0026-895X/91/010080-05$03.00/0

MOLECULAR PHARMACOLOGY, 40:80-84

Purification and Properties of a Rat Liver Phenobarbital-Inducible 4-Hydroxybiphenyl UDP-Glucuronosyltransferase

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Received November 27, 1990; Accepted April 1, 1991

SUMMARY

A phenobarbital-inducible rat hepatic microsomal UDP-glucuronosyltransferase (UDPGT) that catalyzes the glucuronidation of 4-hydroxybiphenyl (4-HBP) has been purified to homogeneity. This UDPGT has an apparent subunit molecular weight of 52,500, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 4-HBP UDPGT was shown to catalyze the glucuronidation of 4-HBP, 4-methylumbelliferone, and *p*-nitrophenol but did not react with testosterone, androsterone, morphine, chloramphenicol, 4-hydroxycoumarin, or 7-methoxycoumarin. The apparent K_m of 4-HBP UDPGT for 4-HBP was determined to be 0.26 mM and for UDPGA was 1.0 mM. Upon treatment with endoglycosidase H, the 4-HBP UDPGT underwent about a 2000-dalton decrease in subunit molecular weight, suggesting that this protein is *N*-glycosylated. Additionally, this protein demonstrated immunoreactivity with antibodies raised in rabbit against rat 17β-hydroxysteroid and 3α-hydroxysteroid UDPGTs. This work describes the purification and characterization of a 4-HBP UDPGT from rat liver microsomes and, furthermore, provides evidence that suggests that this UDPGT is different from another UDPGT previously shown to react with 4-HBP and chloramphenicol.

Glucuronidation is an important and versatile process whereby many xenobiotics and endobiotics are metabolized *in vivo* to polar metabolites, which are readily excreted from the body in the bile or urine. A family of isoenzymes catalyze the formation of glucuronides, the UDPGTs (EC 2.4.1.17). These isoenzymes have been distinguished from each other by differences in functional properties (e.g., substrate specificities) and physical features (e.g., subunit molecular weights, isoelectric points, NH_2-terminal amino acid sequences, and cDNA analysis) (1–5). However, a number of these UDPGTs possess overlapping substrate specificities.

Bock *et al.* (6) reported on the isolation of a UDPGT from liver microsomes of phenobarbital-treated rats, which catalyzed the glucuronidation of morphine, chloramphenicol, and 4-HBP. Later, Puig and Tephly (7) purified a UDPGT to apparent homogeneity, which catalyzed morphine glucuronidation but not 4-HBP glucuronidation. In addition, they showed the separation of a 4-HBP UDPGT from the morphine UDPGT. Mackenzie (8) has reported on the expression of a cDNA from a rat liver library ($UDPGT_r$-2), which demonstrated glucuronidation of testosterone, chloramphenicol, 4-HBP, and 4-methylumbelliferone. However, the $UDPGT_r$-2, as expressed, appears to differ from the 4-HBP UDPGT isolated by Puig and Tephly (7), because testosterone glucuronidation was not observed in the latter preparation. This would suggest the possibility that several UDPGTs that catalyze 4-HBP glucuronidation may be present in rat hepatic microsomes.

In the current report, procedures for the purification to homogeneity of 4-HBP UDPGT are described. In addition, the substrate specificity and immunoreactivity of this protein, using antibodies raised against other rat liver UDPGTs, have been studied.

Experimental Procedures

Chemicals. UDP-D-[U-^{14}C]glucuronic acid (225 mCi/mmol) and *p*-[2,6-^{14}C]nitrophenol (30 mCi/mmol) were purchased from ICN Radiochemicals (Irvine, CA). [4-^{14}C]Testosterone (50 mCi/mmol) and D-*t*-[*dichloroacetyl*-1,2-^{14}C]chloramphenicol (58.2 mCi/mmol) were obtained from Du Pont NEN (Boston, MA). 4-HBP, dithiothreitol, testosterone, 4-methylumbelliferone, *p*-nitrophenol, L-α-phosphatidylcholine, and UDP-hexanolamine-Sepharose 4B were acquired from Sigma Chemical Co. (St. Louis, MO). Morphine sulfate was obtained from Merck and Co., Inc. (Rahway, NJ). Emulgen 911 was a gift from KAO Atlas, Ltd. (Tokyo, Japan). Trisacryl-DEAE was purchased from LKB-Produkter AB (Bromma, Sweden). Chromatofocusing materials and commercial molecular weight standards for SDS-PAGE were purchased from Pharmacia, Inc. (Piscataway, NJ). Protein assay and silver staining reagents were obtained from Bio-Rad (Richmond, CA). Sep-Pak C_{18} cartridges were purchased from Millipore (Bedford, MA). Endo H was purchased from ICN Biomedicals (Costa Mesa, CA).

Animals. Female Wistar rats, weighing approximately 200 g, were

This work was supported by National Institutes of Health Grant GM-26221.

ABBREVIATIONS: UDPGT, UDP-glucuronosyltransferase; 4-HBP, 4-hydroxybiphenyl; UDPGA, UDP-glucuronic acid; Endo H, endo-*N*-acetyl-β-D-glucosaminidase H; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

acquired from the Charles River Breeding Laboratories (Wilmington, MA) and injected intraperitoneally with 80 mg/kg sodium phenobarbital for 4 days. Within 24 hr after the final treatment, the rats were killed and the livers were removed and placed in cold 1.15% KCl.

Preparation of microsomes. Rat liver microsomes were prepared from Wistar rats as described by Puig and Tephly (7). Only hepatic microsomes from livers of Wistar rats that displayed low activity towards androsterone were used. This was done to eliminate the 3α-hydroxysteroid UDPGT from subsequent purification procedures (7). Microsomal preparations were stored at −70° until used for experiments.

Solubilization of microsomes. The microsomal pellets were thawed and resuspended in 5–10 ml of a buffer containing 25 mM Tris-acetate, pH 8.9, 1 mM dithiothreitol, and 20% glycerol. The microsomes were stirred at 4° and solubilized by slow addition of Emulgen 911 until a concentration of 0.85 mg of detergent/mg of protein was achieved. This suspension was stirred for 30 min and then centrifuged for 60 min at 105,000 × *g*. The supernatant fraction was assayed for protein content (9), and 75–85% of the protein was usually recovered.

Separation and purification of 4-HBP UDPGT. A 40-ml column was packed with degassed trisacryl-DEAE anion exchange resin, at 4°. The column was equilibrated with approximately 1.5 liters of buffer containing 25 mM Tris-acetate, pH 8.9, 1 mM dithiothreitol, 20% glycerol, and 0.05% Emulgen 911. About 250 mg of solubilized microsomes (10 mg/ml) were applied to the column and washed with 2 column volumes of the equilibration buffer, which contained 175 μg/ml phosphatidylcholine, as did subsequent buffers used in the column purifications. A linear NaCl gradient from 0–175 mM was generated in a volume of 250 ml. The 4-HBP UDPGT eluted between 100 and 150 mM NaCl. These fractions were pooled and dialyzed against 4 liters of equilibration buffer, pH 8.3, for 12 hr, to remove the NaCl.

After dialysis, the pool was applied to a preequilibrated chromatofocusing column packed with PBE-94 ion exchange resin. The column was washed with 2 column volumes of equilibration buffer containing 175 μg/ml phosphatidylcholine, and a pH gradient from 8.3 to 5.0 was generated using polybuffer. The 4-HBP UDPGT eluted from the column at about pH 5.8. These fractions were pooled, and $MgCl_2$ was added to a final concentration of 5 mM.

The pool from the chromatofocusing step was then applied to a column packed with UDP-hexanolamine-Sepharose 4B, which had been preequilibrated with 25 mM bis-Tris-HCl, pH 6.2, 1 mM dithiothreitol, 20% glycerol, 0.05% Emulgen 911. The column was washed with 10 column volumes of the bis-Tris buffer containing 10 mM KCl. The 4-HBP UDPGT activity eluted from the column in the bis-Tris-

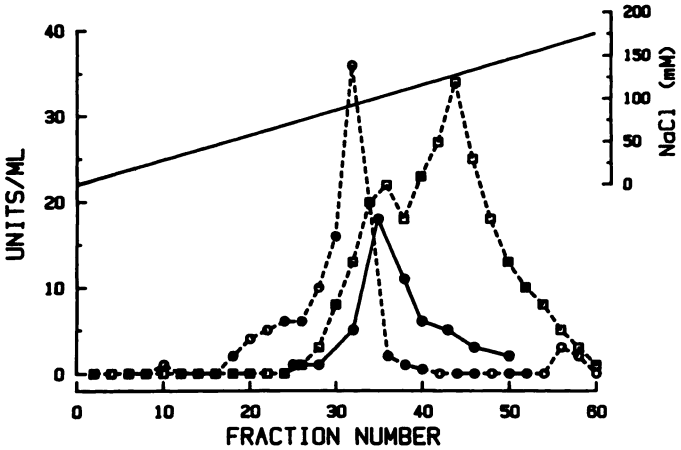

Fig. 1. Trisacryl-DEAE column chromatography of liver microsomes from low-androsterone, phenobarbital-treated, female Wistar rats. Solubilized protein (250 mg) was applied to a 25- × 45-cm Trisacryl-DEAE column equilibrated at pH 8.9. The column was eluted with a linear NaCl gradient (0–175 mM). □– – –□, 4-HBP; ○– – –○, morphine; ●——●, chloramphenicol UDPGT activities. Units represent nmol of glucuronide formed/min.

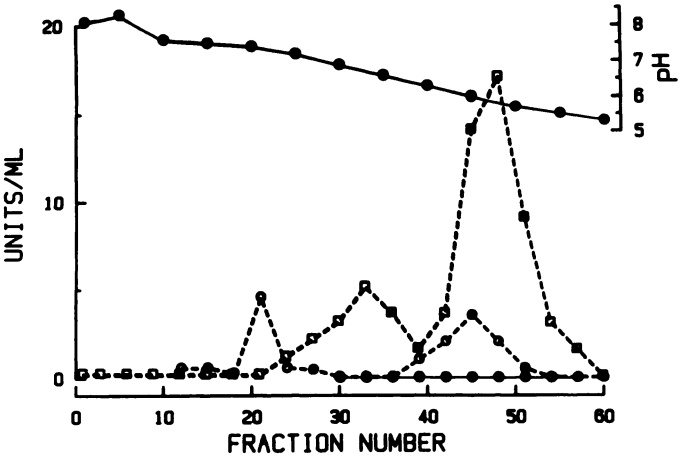

Fig. 2. Chromatofocusing chromatography of dialyzed pooled fractions from a Trisacryl-DEAE column. About 35 mg of protein were applied to a 1.0- × 40-cm chromatofocusing column equilibrated at pH 8.3. The column was eluted with a pH gradient from 8.3–5.0. Fractions 42–54 were pooled and applied to a UDP-hexanolamine Sepharose 4-B affinity column for further purification of 4-HBP UDPGT. □– – –□, 4-HBP; ○– – –○, morphine; ●——●, chloramphenicol UDPGT activities. Units represent nmol of glucuronide formed/min.

KCl buffer containing 3 mM UDPGA. If 17β- or 3α-hydroxysteroid UDPGTs were detected in the chromatofocusing pool, the affinity column was washed with 100 μM UDPGA before the 3 mM UDPGA buffer, to remove these contaminating UDPGTs (10).

SDS-PAGE. SDS-PAGE was carried out as previously described by Puig and Tephly (7).

Glucuronidation assays. Assays for morphine and 4-HBP glucuronidation were performed as previously described by Puig and Tephly (7). *p*-Nitrophenol, testosterone, and androsterone glucuronidations were analyzed as described previously (10). 4-Methylumbelliferone glucuronidation was measured by the method of Frei *et al.* (11). Chloramphenicol glucuronidation was determined by the method described by Young and Lietman (12). Kinetic constants were determined using ENZFITTER (Elsevier).

Glycosylation studies. A sample (50 μl) of the purified 4-HBP UDPGT was denatured by heating to 100° in 150 μl of 0.1% SDS, for 2 min. Upon cooling, the sample was diluted in a 50 mM sodium phosphate buffer, pH 6.5, until the SDS concentration was less than 0.02%. Endo H was added (20 mU), and the mixture was incubated at 37° overnight. The sample was then analyzed by SDS-PAGE and visualized by silver stain.

Immunoreactivity experiments. Antibodies raised in female rabbits against rat liver *p*-nitrophenol UDPGT, 3α-hydroxysteroid UDPGT, and 17β-hydroxysteroid UDPGT and in sheep against rabbit *p*-nitrophenol UDPGT were used in immunoblotting procedures with purified 4-HBP UDPGT, according to methods previously published (13, 14).

NH_2-terminal amino acid analysis. Purified rat liver 4-HBP UDPGT was sequenced using an Applied Biosystems 470A gas phase sequencer with an on-line 120A phenylthiohydantoin analyzer. The protein was applied to a 10% SDS-polyacrylamide gel, copper stained, and electroeluted from the gel. An aliquot of the electroeluted sample was reapplied to a 10% SDS-polyacrylamide gel, to confirm recovery of the protein. The eluate (0.5 ml) was placed in a Centricon-30 microconcentrator, 1.5 ml of 0.1% SDS were added, and the tube was centrifugated at 3000 × *g* for 30 min. This procedure was repeated six times to remove residual Tris and glycine from the preparation. The sequence was performed twice from separate preparations.

Results

Separation and purification of 4-HBP UDPGT. Hepatic microsomes from phenobarbital-treated, low-androsterone,

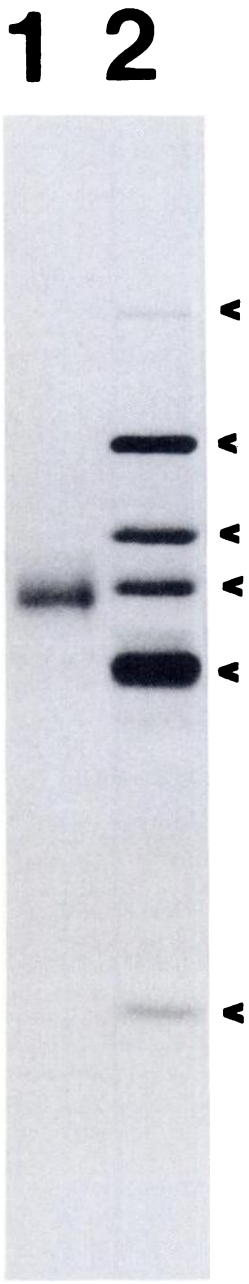

Fig. 3. SDS-PAGE of homogeneous 4-HBP UDPGT after affinity chromatography. *Lane 1*, 4-HBP UDPGT. *Lane 2*, molecular weight standards of 94,000, 67,000, 58,000, 53,000, 43,000, and 20,000 (7). Migration is from *top* to *bottom*. Proteins were visualized using silver stain.

Wistar rats were solubilized with Emulgen 911 and applied to a trisacryl-DEAE anion exchange column. Fig. 1 shows a typical elution profile. UDPGT activities toward morphine, chloramphenicol, and 4-HBP eluted from the column between 100 and 150 mM NaCl. In this step, a partial separation of chloramphenicol, morphine, and 4-HBP UDPGTs was observed. Testosterone glucuronidation followed the activity seen with chloramphenicol (data not shown). The 4-HBP UDPGT activity eluted in two peaks, one of which coincided with chloramphenicol glucuronidation and the other of which did not catalyze the glucuronidation of chloramphenicol. Fractions 40–48 were pooled, dialyzed, and applied to a preequilibrated chromatofocusing column. Fig. 2 shows the 4-HBP UDPGT elution profile over a pH gradient from 8 to 5. 4-HBP UDPGT eluted maximally at about pH 5.8. This procedure resolved this enzyme from most of the morphine UDPGT, which eluted maximally at pH 6.8. Fractions 42–54 were pooled and applied to a preequilibrated UDP-hexanolamine-Sepharose 4B affinity column. This procedure provided a complete separation of the 4-HBP UDPGT from morphine UDPGT, which was recovered in the void volume, and the 17β-hydroxysteroid UDPGT, which eluted from the affinity column at a UDPGA concentration of 100 μM. The 4-HBP UDPGT eluted from the affinity column at 3 mM UDPGA. The peak fractions containing 4-HBP UDPGT were analyzed by SDS-PAGE, and a single band was observed, with an apparent subunit molecular weight of 52,500 (Fig. 3).

TABLE 1

Summary of 4-HBP UDPGT purification

	Total protein	Specific activity[a]	Total activity[a]	Yield	Purification factor	K_m 4-HBP	K_m UDPGA
	mg	*units/mg*	*units*	%		*mM*	
Native microsomes	320	10	3200	100	1	0.6	
Solubilized microsomes	250	21	5250	164	2.1	0.23	
Trisacryl-DEAE chromatography	38.5	33	1271	40	3.3	0.15	
Chromatofocusing	7.2	115	828	26	11.5		
Affinity chromatography	0.2	516	103	3.2	51.6	0.26	1

[a] 1 unit represents 1 nmol of glucuronide formed/min.

TABLE 2

Substrate specificity of the purified rat hepatic 4-HBP UDPGT

Values represent the mean ± standard deviation as determined from three separate preparations.

Substrate	Product formation
	nmol/min/mg
4-HBP	657 ± 175
4-Methylumbelliferone	514 ± 157
p-Nitrophenol	214 ± 132
Morphine	0
Chloramphenicol	0
Androsterone	0
Testosterone	0
4-Hydroxycoumarin	0
7-Methoxycoumarin	0

Table 1 summarizes the procedure for purification of the 4-HBP UDPGT to homogeneity. Based on this typical purification, an apparent 51-fold purification of the enzyme was achieved.

Substrate specificity of 4-HBP UDPGT. The substrate specificity of 4-HBP UDPGT was determined (Table 2). This enzyme was found to catalyze 4-methylumbelliferone and *p*-nitrophenol glucuronidation but did not mediate morphine, testosterone, androsterone, 4-hydroxycoumarin, 7-methoxycoumarin, or chloramphenicol glucuronidation. Kinetic analysis of the 4-HBP UDPGT revealed little change in the apparent K_m throughout the purification. The apparent K_m for 4-HBP with the purified enzyme preparation was determined to be 0.26 ± 0.03 mM. In intact microsomes, the apparent K_m was 0.6 ± 0.01 mM, and in solubilized microsomes the apparent K_m was 0.23 ± 0.06 mM. These values were determined in the presence of 5 mM UDPGA. The apparent K_m for UDPGA was 1 ± 0.14 mM (Table 1), using 0.5 mM 4-HBP.

Glycosylation studies. Fig. 4 shows the results of deglycosylation studies on the purified 4-HBP UDPGT. After treatment with Endo H, the subunit molecular weight of the protein

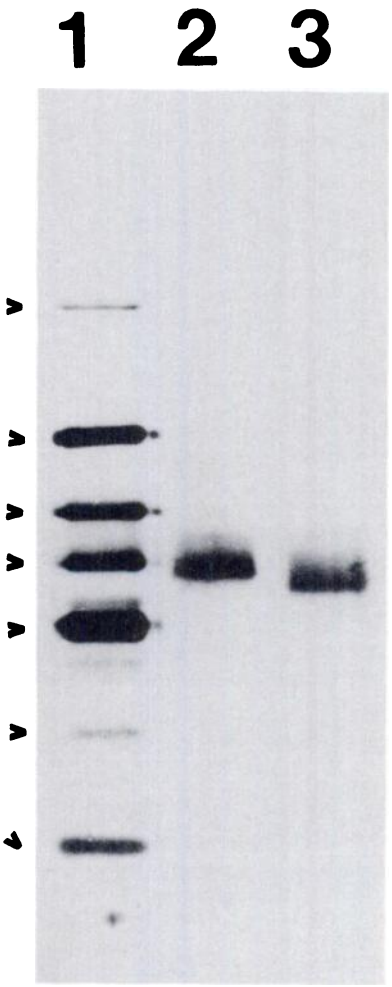

Fig. 4. Glycosylation of 4-HBP UDPGT. *Lane 1*, molecular weight standards of 94,000, 67,000, 58,000, 53,000, 43,000, 30,000, and 20,000 (7). *Lane 2*, 4-HBP UDPGT. *Lane 3*, 4-HBP UDPGT after treatment with Endo H. Migration is from *top* to *bottom*. Proteins were visualized using silver stain.

TABLE 3
Antibody reactivity with 4-HPB UDPGT

Antibody	Reactivity
17β-Hydroxysteroid UDPGT	+
3α-Hydroxysteroid UDPGT	+
Rabbit anti-rat *p*-nitrophenol UDPGT	−
Sheep anti-rabbit *p*-nitrophenol UDPGT	−

decreased from 52,500 to 50,000. These results show that the 4-HBP UDPGT is probably *N*-glycosylated. Previous studies have shown that deglycosylation of UDPGTs does not affect rates of glucuronidation (15).

Immunoreactivity of the 4-HBP UDPGT. Four antibodies raised against UDPGTs were used to determine the immunoreactivity of the 4-HBP UDPGT. Table 3 summarizes the Western blot analysis. Rabbit anti-rat 17β-hydroxysteroid UDPGT and 3α-hydroxysteroid UDPGT antibodies recognized 4-HBP UDPGT, but no immunoreactivity was detected with sheep anti-rabbit *p*-nitrophenol or rabbit anti-rat *p*-nitrophenol UDPGT antibodies.

NH_2-terminal amino acid sequence. NH_2-terminal amino acid analysis was performed, and the sequence was found to be GKVLVWPMEYSHWMN.

Discussion

Phenobarbital treatment of rats leads to the induction of a number of UDPGTs in hepatic microsomes. Bock *et al.* (6) termed a purified UDPGT preparation isolated from rat liver microsomes of phenobarbital-treated rats "GT-2" and noted that this preparation catalyzed the glucuronidation of morphine, 4-HBP, and chloramphenicol. Recently, Puig and Tephly (7) showed that morphine UDPGT could be purified to homogeneity from hepatic microsomes of phenobarbital-treated rats and that it possessed no reactivity with 4-HBP. In addition, these studies showed that a 4-HBP UDPGT could also be separated from morphine UDPGT. However, the 4-HBP UDPGT was not studied further. The current report shows that 4-HBP UDPGT can be purified to homogeneity and that it is functionally and physically different from other rat hepatic UDPGTs.

The 4-HBP UDPGT, as isolated, catalyzed 4-HBP, *p*-nitrophenol, and 4-methylumbelliferone glucuronidation but did not catalyze chloramphenicol, morphine, androsterone, testosterone, 4-hydroxycoumarin, or 7-methoxycoumarin glucuronidation. The 4-HBP UDPGT isolated here is different in its substrate specificity from the protein expressed by the cDNA isolated by Mackenzie (8), UDPGT$_r$-2, which was shown to catalyze 4-HBP, *p*-nitrophenol, 4-methylumbelliferone, testosterone, and chloramphenicol glucuronidation. The NH_2-terminal amino acid sequence of 4-HBP UDPGT is different from that of UDPGT$_r$-2 (3). Evidence for the presence of UDPGT$_r$-2 was shown using trisacryl-DEAE chromatography, where fractions were obtained that possessed activity for both 4-HBP and chloramphenicol. Thus, there are two UDPGTs capable of catalyzing the glucuronidation of 4-HBP.

The apparent K_m values for 4-HBP with fractions of 4-HBP UDPGT obtained from various columns and with the homogeneous protein were similar. The apparent K_m with the pure protein was 0.26 mM, and the apparent K_m for UDPGA was about 1.0 mM. These results are similar to those reported previously with *p*-nitrophenol, 17β-hydroxysteroid, and 3α-hydroxysteroid UDPGTs (10, 16), but not with the morphine UDPGT (7). Another important observation that should be noted is that the 4-HBP UDPGT appears to retain its activity on prolonged storage (weeks), unlike the morphine UDPGT, which is extremely labile after purification procedures are instituted.

The immunoreactivity of 4-HBP UDPGT with antibodies raised against steroid-reactive UDPGTs was somewhat surprising and suggests that one or more endobiotic substrates may react with this protein, presumably, but not necessarily, steroid-type substrates. There was no immunoreactivity with antibodies raised against rabbit or rat *p*-nitrophenol UDPGTs. The anti-rat *p*-nitrophenol UDPGT antibodies used in these experiments have been explored previously in immunoblotting experiments of rat hepatic microsomal preparations and have been shown to be quite specific in recognizing *p*-nitrophenol UDPGT (13, 14). The antibodies raised against the steroid-reactive UDPGTs are known to be less specific and have been shown to be immunoreactive with a steroid-reactive UDPGT in human liver microsomal preparations, the human hepatic estriol (16α-hydroxysteroid) UDPGT, in addition to their cross-reactivity with the 17β- and 3α-hydroxysteroid UDPGTs of rat liver microsomes.

Not uncommonly, investigators interested in glucuronidation

report rates of glucuronidation of aglycone substrates in liver microsomes from untreated and induced animals. The present study, as well as other recent reports, shows that such comparisons may or may not be meaningful. To draw conclusions based on substrates known to react with multiple UDPGTs and to note an increase (or decrease) in "UDPGT activity" seems inappropriate, given the state of our knowledge about multiple UDPGTs, their substrate specificities, and the kinetics of reaction. For example, *p*-nitrophenol is glucuronidated in hepatic microsomes from untreated rats by at least three UDPGTs, the 17β-hydroxysteroid, the *p*-nitrophenol, and the 4-HBP UDPGT. In 3-methylcholanthrene-treated rats hepatic microsomes are enriched in the *p*-nitrophenol UDPGT (10), and in phenobarbital-treated rats there is an increased abundance of 4-HBP UDPGT and the UDPGT related to the UDPGT$_r$-2 cDNA clone (3, 8). A similar situation for 4-methylumbelliferone, testosterone, 1-naphthol, and 4-HBP also exists. Thus, unless a substrate is used that reacts with a single UDPGT, a degree of caution must be exercised when interpreting data obtained from animals where inducing agents have been employed.

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