaining band is in fact due to the presence of both bilirubin UDPGT and phenol UDPGT [5], and the data presented in the present report indicate that a similar situation exists in the rat kidney.

Effect of treatment of rats with xenobiotics

The effect of treatment of rats with β-naphthoflavone and phenobarbital on hepatic UDPGT activities towards various substrates is shown in Fig. 2a. Hepatic transferase activities towards morphine, testosterone and β-estradiol were specifically stimulated by phenobarbital, whereas the glucuronidation of 1-naphthol, 4-nitrophenol, phenol and bilirubin were stimulated to varying extents by both inducers. These results are in contrast to data observed with renal microsomes, where phenobarbital had no stimulatory effect and activities towards β-estradiol and bilirubin were suppressed (Fig. 2b).

Transferase activity in kidney towards 1-naphthol, 4-nitrophenol and phenol was induced about 3-fold after β-naphthoflavone treatment, and towards bilirubin and β-estradiol about 2-fold (Fig. 2b). Glucuronidation of morphine and testosterone was not observed in untreated rats (Table 1), and no measurable activity was detectable following treatment with either phenobarbital or β-naphthoflavone. Interestingly, hepatic glucuronidation of testosterone and morphine, two of the UDPGT substrates not glucuronidated by rat kidney, is selectively induced by phenobarbital. The hypolipidaemic drug clofibrate is known to be a specific inducer of bilirubin UDPGT activity in rat liver microsomes [5]. When kidney microsomes from clofibrate-treated rats were analysed, a 2-fold induction of bilirubin UDPGT activity was observed (M. Coughtrie *et al.*, unpublished observations), whereas activity towards 1-naphthol was suppressed slightly. These data agree well with those obtained with rat liver [5], and demonstrate that bilirubin UDPGT activity is specifically induced by clofibrate in both rat liver and kidney. These differences in substrate specificity and selective induction suggested: (a) there are two distinct UDPGT isoenzymes in rat kidney catalysing conjugation of planar phenols and of bilirubin; and (b) that purification of UDPGT from rat kidney would yield only bilirubin and 1-naphthol/phenol isoenzymes equivalent to the hepatic forms described by Scragg *et al.* [5].

Purification of renal UDP-glucuronosyltransferase

The purification of 1-naphthol UDPGT from Lubrol PX-solubilised rat kidney microsomes is shown in Table 2. The pellet obtained after 25–60% ammonium sulphate fractionation of solubilised

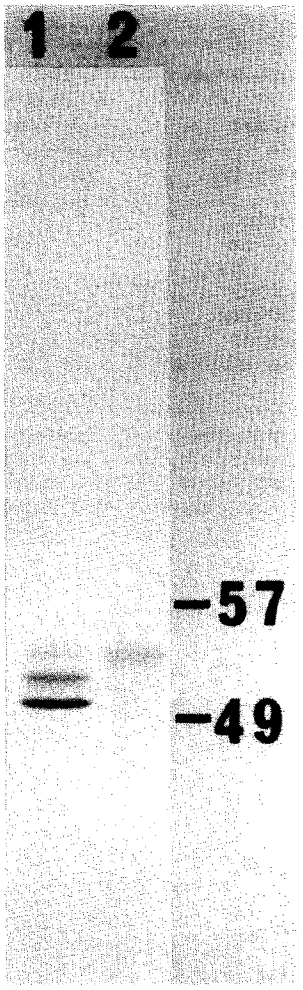

Fig. 1. Immunoblot analysis of rat liver and kidney microsomes. Rat liver (lane 1, 10 μg) and kidney (lane 2, 50 μg) microsomal proteins were resolved on 7.5% polyacrylamide gels in the presence of 0.1% SDS. Proteins were electrophoretically transferred onto nitrocellulose and incubated with anti-rat liver testosterone UDPGT antibody. Chromogenic visualisation of immuno-reactive polypeptides was performed using the immunoperoxidase method with diaminobenzidine as substrate. The relative mobilities of the molecular weight standards fumarase (49 kDa) and pyruvate kinase (57 kDa) are shown.

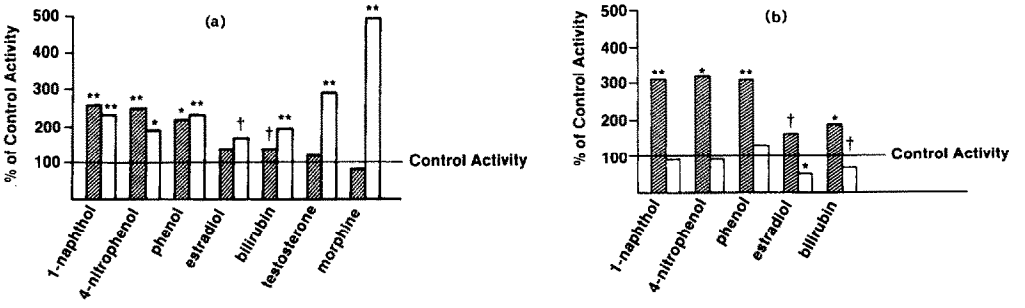

Fig. 2. Effects of the inducing agents phenobarbital (open bars) and β-naphthoflavone (hatched bars) on hepatic (a) and renal (b) microsomal UDP-glucuronosyltransferase activities towards a variety of substrates. Animal treatment regimens and assay conditions are described in Materials and Methods. Data represent the mean of at least three separate experiments. Statistical analysis was performed using Student's t test (†$P < 0.05$, *$P < 0.01$, **$P < 0.001$).

Table 2. Purification of UDP-glucuronosyltransferase towards 1-naphthol from Lubrol PX-solubilised rat kidney microsomes

Fraction	Total protein (mg)	Activity (nmol/min)	Specific activity (u*/mg)	Yield (%)	Purification (fold)
Lubrol PX-solubilised microsomes	1363	36528	26.8 (6.25–26.8)†	100	1
25–60% ammonium sulfate precipitate	649	13759	21.2 (11.6–21.2)	38	0.8
DEAE-cellulose eluate‡	240	6936	28.9 (23.2–28.9)	19	1.1
CM-cellulose eluate	126	3931	31.2 (24.7–36.7)	11	1.2
Sephacryl S-200 eluate	46	3169	68.9 (42.4–68.9)	8.7	2.6
UDP hexanolamine Sepharose 4B, UDPGA eluate	0.122	336	2750 (789–2760)	0.9	103

* 1 unit (u) of enzyme activity represents the formation of 1 nmol of product per minute of incubation.
† Values in parenthesis indicate the range obtained over four separate preparations.
‡ Column fractions were pooled, concentrated and dialysed before reconstitution.
Fractions were reconstituted with L-α-dioleoyl phosphatidylcholine liposomes (1 mg/g protein) prior to assay.

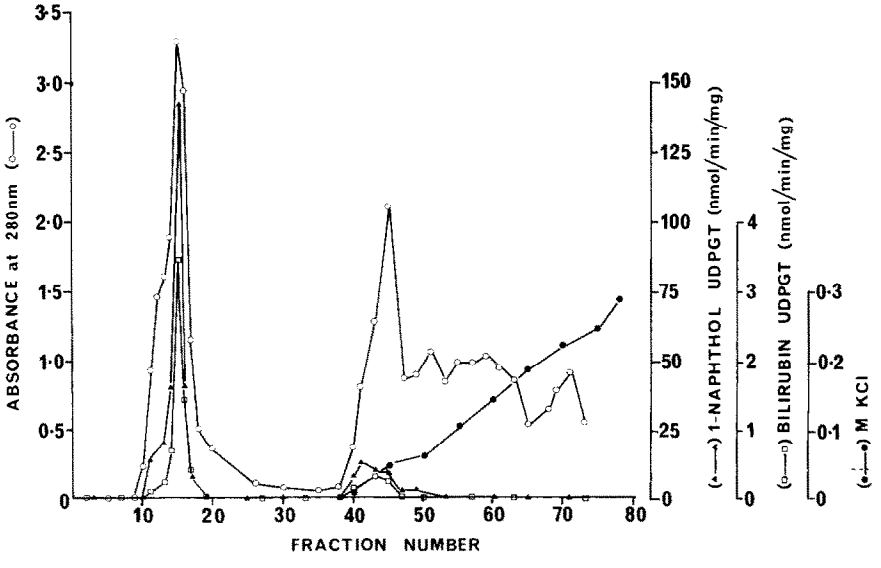

Fig. 3. DEAE-cellulose chromatography of rat kidney UDP-glucuronosyltransferase. Chromatographic conditions are described in Materials and Methods. Fractions (10 ml) were collected at a flow rate of 45 ml/hr, and assayed for 1-naphthol (▲) and bilirubin (□) UDP-glucuronosyltransferase activity in the presence of phosphatidylcholine liposomes (1 mg/mg protein). Protein was monitored by absorbance at 280 nm (○).

Table 3. Co-purification of 1-naphthol and bilirubin UDP-glucuronosyltransferase from Lubrol PX-solubilised rat kidney microsomes

Fraction	1-Naphthol				Bilirubin			
	Activity* (nmol/min)	Specific Activity (nmol/min/mg protein)	Yield (%)	Purification (fold)	Activity (nmol/min)	Specific Activity (nmol/min/mg protein)	Yield (%)	Purification (fold)
Lubrol PX-solubilised microsomes	19646	20.9	100	1	645	0.686	100	1
25–60% ammonium sulphate precipitate	12020	25.2	61	1.2	461	0.966	72	1.4
DEAE-cellulose eluate*	5214	26.6	27	1.3	118	0.604	19	0.88
CM-cellulose eluate	1240	31.8	6.3	1.5	41.7	1.07	6.5	1.6
Sephacryl S-200 eluate	806	42.4	4.1	2.0	24.7	1.30	3.8	1.9
UDP hexanolamine-Sepharose UDPGA eluate	75.7	789	0.4	38	0.653	6.8	0.1	9.9

Protein samples were reconstituted with L-α-dioleoylphosphatidylcholine liposomes (1 mg/mg protein) prior to assay.
* Column fractions were pooled, concentrated and dialysed prior to reconstitution (see Materials and Methods).

microsomes was subjected to chromatography on DEAE-cellulose. Enzyme activity towards 1-naphthol and bilirubin co-purify during this procedure (Fig. 3 and Table 3). The majority (70–80%) of 1-naphthol and bilirubin UDPGT activity recovered was not retained by DEAE-cellulose under these conditions. When ammonium sulphate fractions from rat liver microsomes were subjected to similar analysis, more than 90% of the bilirubin UDGPT activity recovered from DEAE-cellulose was retained by the column, and could be separated from bound 1-naphthol/4-nitrophenol activity by elution with a salt gradient (0–0.3 M KCl) [8, and M. Coughtrie, unpublished observations]. These results suggest that renal bilirubin UDPGT exhibits different charge properties to the hepatic enzyme under these conditions.

Pooled fraction containing high specific activity UDPGT were concentrated and, after dialysis, applied to a column of CM-cellulose equilibrated to pH 6.5. Active, unbound fractions recovered from the column were subjected to gel filtration on Sephacryl S-200. This chromatographic step produced good yields (70–80%) of UDPGT activities, and allowed their separation from the bulk of the protein remaining after chromatography on CM-cellulose. Fractions eluted at the leading edge of the protein peak were further purified by affinity chromatography on UDP-hexanolamine Sepharose 4B. UDPGT was specifically eluted from the affinity column with buffer C containing 1 mM UDP-glucuronic acid (Materials and Methods). Activity towards 1-naphthol was routinely purifed 40- to 100-fold (Table 2) and towards bilirubin, 10-fold (Table 3). The low specific activity of bilirubin UDPGT obtained from affinity chromatography suggests incomplete reconstitution with phospholipid (see below).

Properties of purified renal UDP-glucuronosyltransferase

Purified preparations possessed UDPGT activity towards 1-naphthol, 4-nitrophenol and bilirubin, but glucuronidation of β-estradiol, testosterone or morphine could not be detected in these fractions (data not shown). Transferase activity towards bilirubin in purified fractions was wholly dependent on the presence of phospholipid in the incubation, with maximum activity observed at a concentration of 1 mg phospholipid/mg protein. Highly purified 1-naphthol UDPGT activity exhibited less phospholipid dependence, with 50–70% of the activity being observed in the absence of phospholipid (data not shown).

Incomplete reconstitution of bilirubin UDPGT activity has been demonstrated to result in low apparent specific activity of this enzyme on purification [33]. Reactivation of purified bilirubin UDPGT by interaction with Gunn rat liver microsomes, which possess no bilirubin UDPGT activity [8], resulted in a 4- to 6-fold increase in specific activity over the phosphatidylcholine-reconstituted enzyme [33]. Preliminary studies showed that incubation of purified rat renal bilirubin UDPGT with rat lung microsomes, which also possess no bilirubin UDPGT activity [34], resulted in a 2- to 3-fold increase in

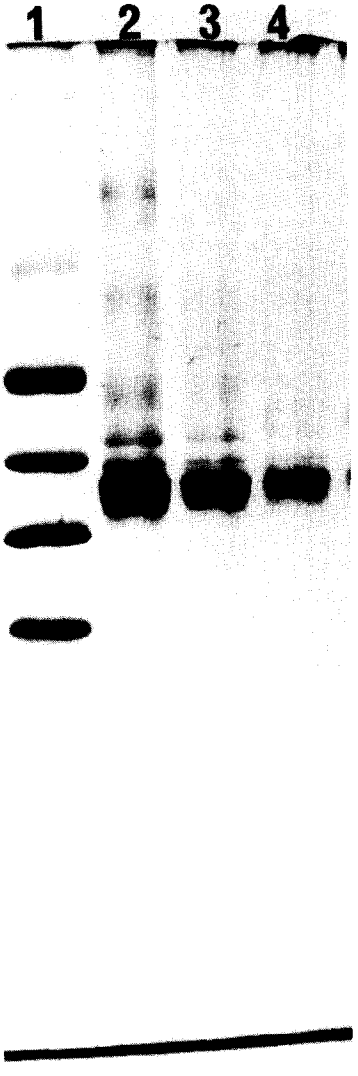

Fig. 4. Electrophoresis of purified rat kidney UDPGTs. Affinity purified fractions were electrophoresed on 7.5% SDS–polyacrylamide gels and proteins were visualised using silver stain. Lane 1, protein molecular weight standards, 1 μg of each protein (albumin, 68 kDa; pyruvate kinase, 57 kDa; fumarase, 50 kDa; ovalbumin, 43 kDa). Lanes 2, 3 and 4, purified rat kidney UDPGT possessing activity towards 1-naphthol and bilirubin (1.0, 0.5 and 0.25 μg of protein, respectively).

Fig. 5. Immunoblot analysis of purified rat kidney UDPGTs. Purified proteins electrophoresed on a SDS–polyacrylamide gel identical to that in Fig. 3 were electrophoretically transferred onto nitrocellulose, and exposed to anti-rat liver testosterone UDPGT antibody. Immuno-reactive polypeptides were visualised using the immunoperoxidase method with diaminobenzidine as substrate. Lanes 1, 2 and 3, purified rat kidney UDPGT with activity towards 1-naphthol and bilirubin (1.0, 0.5 and 0.25 μg of protein, respectively).

specific activity compared with the phosphatidylcholine-reconstituted protein, such that bilirubin GT was purified up to 30-fold (M. Coughtrie, unpublished results).

Electrophoresis of purified renal UDPGT, with activity towards 1-naphthol and bilirubin, on 7.5% SDS–polyacylamide gels followed by silver staining, yielded a major protein-staining polypeptide of subunit molecular weight 55,000 Da, and a minor 54,000 Da polypeptide was detectable when the amount of protein applied to the gel was decreased (Fig. 4, lane 4). When an identical gel was electroblotted onto nitrocellulose and immunostained with antibody raised in sheep against rat liver testosterone UDPGT [29], two immuno-reactive polypeptides were observed, corresponding to subunit molecular weights 55,000 Da and 54,000 Da (Fig. 5). In contrast, immunoblot analysis of Wistar rat liver microsomes has demonstrated the presence of at least four major immuno-reactive polypeptides with molecular weights 50,000–54,000 Da (ref. [5] and Fig. 1). Purification of bilirubin and 4-nitrophenol UDPGTs from Wistar rat liver microsomes yielded polypeptides with subunit molecular weights 54,000 and 53,000 Da, respectively [5]. Our data suggest two UDPGT isoenzymes are present in male Sprague–

Dawley rat kidney, corresponding to bilirubin and 1-naphthol activities, and the differences in molecular weight from previously reported purification of these transferases could be a result of strain differences, of small differences in primary structure of the renal enzymes, or of variations in the conditions of electrophoresis used to analyse purified preparations.

Here we have reported the first purification of UDPGT from rat kidney, capable of glucuronidating bilirubin and 1-naphthol. These activities co-purify using the procedure described, but evidence from induction studies (Fig. 2b), SDS–polyacrylamide gel electrophoresis (Fig. 4) and immunoblot analysis (Figs 1 and 5) suggests they are separate isoenzymes with molecular weights 55,000 and 54,000 Da, which differ from corresponding hepatic isoenzymes when chromatographed on DEAE-cellulose. Current investigations are aimed at separation and further characterisation of renal UDPGTs.

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