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Defective Induction of Phenol Glucuronidation by 3-Methylcholanthrene in Gunn Rats Is Due to the Absence of a Specific UDP-Glucuronosyltransferase Isoenzyme

MICHAEL W. H. COUGHTRIE, BRIAN BURCHELL, ISOBEL M. SHEPHERD,[1] and JOHN R. BEND

Department of Biochemistry, Medical Sciences Institute, The University, Dundee DD1 4HN, Scotland (M.W.H.C., B.B., I.M.S.), Department of Pharmacology and Toxicology, University of Western Ontario, London, Ontario, Canada N6A 5C1 (J.R.B.)

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SUMMARY

Antiserum directed against purified rat kidney UDP-glucuronosyltransferase (UDPGT) was raised in goats. IgG prepared from this antiserum exhibited specificity for only two UDPGT isoenzymes (bilirubin and phenol) on immunoblot analysis of Wistar rat liver microsomes. Use of this antibody preparation to probe Western blots of liver microsomes prepared from Gunn rats confirmed that the defective phenol glucuronidation was due to the absence of a 53-kDa, 3-methylcholanthrene-inducible UDPGT isoenzyme. Results obtained from enzyme activity measurements and immunoblot analysis of microsomes isolated from xenobiotic-treated Wistar and Gunn rat liver are consistent with the 3-methylcholanthrene/UDPGT induction deficiency in the Gunn rat being due to the absence of this phenol UDPGT isoenzyme. The contribution of other UDPGT isoenzymes to the greatly reduced glucuronidation of planar phenols observed in the Gunn rat is discussed.

Microsomal UDPGTs (EC 2.4.1.17) are a family of closely related isoenzymes catalyzing the conjugation of a variety of endogenous and exogenous lipophilic compounds with uridine diphosphoglucuronic acid, thus facilitating their elimination (1, 2). The heterogeneity of UDPGT has been well characterized by differential inducibility with various xenobiotics (3), perinatal development studies (4, 5), and purification of various isoenzymes (2, 6–10).

Several examples of inherited deficiency of UDPGT activities have been described, in particular, the discontinuous variation of androsterone (i.e., 3α-hydroxysteroid) UDPGT in the Wistar rat (11) and the lack of bilirubin UDPGT activity in the Gunn rat (12, 13). The hyperbilirubinemic Gunn rat has been used as a model for human Crigler-Najjar type I syndrome as a result of this total absence of bilirubin UDPGT activity (14). Recently, the inability of the Gunn rat to conjugate bilirubin has been shown to be due to the absence of the bilirubin UDPGT isoenzyme (15). The glucuronidation of planar phenols such as 2-aminophenol, 1-naphthol, and 4-nitrophenol is also severely impaired in the Gunn rat (see Ref. 15).

It has been reported that the Gunn rat exhibits a deficiency in 3-methylcholanthrene induction of the impaired UDPGT activity toward 4-nitrophenol (16) and also toward 1-naphthol (17), an observation which was attributed to a constitutive defect in the regulation of UDPGT activity by 3-methylcholanthrene (16). A similar induction deficiency also exists for the 3-methylcholanthrene-type inducer β-naphthoflavone (18).

In order to investigate this induction deficiency further, and to try to resolve confusion over the molecular basis of the Gunn rat genetic deficiencies, we have used immunoblot analysis with two polyclonal anti-UDPGT antibodies, exhibiting different specificity, raised against purified rat liver testosterone/4-nitrophenol UDPGT (19) and 1-napthhthol/bilirubin UDPGT purified from rat kidney (20). The data presented indicate that the genetic deficiency of UDPGT activities in the Gunn rat is due to the absence of at least two UDPGT isoenzymes, one of which is inducible by 3-methylcholanthrene and β-naphthoflavone, and suggests that the 3-methylcholanthrene induction deficiency is linked to the absence of this protein, probably through a large deletion in the Gunn rat genome involving the area(s) encoding this UDPGT isoenzyme.

Experimental Procedures

Materials

1-[1-^{14}C]Naphthol (58 mCi/mmol) and [4-^{14}C]testosterone (57 mCi/

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[1] Present address: Department of Pharmacological Sciences, Medical School, University of Newcastle-Upon-Tyne, England.

ABBREVIATIONS: UDPGT, uridine 5′-diphosphoglucuronosyltransferase; SDS, sodium dodecyl sulfate; LA, HA, Wistar rats exhibiting the low and high androsterone UDPGT activity phenotype, respectively.

mmol) were purchased from Amersham, England and were determined by the manufacturer to be of ≥97% radiochemical purity. 1-Naphthol, testosterone, β-estradiol, bilirubin, Lubrol PX, UDP-glucuronic acid (sodium salt), 4-chloro-1-naphthol, β-naphthoflavone, and 3-methylcholanthrene were purchased from Sigma Chemical Co., Poole, England. 2-Aminophenol (twice resublimed before use) and phenobarbitone sodium were obtained from BDH Ltd., Poole, England. Ethyl2-(4-chlorphenoxy)-2-methyl-propionate (clofibrate) was from Fluka. All other reagents were from commonly used suppliers and were of analytical grade or better. Donkey anti-goat/sheep IgG and sheep peroxidase-antiperoxidase complex were generously provided by the Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland.

Treatment of Animals

Adult male Wistar and homozygous Gunn rats (6–10 weeks old, 100–200 g) were from colonies maintained in the Medical Sciences Institute, University of Dundee. For induction studies, animals received either phenobarbitone sodium—100 mg/kg intraperitoneally in 0.9% NaCl daily for 4 days (animals were sacrificed on the fifth day); 3-methylcholanthrene—once, 80 mg/kg intraperitoneally in corn oil (animals were sacrificed after 3 days); β-naphthoflavone—100 mg/kg intraperitoneally in corn oil on day 1 and day 4 (animals were sacrificed on day 6); or clofibrate—200 mg/kg intraperitoneally in corn oil twice daily for 4 days (animals were sacrificed on the fifth day). Control animals received the appropriate injection vehicle only.

Preparation of Microsomal Fractions

Livers were removed, finely chopped, and homogenized in ice-cold 0.25 M sucrose to give a 20% (w/v) homogenate. All further operations were performed at 0–4°. The homogenate was centrifuged at 10,000 × *g* for 15 min and the resulting supernatant was centrifuged for 1 hr at 105,000 × *g*. The pellets were resuspended in 0.25 M sucrose and centrifuged for a further 45 min at 105,000 × *g*. The washed microsomal pellets were resuspended in 0.25 M sucrose to a protein content of approximately 20 mg/ml, and stored as 0.5-ml aliquots at −80° until use (within 2 months).

Enzyme Assays

In order to counteract the well known latency of UDPGT (1), enzyme activities were measured in the presence of optimally activating concentrations of the detergent Lubrol PX, determined for each substrate and for each strain of rat.[2] Microsomes which had been frozen and thawed only once were used. Established assay procedures for the following acceptor substrates were employed: 1-naphthol (21), testosterone (22), bilirubin (23), and 2-aminophenol (24).

Production of Anti-UDPGT Antibodies[3]

Anti-rat liver testosterone/4-nitrophenol UDPGT has been previously described (19). Antibodies directed against purified rat kidney 1-naphthol/bilirubin UDPGT (20) were raised in goats by a combination of multisite intradermal and subcutaneous injections. An initial immunization of 100 μg of purified antigen, in Freund's complete adjuvant, was followed by two subsequent immunizations 2 weeks apart of 50 μg each in Freund's incomplete adjuvant. Blood was collected at 2-week intervals for the first 4 weeks after the final immunization, and thereafter weekly until the peak titer was achieved (about 12 weeks after the first immunization), at which time the goat was bled to yield about 500 ml of antiserum. IgG was prepared from antiserum by a combination of ammonium sulfate precipitation and DEAE-cellulose chromatography, essentially as described in Ref. 25. Preimmune serum was collected prior to the first immunization. Production of specific antisera was monitored by dot-blot and immunoblot analyses using both microsomes and purified UDPGT.

Immunoinhibition and Immunoprecipitation of Microsomal UDPGT Activity with Anti-UDPGT Antibodies

Immunoinhibition. Microsomal protein (10 mg/ml) was solubilized with Lubrol PX (at a ratio of 1 mg/mg of protein) for 1 hr on ice, and then centrifuged at 105,000 × *g* for 30 min. Aliquots of this solubilized fraction were incubated with various amounts of anti-rat UDPGT IgG. The total amount of IgG in the incubation was held constant by adding the required amount of preimmune IgG. After incubation for 5 min at room temperature, aliquots were immediately assayed for UDPGT activity toward various acceptor substrates characteristic of individual UDPGT isoenzymes.

Immunoprecipitation of microsomal 1-naphthol UDPGT from Wistar rat liver and kidney. Solubilized microsomal fractions, prepared as described above, were incubated with various amounts of sheep anti-rat liver or goat anti-rat kidney UDPGT IgG for 1 hr at room temperature. As the goat antibody alone did not precipitate UDPGT activity, 100 μg of donkey anti-sheep/goat IgG were added to aid precipitation and incubated for 30 min at room temperature. Precipitates were removed by centrifugation at 16,000 × *g* for 10 min, and aliquots (60 μg) of the supernatant were assayed for 1-naphthol UDPGT activity.

Electrophoresis and Immunoblot Analysis

All electrophoresis and blotting equipment was from Bio-Rad, and electrophoresis chemicals were from BDH. Microsomal protein samples were subjected to polyacrylamide gel electrophoresis on 7% gels in the presence of 0.1% SDS, by the method of Laemmli (26). For immunoblot analysis, proteins resolved on SDS-gels were electrophoretically transferred to nitrocellulose (Schleicher and Schuell) as described by Towbin *et al.* (27), and chromogenic detection of immunoreactive polypeptides was performed using the peroxidase-linked method as outlined in Ref. 28, with the exception that 4-chloro-1-naphthol replaced diaminobenzidine as peroxidase co-substrate.

Protein Determination

Protein in microsomal samples was estimated by the method of Lowry *et al.* (29) with bovine serum albumin as standard. Protein determinations were performed on microsomal samples which had been frozen and thawed once.

Results

Comparison of UDPGT activities in Wistar and Gunn rat liver microsomes. Rat liver microsomes were assayed for UDPGT activity toward four substrates to illustrate the existence of multiple defective glucuronidation reactions in the Gunn rat (Table 1). These results showed the complete absence of UDPGT activity toward bilirubin and very reduced levels of activity toward 2-aminophenol and 1-naphthol, whereas activity toward testosterone was nearly normal in Gunn rat liver. These results confirm earlier work from this laboratory (see Ref. 15).

Induction of rat liver microsomal UDPGT activities by various xenobiotic compounds. Hepatic microsomal fractions were prepared from xenobiotic-pretreated Wistar and Gunn rats to facilitate the examination of different profiles of UDPGT activities, which are representative of several different isoenzymes (Table 1). The results show that in Wistar rat liver microsomes, UDPGT activities toward testosterone, bilirubin,

[2] Lubrol PX/protein ratios (as mg of detergent/mg of protein) for the optimal activation of Wistar and Gunn rat liver microsomal UDPGT activities were as follows: 1-naphthol (Wistar, 0.25; Gunn, 0.10), 2-aminophenol (Wistar, 0.20; Gunn 0.10), testosterone (Wistar, 0.20; Gunn, 0.05). For assay of bilirubin UDPGT activity in Wistar rat liver microsomes, a Lubrol PX/protein ratio of 0.15 was used.

[3] The nomenclature used for the anti-UDPGT antibody preparations described in this paper is as follows: sheep antibody (sheep anti-rat liver testosterone/4-nitrophenol UDPGT antibody); goat antibody (goat anti-rat kidney 1-naphthol/bilirubin UDPGT).

TABLE 1

The effect of various xenobiotic inducers on UDPGT activities in Wistar and Gunn Rat Liver Microsomes

Inducer	UDP-Glucuronosyltransferase activities[a]							
	2-Aminophenol	-Fold Change	1-Naphthol	-Fold Change	Testosterone	-Fold Change	Bilirubin	-Fold Change
Wistar rats								
None (n = 15)	1.40 ± 0.7		30.0 ± 7.2		2.09 ± 1.2		0.68 ± 0.24	
3-Methylcholanthrene (n = 6)	6.45 ± 3.8	4.6	69.1 ± 10.1	2.3	1.45 ± 0.6	0.7	0.6	
β-Naphthoflavone (n = 9)	4.73 ± 2.2	3.4	71.7 ± 38	2.4	2.19 ± 1.5	1.1	0.61 ± 0.1	
Phenobarbitone (n = 12)	1.85 ± 0.7	1.3	37.8 ± 14	1.3	4.09 ± 1.7	2.0	0.92 ± 0.26	1.35
Clofibrate (n = 12)	0.63 ± 0.2	0.4	13.1 ± 5	0.4	1.96 ± 1.6	0.7	1.23 ± 0.37	1.8
Gunn rats								
None (n = 12)	0.09 ± 0.04		3.8 ± 1.0		1.86 ± 0.6		ND[b]	
3-Methylcholanthrene (n = 6)	0.11 ± 0.07	1.2	3.4 ± 0.6	0.9	1.70 ± 0.5	0.9	ND	
β-Naphthoflavone (n = 3)	0.13 ± 0.01	1.4	4.3 ± 0.7	1.1	2.24 ± 0.8	1.2	ND	
Phenobarbitone (n = 6)	0.07 ± 0.04	0.8	4.5 ± 2.2	1.2	3.87 ± 1.2	2.1	ND	

[a] UDPGT activities were determined in the presence of optimally activating concentrations of Lubrol PX, determined for each strain and aglycone substrate (see Footnote 2 in the text). Activities are expressed as nmol/min/mg of protein and are the mean ± SD for the number of different microsomal samples shown in parentheses.
[b] ND, no detectable UDPGT activity.

1-naphthol, and 2-aminophenol were increased up to 2-fold by phenobarbitone, whereas only testosterone UDPGT activity was increased 2-fold in Gunn rat liver microsomes by this drug. 3-Methylcholanthrene and β-naphthoflavone specifically increased UDPGT activities toward 2-aminophenol and 1-naphthol in Wistar rat liver up to 5-fold, but no induction of these activities was observed in Gunn rat liver in response to treatment with these compounds (Table 1). However, the cytochrome P-450-dependent *O*-deethylation of 7-ethoxycoumarin (30) was increased some 10-fold in both the Gunn and Wistar rat liver microsomes by the 3-methylcholanthrene pretreatment (Table 2). Note that in Wistar rat liver, clofibrate specifically increased bilirubin UDPGT activity only, and decreased the glucuronidation of the three other substrates assayed (Table 1).

Inhibition and immunoprecipitation of UDPGT activities by anti-rat UDPGT IgG. Purified IgG fractions were used to determine whether the antibodies raised against purified UDPGTs would cross-react with different UDPGT isoenzymes, identified by their substrate specificities. Aliquots of solubilized microsomes from untreated rat liver were incubated with sheep anti-rat UDPGT IgG and then assayed for inhibition of various UDPGT enzyme activities. Purified preimmune IgGs did not inhibit the UDPGT activities measured, whereas sheep anti-rat UDPGT IgG inhibited UDPGT activity toward bilirubin, testosterone, and 1-naphthol in solubilized Wistar rat liver microsomes (Fig. 1). Transferase activity toward androsterone, estrone, and morphine was also inhibited >50% following incubation with 0.7 mg of IgG/mg of protein (data not shown). Assay of these activities suggested that the sheep

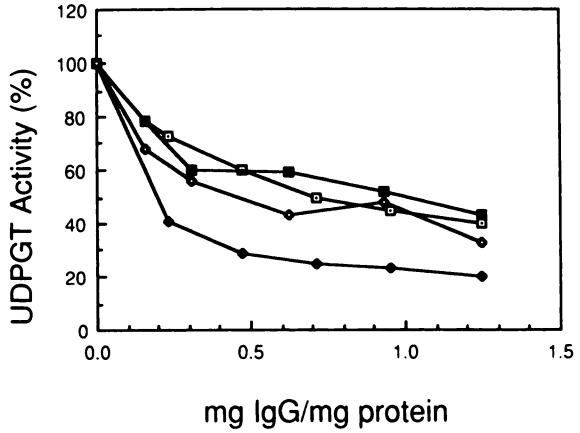

Fig. 1. Inhibition of rat liver microsomal UDPGT activities by reaction with sheep anti-rat UDPGT IgG. Aliquots of Lubrol PX-solubilized rat liver microsomes were incubated with various amounts of sheep anti-rat UDPGT IgG as detailed in Experimental Procedures. Incubation mixtures were assayed for UDPGT activity toward the following aglycone substrates: 1-naphthol (Wistar rat, ⊡; Gunn rat ◆), testosterone (Wistar rat, ◇), and bilirubin (Wistar rat, ■). Data points represent the mean of determinations on three separate microsomal samples.

antibody interacts with at least five different transferase isoenzymes (5).

1-Naphthol UDPGT activity in Gunn rat liver microsomes was inhibited to a much greater extent (up to 80%) by equivalent amounts of sheep antibody (Fig. 1), suggesting that the total amount of UDPGT proteins was reduced in Gunn rat liver microsomes and confirming earlier results obtained with a different antibody preparation (25).

Goat antibody did not inhibit UDPGT activities under any of the conditions tried. Therefore we used immunoprecipitation as a criterion of interaction with UDPGT. Aliquots of solubilized hepatic and renal microsomes from Wistar rats were incubated with different amounts of either goat or sheep antibodies. The supernatants from the incubation mixtures were assayed for 1-naphthol UDPGT activity after addition of donkey anti-goat/sheep antibody (to aid precipitation of immune complexes) and centrifugation to remove the precipitates (see Experimental Procedures). The results of these experiments are shown in Fig. 2. Up to 50% of the enzyme in kidney microsomes and 25% of the enzyme from liver microsomes was immunoprecipitated using the goat antibody, whereas the sheep

TABLE 2

The effect of pretreatment of Gunn and Wistar rats with 3-methylcholanthrene on hepatic microsomal 7-ethoxycoumarin O-deethylase activity

Strain	7-Ethoxycoumarin O-deethylase activity[a]		
	Control	3-MC[b]	-Fold induction
	nmol/min/mg protein		
Gunn	1.20 ± 0.08	13.4 ± 0.68	11.1
Wistar	1.46 ± 0.07	15.7 ± 0.58	10.7

[a] The results show the mean triplicate assays of three different microsomal preparations ± SD. Assays were performed as described in Ref. 30.
[b] 3-MC, 3-methylcholanthrene-treated.

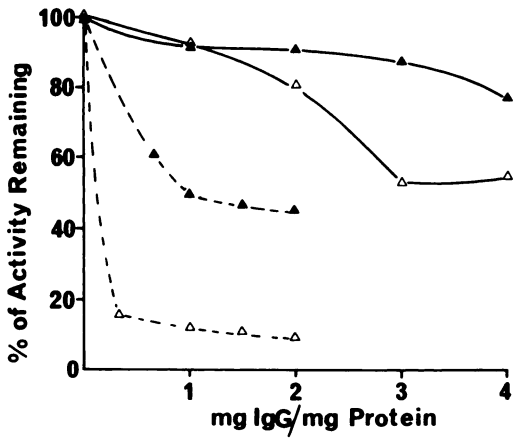

Fig. 2. Immunoprecipitation of rat liver and kidney microsomal UDPGT activity by goat anti-rat kidney UDPGT antibody. Solubilized Wistar rat liver microsomes were incubated with various concentrations of anti-rat kidney UDPGT as described in Experimental Procedures. After addition of donkey anti-goat IgG to aid precipitation, precipitates were centrifuged down at 16,000 × *g* for 10 min, and aliquots of the supernatant were assayed for 1-naphthol UDPGT activity. —, results obtained with anti-rat kidney UDPGT antibody and rat liver (▲) and rat kidney (△) microsomes. Data obtained with anti-rat liver UDPGT antibody are included for comparison (– – –). Values represent the percentage of activity in the post-precipitate supernatant compared with a control sample which contained only preimmune IgG.

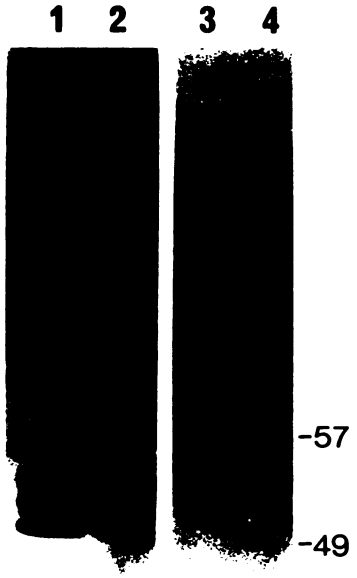

Fig. 3. Immunoblot analysis of liver microsomes prepared from Wistar and Gunn rats. Following electrophoresis (7% SDS-polyacrylamide gel) and transfer to nitrocellulose, strips were immunostained for anti-UDPGT-reactive polypeptides by the immunoperoxidase method (visualization was with 4-chloro-1-naphthol as peroxidase co-substrate). *Lanes 1* and *2* were exposed to sheep anti-rat liver UDPGT, and *lanes 3* and *4* to goat anti-rat kidney UDPGT. Microsomal proteins were prepared from: Wistar rat liver (*lanes 1* and *3*, 15 μg) and Gunn rat liver (*lanes 2* and *4*, 15 μg). The relative mobilities of two molecular weight marker proteins (pyruvate kinase, 57 kDa; fumarase, 49 kDa), determined on an identical Coomassie Blue-stained gel, are shown.

antibody immunoprecipitated 90% and 55% of the enzyme from equivalent aliquots of kidney and hepatic microsomes, respectively, using half the amount of antibody. These data show that both antibodies bound 1-naphthol UDPGT and suggest that lower overall levels of UDPGT proteins are present in kidney microsomes. This has been confirmed by immunoblot of rat kidney and liver microsomes (20).

Immunoblot analysis of rat liver microsomes from untreated and xenobiotic-treated Gunn and Wistar rats. Hepatic microsomes from untreated and xenobiotic-treated Gunn rats were examined in order to confirm that the isoenzyme corresponding to the 3-methylcholanthrene-inducible phenol UDPGT was absent from the Gunn rat. Microsomal fractions from untreated Gunn and Wistar rats, which exhibited the UDPGT activities reported in Table 1, were resolved by SDS-polyacrylamide gel electrophoresis and examined by immunoblotting using the two polyclonal anti-UDPGT antibodies described. The more specific goat antibody, which recognizes only bilirubin and phenol UDPGTs in Wistar liver microsomes (see Figs. 3 and 4; Discussion), did not interact with any of the UDPGTs in Gunn rat liver microsomes (Fig. 3). Use of the sheep antibody, which interacts with several UDPGTs (Fig. 1), reveals five immunoreactive polypeptides in Wistar liver microsomes and confirms the absence of the immunostaining region of M_r = 53,000–54,000 in Gunn liver microsomes (Fig. 3). This is in fact due to the absence of two immunoreactive UDPGT polypeptides, based on evidence from differential induction (Table 1, Fig. 4; see also below).

The effect of xenobiotic treatment on the presence of immunoreactive UDPGTs in Wistar and Gunn liver microsomes was also examined by immunoblotting to determine whether UDPGT isoenzymes apparently absent from the Gunn rat can be induced to appear by the xenobiotics. Analysis of the differential induction of Wistar rat liver microsomal UDPGTs by immunoblotting with the goat antibody confirmed that the heavily staining region at 53–54 kDa was in fact due to two UDPGT isoenzymes (Fig. 4). One of these polypeptides (53 kDa) was induced by 3-methylcholanthrene (Fig. 4A, *lanes 4* and *13*) and to a lesser extent by β-naphthoflavone (Fig. 4A, *lane 10*), and corresponds to the glucuronidation of planar phenols such as 1-naphthol and 2-aminophenol, whereas the other (54 kDa), representing bilirubin UDPGT activity, was induced by clofibrate (Fig. 4A, *lane 12*). The induction of bilirubin UDPGT by clofibrate is not as dramatic as that observed for 3-methylcholanthrene induction of phenol UDPGT. Clofibrate treatment of Wistar rats results in only a 2-fold induction of bilirubin UDPGT (compared with the 4–5-fold observed with phenol UDPGT after 3-MC treatment—see Table 1); thus, the visual effect on immunoblot analysis of microsomes from clofibrate-treated animals is of a decreased relative mobility of the 53/54-kDa "band" following SDS-polyacrylamide gel electrophoresis. This phenomenon results from (a) a 2-fold increase in bilirubin UDPGT activity, and (b) a 2-fold decrease in phenol UDPGT activity, thus shifting the emphasis toward the 54-kDa band. These observations were confirmed by probing an identical immunoblot with the sheep antibody (Fig. 4B). Note that the phenol UDPGT was not detected in Gunn rat liver microsomes even after treatment of the animals with 3-methylcholanthrene or β-naphthoflavone (Fig. 4). Our previous studies have shown that bilirubin UDPGT was not induced to appear in Gunn rat liver microsomes by pretreatment of animals with clofibrate (15).

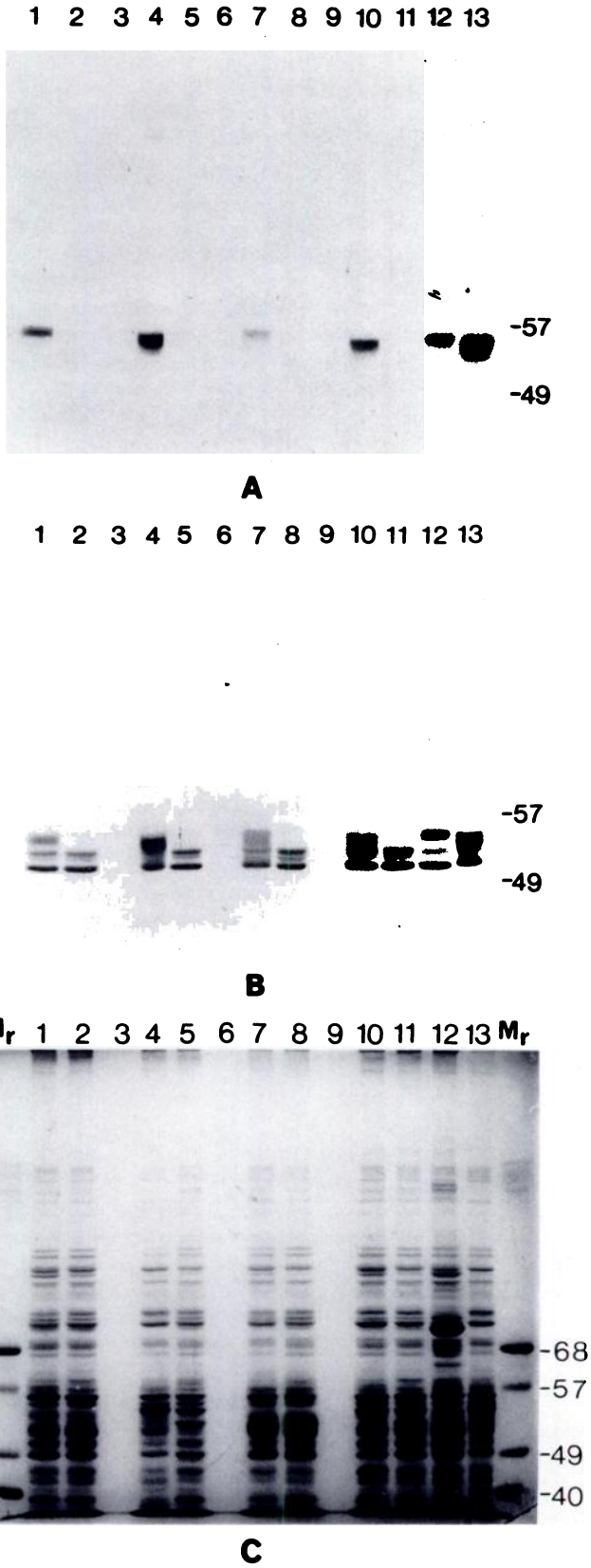

Fig. 4. Immunoblot analysis of liver microsomes prepared from Wistar and Gunn rats treated with various xenobiotic inducers. Microsomes were subjected to electrophoresis on SDS-polyacrylamide (7%) gels, followed by electrophoretic transfer to nitrocellulose. Immunostaining with goat anti-rat kidney UDPGT (A) or sheep anti-rat liver UDPGT (B) was performed using the immunoperoxidase method with 4-chloro-1-naphthol as chromogenic substrate. An identical gel, stained with Coomassie Blue, is shown for comparison (C). Liver microsomal samples from the following sources were applied to all three gels: *lane 1*, untreated Wistar rat; *lane 2*, untreated Gunn rat; *lane 4* and *13*, 3-methylcholanthrene-treated Wistar rat; *lane 5*, 3-methylcholanthrene-treated Gunn rat; *lane 7*, phenobarbitone-treated Wistar rat; *lane 8*, phenobarbitone-treated Gunn rat; *lane 10*, β-naphthoflavone-treated Wistar rat; *lane 11*, β-naphthoflavone-treated Gunn rat; *lane 12*, clofibrate-treated Wistar rat. *Lanes 3*, *6*, and *9* were empty. Twenty μg of each microsomal protein sample were loaded. Only microsomal samples from animals exhibiting the LA UDPGT phenotype were used. In gel C, lanes marked M_r contained a mixture of the following molecular weight marker proteins (2 μg of each): albumin (68 kDa), pyruvate kinase (57 kDa), fumarase (49 kDa), and aldolase (40 kDa).

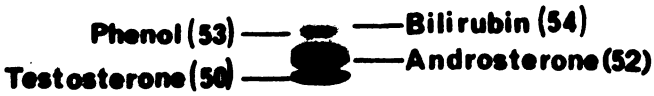

Fig. 5. Identification of UDPGT isoenzymes on immunoblot analysis of Wistar rat liver microsomes. Liver microsomes prepared from an adult male Wistar rat exhibiting the HA UDPGT phenotype were subjected to immunoblot analysis using sheep anti-rat liver UDPGT antibody. The assignation of immunoreactive polypeptides to UDPGT enzyme activities was based on analysis of microsomes from developing, genetically deficient, and xenobiotic-treated rats in comparison with purified UDPGTs, as described in Discussion. Twenty μg of microsomal protein were applied to a 7% polyacrylamide gel, in the presence of 0.1% SDS. Following electrophoresis, the resolved proteins were transferred to nitrocellulose and exposed to antibody. Antibody/antigen complexes were visualized by the immunoperoxidase method, with 4-chloro-1-naphthol as peroxidase co-substrate.

The results presented here demonstrate that the 53-kDa phenol UDPGT isoenzyme is not present in Gunn rat liver microsomal fractions and that neither the corresponding enzyme activities nor enzyme protein were induced by 3-methylcholanthrene, although the cytochrome P-450-dependent 7-ethoxycoumarin *O*-deethylase activity was induced by this xenobiotic in the same animals (Table 2).

Discussion

Immunochemical identification of individual UDPGTs in hepatic microsomes. Many anti-UDPGT antibodies have been raised in rabbits, sheep, chickens, goats, and mice in this laboratory using purified preparations of individual UDPGTs or a combination of UDPGT isoenzymes as antigens. To date, an antibody preparation which will recognize a single isoenzyme has not been obtained by this laboratory or reported in the literature. The extensive sequence homology predicted from our cDNA clones (31) indicates the difficulties that will be encountered in raising antibodies even with a homogenous preparation of antigen, due to the presence of common (possibly predominant) epitopes on different UDPGTs. However, the UDPGT sequences may allow the identification of unique peptides which could be synthesized and used for preparation of monospecific antibodies. This specificity problem has been partially overcome by the use of purified rat kidney UDPGT (20) as antigen to elicit an antibody preparation exhibiting increased specificity, as was described here.

Immunoblotting of Wistar rat liver microsomes reveals the presence of at least five stained bands as shown in Fig. 5 (M_r = 50,000–54,000). The labeling of the immunoreactive polypeptides in Fig. 5 summarizes our current knowledge and is deduced from analysis of microsomes from developing, genetically defi-

cient, and xenobiotic-treated rats in comparison with purified UDPGTs (Refs. 5, 15, and 32; this study).

The enzymological defect in Gunn rat liver. The exact nature of the molecular basis of the genetic deficiency of Gunn rat UDPGT has been a controversial problem for more than 20 years. The well recognized loss of bilirubin UDPGT activity has been complicated by the proposed existence of a functionally defective phenol UDPGT (33) and the additional 3-methylcholanthrene "induction deficiency" (16), which has confused many investigators for a number of years (2). We have sought to rationalize this impossibly complicated issue by a molecular/immunological approach.

We have previously reported the absence of bilirubin UDPGT enzyme protein from Gunn rat liver microsomes (15). The results presented here show that phenol UDPGT, a 53-kDa protein when purified from Wistar rat liver (15) and kidney (20), was completely absent from Gunn rat liver microsomes as determined by immunoblot analysis with an antibody preparation exhibiting high specificity for this isoenzyme. This absent protein was not inducible by 3-methylcholanthrene or by β-naphthoflavone in the Gunn rat. Furthermore, the fetal phenol UDPGT was not present in microsomes prepared from fetal Gunn rat liver (15) and no immunoreactive polypeptides were observed when Western blots of Gunn rat kidney microsomes were probed with the goat antibody (34). The residual phenol glucuronidation in the Gunn rat would appear to be catalyzed by testosterone (i.e., 17β-hydroxysteroid) UDPGT as (a) increases of testosterone UDPGT activity in Gunn rat liver microsomes caused by treatment of the rats with phenobarbitone result in a slight increase in 1-naphthol glucuronidation (Ref. 17; Table 1) and up to a 2-fold increase in 2-aminophenol glucuronidation in the presence of 3-pentanone (18) [3-pentanone specifically stimulates the defective glucuronidation of phenols in Gunn rat liver (35, 36)]; (b) 2-aminophenol inhibits the glucuronidation of testosterone by Gunn rat liver microsomes and vice versa (data not shown); (c) the glucuronidation of 2-aminophenol by purified Wistar rat liver testosterone UDPGT was stimulated by the presence of 3-pentanone, whereas no such effect was observed with purified phenol UDPGT (37); (d) purified Wistar rat liver testosterone UDPGT conjugates 4-nitrophenol at about 20% of the rate of 4-nitrophenol UDPGT purified from Wistar rat liver (6), and Gunn rat liver microsomes are only able to glucuronidate such planar phenolic aglycones at 5–20% of the rate demonstrated by Wistar rat liver microsomes (Table 1); and (e) the glucuronidation of 2-aminophenol by Wistar rat kidney and lung microsomes is not affected by the presence of 3-pentanone—these tissues do not possess testosterone UDPGT activity (34).

The data presented in this paper have shown that the deficiencies of UDPGT activity in the Gunn rat are less complex than originally imagined, being the result of the absence of at least two UDPGT isoenzymes. Confusion in the literature over the nature of this lesion has arisen from kinetic analysis of UDPGT activity in the microsomal fraction with substrates such as 4-nitrophenol (e.g. Ref. 33) which are now known to be glucuronidated by at least two UDPGT isoenzymes (10), one of which is absent in the Gunn rat (i.e., phenol UDPGT). It is thus necessary to define the boundaries of substrate specificity of the purified individual UDPGT isoenzymes in order that valid interpretation of data obtained from kinetic analyses using microsomal fractions can be made.

Monooxygenase activities were induced to a similar extent in Wistar and Gunn rat liver microsomes by 3-methylcholanthrene (Ref. 16; Table 2). No differences in the binding of benzo(*a*)pyrene to hepatic cytosolic proteins have been observed,[4] suggesting that there are no differences in the cytosolic receptors involved in the regulation of UDPGTs between the two strains. Therefore, the genetic lesion in the Gunn rat does not involve a constitutive defect in the regulation of phenol UDPGT activity as suggested by the proposed "induction deficiency," nor is it the result of a functionally defective phenol UDPGT. These proposals are both explained by the loss of this isoenzyme. It could be argued that a "defective" phenol UDPGT protein is altered in such a way that it is no longer recognized by the antibody preparations used in this study. (This would be a stronger possibility had monoclonal antibodies been used.) However, the androsterone UDPGT genetic deficiency in LA Wistar rats, which has also been shown to be due to the absence of the corresponding UDPGT isoenzyme (11), appears to be the result of a deletion in the LA Wistar rat genome, since (a) there is no androsterone UDPGT mRNA synthesized (31), and (b) there is a restriction fragment length difference in genomic DNA from LA and HA Wistar rats when subjected to Southern blot analysis (32).

A single genetic defect accounting for the loss of two different gene products (i.e., bilirubin and phenol UDPGTs from the Gunn rat) is likely to be due to a recombination event between homologous regions of two genes, followed by unequal crossover (38). Such an event could result in the deletion of an area of the Gunn rat genome encoding the bilirubin and phenol UDPGT isoenzymes, giving rise to the observed deficiencies of UDPGT activity, as well as to the apparent induction deficiency in response to 3-methylcholanthrene observed in the Gunn rat.

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Send reprint requests to: Dr. Michael W. H. Coughtrie, Department of Biochemistry, Medical Sciences Institute, The University, Dundee DD1 4HN, Scotland, U.K.