0026-895X/88/010014-08$02.00/0

MOLECULAR PHARMACOLOGY, 33:14-21

Immunohistochemical Demonstration of Isozyme- and Strain-Specific Differences in the Intralobular Localizations and Distributions of UDP-Glucuronosyltransferases in Livers of Untreated Rats

SHIRLEY A. KNAPP, MITCHELL D. GREEN, THOMAS R. TEPHLY, and JEFFREY BARON
Department of Pharmacology, The University of Iowa, Iowa City, Iowa 52242
Received July 7, 1987; Accepted October 13, 1987

SUMMARY

Antibodies directed against three isozymes of rat hepatic microsomal UDP-glucuronosyltransferase (EC 2.4.1.17), *p*-nitrophenol, 3α-hydroxysteroid, and 17β-hydroxysteroid UDP-glucuronosyltransferases were used to localize these enzymes at the light microscopic level in livers of untreated Sprague-Dawley and Wistar rats. Avidin-biotin-peroxidase staining revealed the presence of each isozyme within parenchymal cells throughout the liver lobule in rats of both strains. However, although antibodies to the 3α- and 17β-hydroxysteroid UDP-glucuronosyltransferases appeared to stain hepatocytes across the liver lobule quite uniformly, centrilobular hepatocytes were stained much more intensely for *p*-nitrophenol UDP-glucuronosyltransferase than were midzonal and periportal cells. Additionally, appreciable immunohistochemical staining for *p*-nitrophenol UDP-glucuronosyltransferase, but not for the two hydroxysteroid UDP-glucuronosyltransferases, was detected within the epithelium of the hepatic bile duct and the endothelium of the hepatic artery and portal vein. Another difference was noted in livers of Wistar rats: hepatocytes of rats possessing low 3α-hydroxysteroid (i.e., androsterone) UDP-glucuronosyltransferase activity were stained much less intensely for the 3α-hydroxysteroid UDP-glucuronosyltransferase than were those of rats exhibiting high rates of androsterone glucuronidation, whereas differences in immunoperoxidase staining for *p*-nitrophenol and 17β-hydroxysteroid UDP-glucuronosyltransferases were not apparent between the two subclasses of Wistar rats. These immunohistochemical findings demonstrate that different UDP-glucuronosyltransferase isozymes are distributed across the liver lobule in significantly different manners and, furthermore, suggest that xenobiotics may be glucuronidated within epithelial cells of the hepatic bile duct and endothelial cells of the hepatic artery and portal vein, as well as within hepatocytes. The results of this study also provide evidence that differences in the content of 3α-hydroxysteroid UDP-glucuronosyltransferase within hepatocytes account for genetically determined variations in the rates at which androsterone and certain other xenobiotics are glucuronidated in livers of Wistar rats.

Glucuronidation represents an important process in the mammalian organism whereby the glucuronic acid moiety of UDP-glucuronic acid is covalently linked to xenobiotics and endogenous substances to yield more water-soluble compounds that, in general, are excreted more readily than are the parent chemicals (1). The glucuronidation of xenobiotics and endogenous substances occurs in numerous tissues and is catalyzed by a family of isozymes that are named, collectively, the UDP-glucuronosyltransferases (UDPGTs) (1, 2).

Recently, considerable attention has been focused on the resolution, purification, and characterization of the UDPGT isozymes that are associated with the endoplasmic reticulum of the liver. Results of studies conducted by a number of laboratories have conclusively demonstrated that marked heterogeneity exists in the biophysical properties of hepatic microsomal UDPGTs (1, 3–11). Furthermore, although the isozymes that have been purified and characterized to date generally possess overlapping xenobiotic substrate specificities, they have rather distinct specificities for endogenous substrates (1, 3–6, 12–15). Additionally, genetic variability in glucuronidations catalyzed by one of these isozymes, 3α-hydroxysteroid UDPGT, has been found to occur in Wistar rats (16–20), 50–60% of which possess high (HA) and 40–50% low (LA) glucuronidation activity toward androsterone (16–18) and certain other substrates, including aromatic amines (20). The variability in hepatic microsomal 3α-hydroxysteroid UDPGT activity in Wistar rats primarily appears to be the consequence of a genetically

This investigation was supported by United States Public Health Service Grants GM26221 and GM33253.

ABBREVIATIONS: UDPGT, UDP-glucuronosyltransferase; HA, Wistar rats exhibiting high 3α-hydroxysteroid UDPGT activity; LA, Wistar rats having low 3α-hydroxysteroid UDPGT activity, CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid.

determined difference in the hepatic content of this isozyme (17–19).

Since glucuronidation serves an important role in xenobiotic detoxication (1), knowledge of the precise cellular localizations and intercellular distributions of UDPGTs within the liver lobule could contribute to an increased understanding of the basis for the marked differences that are observed in the susceptibilities of hepatocytes lying within the different regions of the liver lobule to many chemically induced toxicities. Indeed, results of studies conducted with perfused rat liver (21, 22) and of enzymatic determinations in microdissected centrilobular and periportal regions (23, 24) have revealed that xenobiotics are not glucuronidated uniformly across the liver lobule. Although heterogeneity in the intralobular distribution of *p*-nitrophenol UDPGT in rat liver has been demonstrated immunohistochemically (24), virtually nothing is currently known regarding the intrahepatic localizations and intralobular distributions of different UDPGT isozymes. In the present study, antibodies raised against highly purified monomers of rat hepatic microsomal 3α-hydroxysteroid UDPGT, 17β-hydroxysteroid UDPGT, and a 3-methylcholanthrene-inducible *p*-nitrophenol UDPGT were employed to determine immunohistochemically the cellular localizations and intralobular distributions of these three isozymes in livers of untreated rats. Differences in the content of 3α-hydroxysteroid UDPGT within hepatocytes of HA and LA Wistar rats were also investigated immunohistochemically.

Experimental Procedures

Materials. Parabenzoquinone was purchased from Fisher Chemical Co. (Pittsburg, PA), and 3,3′-diaminobenzidine tetrahydrochloride was obtained from Hach Chemical Co. (Loveland, CO). Biotinylated goat anti-rabbit IgG and the avidin-biotinylated horseradish peroxidase complex were purchased from Vector Laboratories, Inc. (Burlingame, CA). Goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Cooper-Biomedical, Inc. (Malvern, PA). 4-Chloro-1-naphthol, obtained from Bio-Rad Laboratories (Richmond, CA), was used as the peroxidase substrate in immunoblotting procedures. [1,2-^{3}H]Androsterone (40–50 Ci/mmol), [4-^{14}C]estrone (57 mCi/mmol), [4-^{14}C]testosterone (50 mCi/mmol), [4-^{14}C]-17β-estradiol (50 mCi/mmol), and [9,11-^{3}H]-5α-androstane-3α,17β-diol (60 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA). [2,6-^{14}C]-*p*-Nitrophenol (30 mCi/mmol) and UDP-[U-^{14}C]glucuronic acid (254 mCi/mmol) were obtained from ICN Radiochemicals (Irvine, CA), and 1-[1-^{14}C]naphthol was purchased from Amersham Corp. (Arlington Heights, IL). UDP-Glucuronic acid (ammonium salt), 4-methylumbelliferone, androsterone, testosterone, estrone, 17β-estradiol, *p*-nitrophenol, 5α-androstane-3α,17β-diol, and CHAPS were purchased from Sigma Chemical Co. (St. Louis, MO). Morphine sulfate was acquired from Merck and Company, Inc. (Rahway, NJ).

Preparation of antigens and antibodies. 3α-Hydroxysteroid, 17β-hydroxysteroid, and 3-methylcholanthrene-inducible *p*-nitrophenol UDPGTs were isolated and purified to apparent homogeneity from hepatic microsomes of untreated or 3-methylcholanthrene-pretreated Sprague-Dawley rats using chromatofocusing and affinity chromatography as described previously (3, 13). Monomeric subunits of each isozyme were isolated by means of preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Falany *et al.* (6).

To elicit the production of antibodies against each UDPGT, female New Zealand White rabbits were initially administered 100 μg of the isozyme's monomeric subunit in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) subcutaneously at multiple sites along the back. Two and 4 weeks later, rabbits received booster injections containing 75 and 45 μg, respectively, of the subunit in incomplete Freund's adjuvant. Whole rabbit antisera and whole rabbit preimmune sera were used in the immunoblotting and immunohistochemical studies. For immunoprecipitation experiments, the IgG fraction was prepared from preimmune and immune sera by means of ammonium sulfate precipitation and DEAE-cellulose chromatography (25).

Immunoblotting and immunoprecipitation procedures. Hepatic microsomal preparations from untreated, male Sprague-Dawley rats were solubilized in 100 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, and 15 mM CHAPS detergent and centrifuged at 100,000 × *g* for 1 hr (26). The ratio of CHAPS to microsomal protein was approximately 1.5. Samples of solubilized microsomal protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the method of Laemmli (27) in 10% acrylamide gels as described previously (3). Electrophoresis was normally conducted at 10 mamp/gel for 14–16 hr. In some cases, however, the current was increased after 16 hr to 50 mamp/gel, and electrophoresis was continued for an additional 2 hr to increase separation in the 40,000–70,000 molecular weight region. Immunoblotting was conducted according to the method of Towbin *et al.* (28) using nitrocellulose membranes (BA85, Schleicher and Schuell, Keene, NH) and a Hoefer Scientific Instruments (San Francisco, CA) Transphor apparatus. Tween 20 was used to block nonspecific binding of antisera to the nitrocellulose membrane.

Since none of the anti-UDPGTs directly inhibited glucuronidation activities catalyzed by hepatic microsomal preparations from untreated rats, immunoprecipitation studies were performed. For these experiments, aliquots of solubilized microsomal protein were incubated with immune or preimmune IgG for 2 hr at 4° with gentle mixing. To precipitate immune complexes, goat anti-rabbit IgG was added, and the samples were incubated for an additional 2 hr at 4°. The samples were then centrifuged, and UDPGT activities were determined in the supernatants. The rates of androsterone, 5α-androstane-3α,17β-diol, estrone, 17β-estradiol, testosterone, and *p*-nitrophenol glucuronidation were determined as described previously (3, 29). 4-Methylumbelliferone glucuronidation was assessed according to the method of Frei *et al.* (30), while morphine glucuronidation was determined as described by Puig and Tephly (10). The addition of less than 10 mg of IgG protein/mg of microsomal protein to the reaction mixture resulted in less than 10% inhibition of the rates at which these substrates were glucuronidated.

Immunohistochemistry of UDPGTs. Male albino Sprague-Dawley (Harlan Sprague-Dawley, Inc., Indianapolis, IN) and Wistar (Charles River Breeding Laboratories, Inc., Wilmington, MA) rats weighing 180–230 g were fasted for 24 hr before decapitation. Blocks of tissue, approximately 5 × 3 × 1 mm, obtained from the median lobe of the liver, were fixed at 4° for a total period of 3 hr by immersion in several changes of a solution containing 0.35% (w/v) parabenzoquinone and 0.02 M $CaCl_2$ in 0.2 M sodium cacodylate buffer, pH 7.4. The remainder of the liver from each Wistar rat was used for determination of androsterone glucuronidation activity as described by Green *et al.* (18). Rats whose hepatic microsomes catalyzed the glucuronidation of androsterone at rates of at least 9 nmol/min/mg of protein were classified as having high 3α-hydroxysteroid UDPGT activity (i.e., HA rats), while those with specific activities of 1–2 nmol of androsterone conjugated/min/mg of hepatic microsomal protein were considered to possess low activity (i.e., LA rats) (18).

Following fixation, the liver blocks were dehydrated, cleared, and embedded in paraffin, and serial sections 7 μm in thickness were prepared. Each UDPGT isozyme was localized at the light microscopic level by means of avidin-biotin-peroxidase staining (31, 32). Nonspecific immunoperoxidase staining was assessed by exposing adjacent sections to preimmune rabbit serum rather than to rabbit antiserum to the UDPGT being studied. Identification of bile ducts, hepatic arteries, and portal veins in immunohistochemically stained sections was confirmed by examining serial sections that had been stained with hematoxylin and eosin.

Results

Antibody characterization. Antibodies raised against electroeluted monomeric subunits of rat hepatic microsomal *p*-nitrophenol, 3α-hydroxysteroid, and 17β-hydroxysteroid UDPGTs were examined for their antigen specificity using purified enzymes as well as solubilized hepatic microsomal preparations obtained from untreated Sprague-Dawley and HA and LA Wistar rats. Fig. 1 shows the results of immunoblotting experiments using anti-*p*-nitrophenol UDPGT. On Western blots, this antibody appears to recognize only one protein in hepatic microsomal preparations from untreated rats and does not react with either 17β-hydroxysteroid or 3α-hydroxysteroid UDPGTs (Fig. 1, *lanes 1* and *9*, respectively). The molecular weight of the peptide recognized by this antibody is approximately 56,000. Staining was not observed when preimmune serum was employed (data not shown).

Results of immunoblotting experiments performed with anti-17β-hydroxysteroid UDPGT are shown in Fig. 2. This antibody reacts with purified 17β-hydroxysteroid UDPGT (*M*r 50,000)

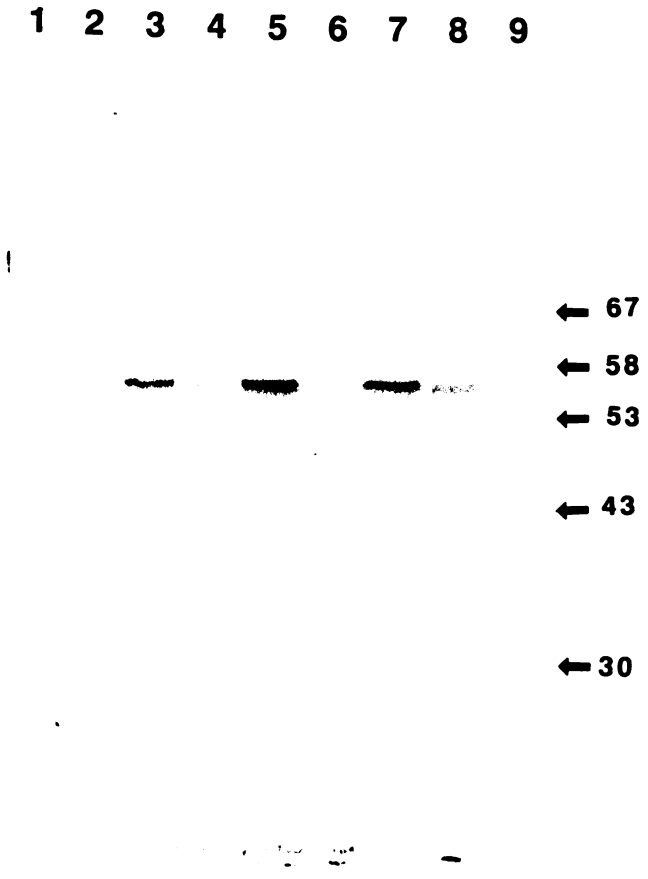

Fig. 1. Immunoblotting analysis of solubilized hepatic microsomal protein of untreated rats using rabbit anti-*p*-nitrophenol UDPGT. Solubilized microsomal protein from livers of Sprague-Dawley [10 μg (*lane 2*) and 50 μg (*lane 3*)], HA Wistar [10 μg (*lane 4*) and 50 μg (*lane 5*)], and LA Wistar [10 μg (*lane 6*) and 50 μg (*lane 7*)] rats were separated on 10% polyacrylamide gels and transferred to nitrocellulose. The blots were incubated overnight with antiserum to *p*-nitrophenol UDPGT (1:5000 dilution) and then for 4 hr with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution) and were developed using 4-chloro-1-naphthol as substrate for the peroxidase. Purified *p*-nitrophenol UDPGT (*lane 8*, 0.3 μg), 17β-hydroxysteroid UDPGT (*lane 1*, 2 μg), and 3α-hydroxysteroid UDPGT (*lane 9*, 2 μg) were also analyzed. Molecular weight markers (×10^3) are indicated by the *arrows* on the right.

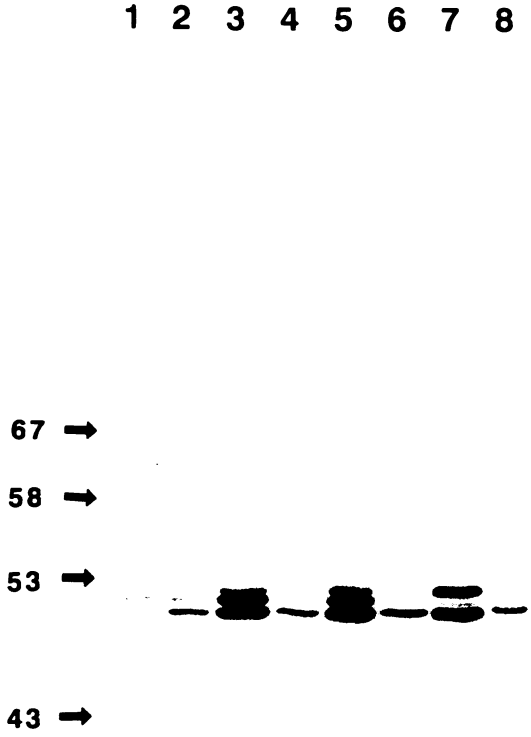

Fig. 2. Immunoblotting analysis of solubilized hepatic microsomal protein of untreated rats using rabbit anti-17β-hydroxysteroid UDPGT. Immunoblotting of solubilized microsomal protein from livers of Sprague-Dawley [5 μg (*lane 2*) and 25 μg (*lane 3*)], HA Wistar [5 μg (*lane 4*) and 25 μg (*lane 5*)], and LA Wistar [5 μg (*lane 6*) and 25 μg (*lane 7*)] rats were conducted using antiserum to 17β-hydroxysteroid UDPGT as described in Fig. 1, except that the dilution of the primary antiserum was 1:2000. Results with purified 3α-hydroxysteroid UDPGT (*lane 1*, 0.1 μg) and 17β-hydroxysteroid UDPGT (*lane 8*, 0.1 μg) are also shown. Molecular weight markers (×10^3) are indicated by the *arrows* on the left.

(Fig. 2, *lane 8*) as well as purified 3α-hydroxysteroid UDPGT (*M*r 52,000) (Fig. 2, *lane 1*). It does not, however, recognize *p*-nitrophenol UDPGT (data not shown). The anti-17β-hydroxysteroid UDPGT also reacts with more than a single protein in hepatic microsomes. As seen in Fig. 2, similar results were obtained using hepatic microsomal preparations from untreated Sprague-Dawley (*lanes 2* and *3*) and HA Wistar (*lanes 4* and *5*) rats. In contrast, the 52,000-dalton subunit corresponding to 3α-hydroxysteroid UDPGT appears to be either absent or greatly diminished in microsomal preparations derived from livers of LA Wistar rats (Fig. 2, *lanes 6* and *7*). This finding is consistent with previous reports (18, 19).

Immunoblotting results obtained using anti-3α-hydroxysteroid UDPGT (Fig. 3) were similar to those obtained with anti-17β-hydroxysteroid UDPGT. As was noted for the anti-17β-hydroxysteroid UDPGT, the antibody to 3α-hydroxysteroid UDPGT also recognizes both of the purified hydroxysteroid UDPGTs on Western blots (Fig. 3, *lanes 7* and *8*), but not *p*-nitrophenol UDPGT (Fig. 3, *lane 9*), and reacts with more than one protein in solubilized hepatic microsomal preparations from Sprague-Dawley (Fig. 3, *lanes 1* and *2*) and HA Wistar (Fig. 3, *lanes 3* and *4*) rats. Again, little if any 3α-hydroxysteroid

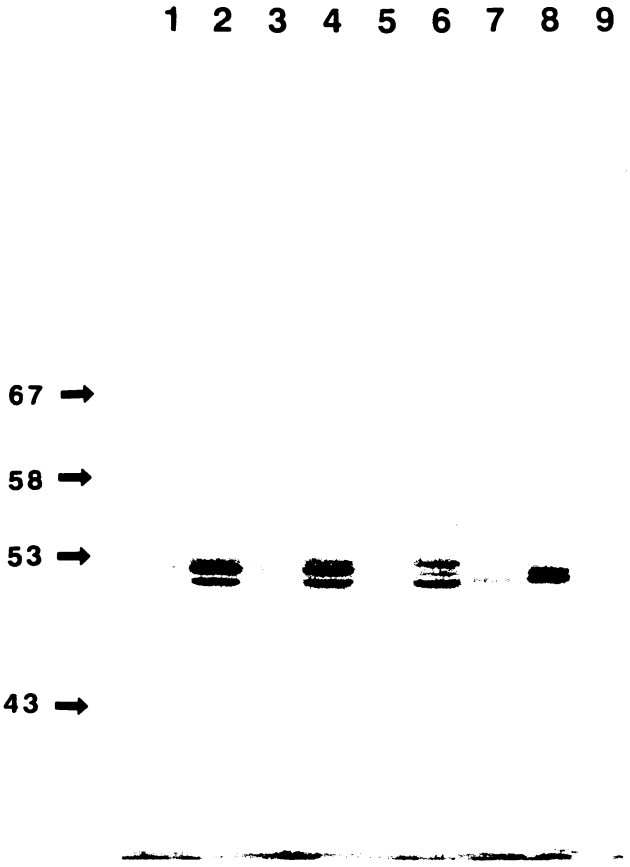

Fig. 3. Immunoblotting analysis of solubilized hepatic microsomal protein of untreated rats using rabbit anti-3α-hydroxysteroid UDPGT. Immunoblotting analysis of solubilized microsomal protein from livers of Sprague-Dawley [5 μg (*lane 1*) and 25 μg (*lane 2*)], HA Wistar [5 μg (*lane 3*) and 25 μg (*lane 4*)], and LA Wistar [5 μg (*lane 5*) and 25 μg (*lane 6*)] rats was conducted using antiserum to 3α-hydroxysteroid UDPGT as described in Fig. 1, except that the dilution of primary antiserum was 1:2000. Results obtained with purified 17β-hydroxysteroid UDPGT (*lane 7*, 0.1 μg), a mixture of 17β- and 3α-hydroxysteroid UDPGTs (*lane 8*, 0.3 μg of each), and *p*-nitrophenol UDPGT (*lane 9*, 0.5 μg) are also shown. Molecular weight markers (×10^3) are indicated by the *arrows* on the left.

UDPGT was detected in hepatic microsomal preparations from LA Wistar rats (Fig. 3, *lanes 5* and *6*).

Immunoprecipitation studies. Anti-*p*-nitrophenol UDPGT IgG did not precipitate any UDPGT activities from solubilized hepatic microsomes of untreated rats. In contrast, the addition of anti-17β-hydroxysteroid UDPGT IgG to solubilized hepatic microsomes resulted in the precipitation of glucuronidation activities catalyzed by 17β-hydroxysteroid UDPGT (Table 1). Immunoprecipitation of these UDPGT activities occurred in a concentration-dependent manner (data not shown). At an antibody protein to microsomal protein ratio of 10, the anti-17β-hydroxysteroid UDPGT precipitated testosterone, 17β-estradiol, androstane-3a,17β-diol, *p*-nitrophenol, and 1-naphthol glucuronidation activities from solubilized Sprague-Dawley hepatic microsomal preparations. It did not, however, precipitate 4-methylumbelliferone glucuronidation activity, which is catalyzed by *p*-nitrophenol UDPGT (3). Similarly, androsterone glucuronidation activity, which is catalyzed by 3α-hydroxysteroid UDPGT, was not precipitated by the antibody to 17β-hydroxysteroid UDPGT under these conditions even though this antibody did cross-react with 3α-hydroxysteroid UDPGT in immunoblotting experiments. Also, the hepatic microsomal glucuronidations of morphine and estrone, substrates for other known UDPGT isozymes, were not affected by the anti-17β-hydroxysteroid UDPGT. When higher concentrations of anti-17β-hydroxysteroid UDPGT were used (e.g., 20 mg of IgG protein/mg of microsomal protein), activities catalyzed by UDPGTs other than 17β-hydroxysteroid UDPGT were precipitated in a nonspecific manner (data not shown). Preimmune IgG did not alter UDPGT activities under these conditions.

TABLE 1

Immunoprecipitation of Sprague-Dawley rat hepatic microsomal UDPGT activities by anti-17β-hydroxysteroid UDPGT IgG

Immunoprecipitation experiments were conducted as described under Experimental Procedures using 10 mg of IgG protein/mg of microsomal protein.

Substrate	Percentage of activity precipitated[a]
Testosterone	56 ± 3
17β-Estradiol	50 ± 2
Androstane-3α,17β-diol	23
p-Nitrophenol	33 ± 10
1-Naphthol	23
4-Methylumbelliferone	5
Androsterone	5 ± 5
Estrone	5
Morphine	7

[a] Values given represent the mean ± standard deviation for determinations made on three different microsomal preparations. Single values represent the mean of two determinations.

TABLE 2

Immunoprecipitation of Sprague-Dawley rat hepatic microsomal UDPGT activities by anti-3α-hydroxysteroid UDPGT IgG

Immunoprecipitation experiments were conducted as described under Experimental Procedures using 20 mg of IgG protein/mg of microsomal protein. Values given repersent the mean of two determinations.

Substrate	Percentage of activity precipitated
Androsterone	51
Testosterone	37
17β-Estradiol	70
Estrone	7
Morphine	1

Results obtained using anti-3α-hydroxysteroid UDPGT are presented in Table 2. In contrast to the effects of anti-17β-hydroxysteroid UDPGT, the antibody to 3α-hydroxysteroid UDPGT nonspecifically precipitated rat hepatic microsomal steroid glucuronidation activities catalyzed by both 3α-hydroxysteroid UDPGT (i.e., androsterone glucuronidation) and 17β-hydroxysteroid UDPGT (i.e., testosterone and 17β-estradiol glucuronidations). However, the anti-3α-hydroxysteroid UDPGT did not affect either estrone or morphine UDPGT activities.

Localization of UDPGTs in livers of Sprague-Dawley rats. When sections of liver from untreated, male Sprague-Dawley rats were exposed to rabbit antisera to *p*-nitrophenol, 3α-hydroxysteroid, and 17β-hydroxysteroid UDPGTs in the avidin-biotin-peroxidase staining technique, the presence of immunohistochemical staining for each isozyme was readily detected within parenchymal cells throughout the lobule but not within either Kupffer or sinusoidal cells (Fig. 4). Immunoperoxidase staining was not evident in sections exposed to preimmune rabbit sera (not shown).

Although all hepatocytes were stained for each UDPGT, it is apparent that centrilobular hepatocytes were stained much more intensely by the anti-*p*-nitrophenol UDPGT than were

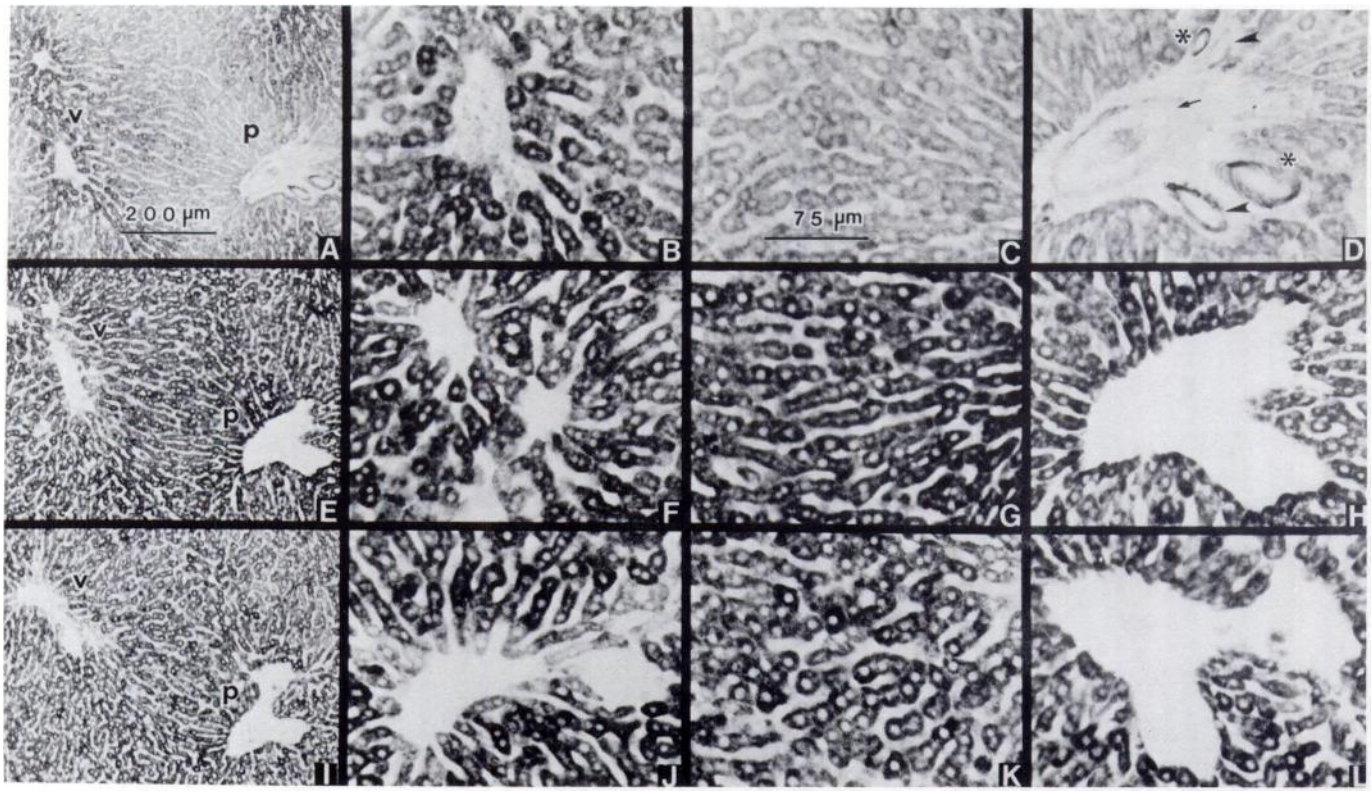

Fig. 4. Immunoperoxidase staining for UDPGTs within the liver of an untreated, male Sprague-Dawley rat. A–D, section exposed to rabbit antiserum to rat hepatic microsomal *p*-nitrophenol UDPGT; E–H, section exposed to rabbit antiserum to rat hepatic microsomal 17β-hydroxysteroid UDPGT; and I–L, section exposed to rabbit antiserum to rat hepatic microsomal 3α-hydroxysteroid UDPGT. A, E, and I show representative immunohistochemical staining within the same area in serial sections. Higher magnification photomicrographs of centrilobular regions are seen in B, F, and J, while midzonal regions are shown in C, G, and K and periportal regions in D, H, and L. The presence of immunoperoxidase staining was not evident in adjacent sections exposed to equal dilutions of preimmune rabbit sera. A central vein (*v*), a portal triad (*p*), bile ducts (◀) and branches of the hepatic artery (•) and portal vein (←) are indicated.

midzonal and periportal hepatocytes (Fig. 4, A–D). In contrast, hepatocytes across the lobule were stained quite uniformly by the antibodies to 17β (Fig. 4, E–H)- and 3α (Fig. 4, I–L)-hydroxysteroid UDPGTs. Additionally, immunohistochemical staining for both 3α- and 17β-hydroxysteroid UDPGTs was restricted to parenchymal cells, whereas staining for *p*-nitrophenol UDPGT was also detected within bile duct epithelial cells and endothelial cells of the hepatic artery and portal vein (Fig. 4D). These findings indicate that, in tissue sections, the anti-*p*-nitrophenol UDPGT used in this study does not cross-react with either of the two hydroxysteroid UDPGTs and, similarly, that antibodies to 3α- and 17β-hydroxysteroid UDPGTs do not cross-react with *p*-nitrophenol UDPGT.

Localizations of UDPGTs in livers of Wistar rats. Immunohistochemical staining for UDPGTs in livers of untreated, male HA Wistar rats closely resembled that observed in livers of untreated, male Sprague-Dawley rats. As seen in Fig. 5, A–D, centrilobular hepatocytes of these rats were stained much more intensely by the anti-*p*-nitrophenol UDPGT than were either midzonal or periportal cells, whereas hepatocytes across the lobule were stained quite uniformly for both 17β (Fig. 5, E–H)- and 3α (Fig. 5, I–L)-hydroxysteroid UDPGTs. Furthermore, in addition to staining parenchymal cells, the anti-*p*-nitrophenol UDPGT stained the bile duct epithelium and endothelial cells of the hepatic artery and portal vein (Fig. 5D), whereas antibodies to the 3α- and 17β-hydroxysteroid UDPGTs solely stained hepatocytes.

Staining for *p*-nitrophenol (Fig. 6, A–D) and 17β-hydroxysteroid (Fig. 6, E–H) UDPGTs in livers of untreated, male LA Wistar rats mirrored that observed in livers of untreated Sprague-Dawley and HA Wistar rats. In marked contrast to these findings, the intensity with which hepatocytes of LA Wistar rats were stained by the anti-3α-hydroxysteroid UDPGT was dramatically less (Fig. 6, I–L) than that seen in either Sprague-Dawley (Fig. 4) or HA Wistar (Fig. 5) rats. However, despite being depressed, the intensity of staining for 3α-hydroxysteroid UDPGT remained quite uniform across the lobule. The fact that immunohistochemical staining for *p*-nitrophenol and 17β-hydroxysteroid UDPGTs was identical in the livers of all rats studied indicates that the antibodies raised against these isozymes do not cross-react with 3α-hydroxysteroid UDPGT in tissue sections.

Discussion

During the past several years, considerable effort has been focused on determinations of how various xenobiotic-metabolizing enzymes are distributed across the liver lobule (33–36), inasmuch as differences in the intralobular distributions of enzymes that catalyze the bioactivation and detoxication of xenobiotics could profoundly influence the susceptibilities of hepatocytes lying within the different regions of the lobule to toxicities resulting from the formation of potentially reactive, electrophilic metabolites. With respect to glucuronidation, results of studies conducted on the perfused rat liver have revealed that xenobiotics are usually glucuronidated at greater

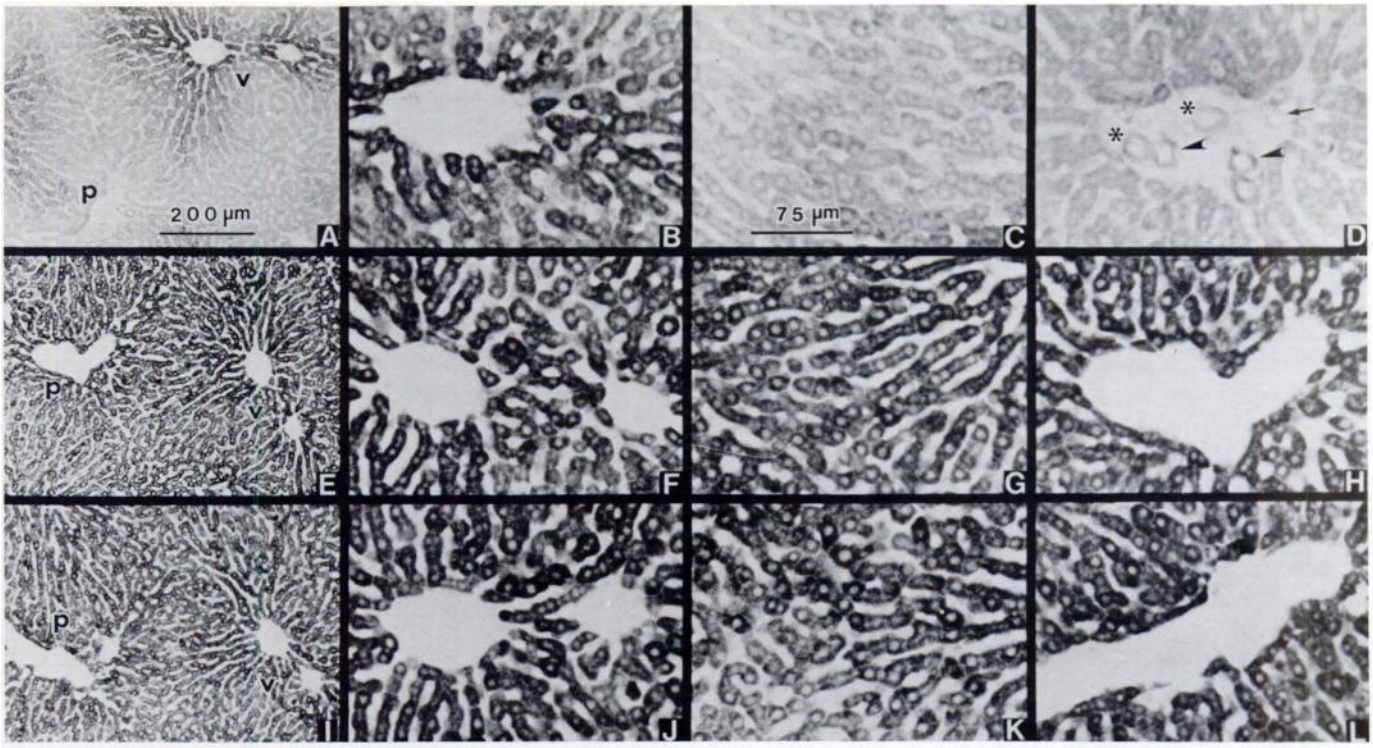

Fig. 5. Immunoperoxidase staining for UDPGTs within the liver of an untreated, male HA Wistar rat. A–D, section exposed to rabbit antiserum to *p*-nitrophenol UDPGT; E–H, section exposed to rabbit antiserum to 17β-hydroxysteroid UDPGT; and I–L, section exposed to rabbit antiserum to 3α-hydroxysteroid UDPGT. A, E, and I show representative immunohistochemical staining within the same area in serial sections. Higher magnification photomicrographs of centrilobular regions are seen in B, F, and J, while midzonal regions are shown in C, G, and K and periportal regions in D, H, and L. Immunohistochemical staining could not be detected in adjacent sections exposed to equal dilutions of preimmune rabbit sera. A central vein (*v*), a portal traid (*p*), bile ducts (◀), and branches of the hepatic artery (•) and portal vein (←) are indicated.

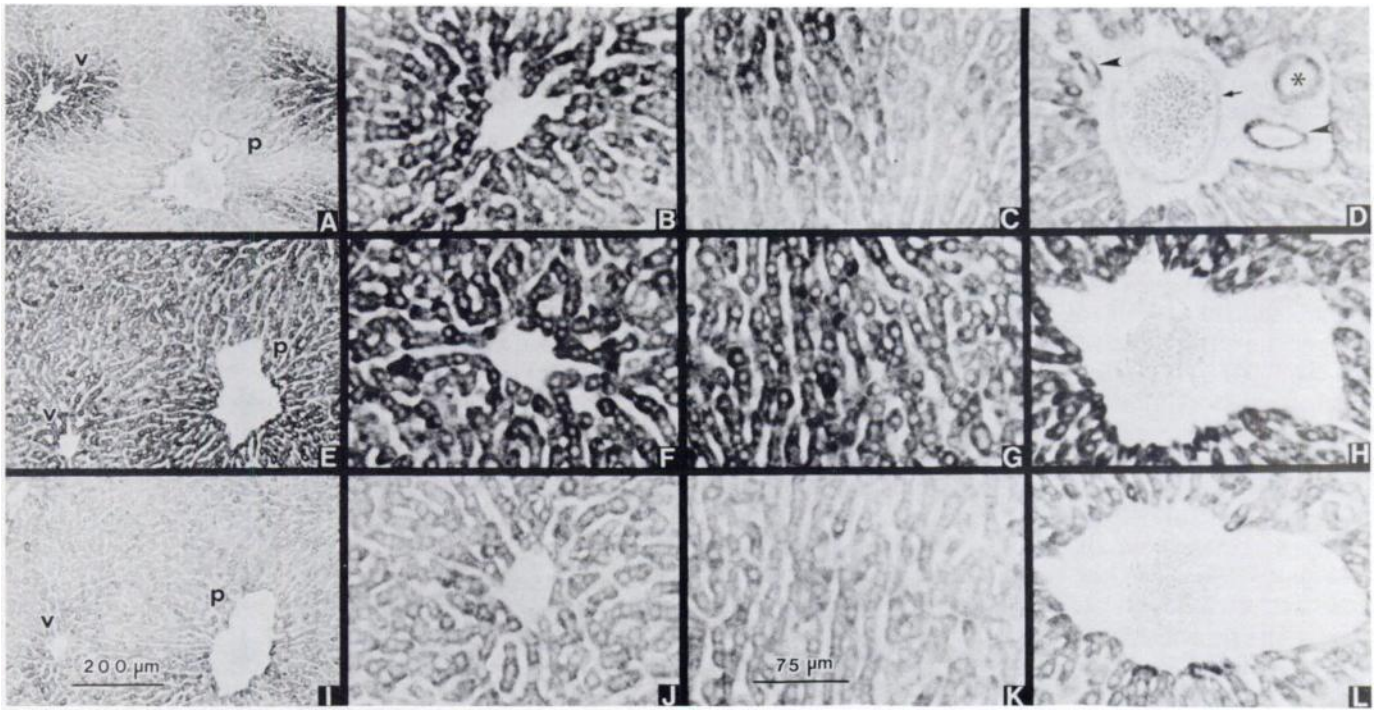

Fig. 6. Immunoperoxidase staining for UDPGTs within the liver of an untreated, male LA Wistar rat. A–D, section exposed to rabbit antiserum to *p*-nitrophenol UDPGT; E–H, section exposed to rabbit antiserum to 17β-hydroxysteroid UDPGT; and I–L, section exposed to rabbit antiserum to 3α-hydroxysteroid UDPGT. A, E, and I show representative immunohistochemical staining within the same area in serial sections. Higher magnification photomicrographs of centrilobular regions are seen in B, F, and J, while midzonal regions are shown in C, G, and K and periportal regions in D, H, and L. Immunohistochemical staining was not evident in adjacent sections exposed to preimmune rabbit sera. A central vein (*v*), a portal triad (*p*), bile ducts (◀), and branches of the hepatic artery (•) and portal vein (←) are indicated.

rates by centrilobular hepatocytes than by those hepatocytes that are situated around the portal triad (21, 22, 36). Microchemical determinations in microdissected centrilobular and periportal regions have also demonstrated that centrilobular cells exhibit significantly greater xenobiotic glucuronidation activity than do periportal cells (23, 24). In addition, immunohistochemical analyses conducted with an antibody to a 3-methylcholanthrene-inducible isozyme of rat hepatic microsomal UDPGT provided evidence for the presence of a greater amount of this enzyme within centrilobular hepatocytes of untreated rats than within periportal cells (24). In contrast, an antibody that recognizes several UDPGT isozymes has been reported to produce staining of uniform intensity across the liver lobule (37).

Significant differences have been shown to exist in the distributions of different isozymes of other xenobiotic-metabolizing enzymes, such as cytochrome P-450 (33–35, 38) and glutathione *S*-transferase (33–35, 39), across the lobule in livers of untreated rats. The results of the present study similarly demonstrated that different UDPGT isozymes are not distributed in identical manners across the liver lobule in untreated rats: the content of *p*-nitrophenol UDPGT is greatest within centrilobular hepatocytes, whereas 3α- and 17β-hydroxysteroid UDPGTs are distributed quite uniformly across the lobule. These findings thus suggest that there would be significant differences in the rates as well as the extents to which different substrates undergo glucuronidation across the liver lobule in untreated rats.

The antibodies raised against the monomeric subunits of rat hepatic microsomal *p*-nitrophenol, 3α-hydroxysteroid, and 17β-hydroxysteroid UDPGTs that were utilized in the immunohistochemical studies were also characterized with respect to their ability to recognize different UDPGT isozymes in solubilized hepatic microsomes of untreated rats and to immunoprecipitate hepatic microsomal activities catalyzed by these isozymes. On Western blots, the anti-*p*-nitrophenol UDPGT recognizes a single protein in solubilized hepatic microsomes that has a subunit molecular weight of 56,000: this is the molecular weight of the monomer of purified *p*-nitrophenol UDPGT (3). Proteins at other molecular weights were not immunochemically detectable by this antibody in hepatic microsomal preparations from untreated rats. However, the antibody to *p*-nitrophenol UDPGT was not capable of precipitating UDPGT activities from solubilized microsomes. In contrast, the anti-17β-hydroxysteroid UDPGT specifically immunoprecipitated 17β-hydroxysteroid UDPGT-catalyzed activities from hepatic microsomal preparations, although it cross-reacted with 3α-hydroxysteroid UDPGT in immunoblotting experiments. Conversely, the anti-3α-hydroxysteroid UDPGT demonstrated cross-reactivity with 17β-hydroxysteroid UDPGT in both immunoblotting and immunoprecipitation experiments.

In view of the above findings, it is remarkable to note that these antibodies specifically recognize distinct UDPGTs in tissue sections. The intralobular pattern of immunohistochemical staining for *p*-nitrophenol UDPGT differs markedly from those observed for the two hydroxysteroid UDPGTs. Although hepatocyte staining for *p*-nitrophenol UDPGT observed in the present study is similar to that noted by Ullrich *et al.* (24), the findings reported in the present communication represent the first description of the localization of this UDPGT isozyme within the bile duct epithelium and the endothelium of the hepatic artery and portal vein. The observation that epithelial cells of the hepatic bile duct and endothelial cells of the hepatic artery and portal vein contain an antigen that is recognized by the anti-*p*-nitrophenol UDPGT was unexpected. However, this finding was reproducibly obtained in livers of Sprague-Dawley and Wistar rats, as well as in rats of other strains, with liver fixed using a variety of fixatives, and when partially purified anti-*p*-nitrophenol UDPGT was employed. Thus, this immunohistochemical result suggests that bile duct epithelial cells and endothelial cells of the hepatic artery and portal vein represent other potentially important sites at which xenobiotics can undergo glucuronidation in the liver. This finding also serves to illustrate another significant difference that exists between the *p*-nitrophenol UDPGT and the two hydroxysteroid UDPGTs which appear to be restricted to hepatocytes.

Despite the fact that antibodies to the 3α- and 17β-hydroxysteroid UDPGTs are capable of cross-reacting with the heterologous antigen in solubilized hepatic microsomes, such cross-reactivity does not occur to any significant degree in tissue sections. For instance, hepatocyte staining for 17β-hydroxysteroid UDPGT was found to be intense and uniformly distributed across the lobule in both HA and LA Wistar rats. This observation is in agreement with the results of biochemical analyses which have demonstrated that this isozyme's activity is similar in livers of HA and LA Wistar rats (16–19). In contrast, hepatocytes of LA Wistar rats were stained much less intensely for 3α-hydroxysteroid UDPGT in comparison to that observed in HA Wistar rats. This finding is entirely consistent with the fact that 3α-hydroxysteroid UDPGT activity is extremely low in LA Wistar rat liver (18, 19, 40). Such correlations indicate that immunohistochemistry provides for a more specific antibody-antigen interaction than is obtained with more conventional immunochemical procedures and, furthermore, are consistent with findings on different hepatic microsomal cytochrome P-450 isozymes (33–35, 38).

The explanation for why antigen specificity is retained to a far greater degree in tissue sections may be related to the presence of the holoprotein in a more organized and physiological state. In immunoblotting and immunoprecipitation procedures, disruption processes associated with the solubilization of microsomal proteins may result in the exposure of epitopes that may not be accessible in the native UDPGT proteins as they exist in tissue sections. Although marked homology has been observed within the UDPGT family [for example, there is an 85% amino acid sequence homology between 3α- and 17β-hydroxysteroid UDPGTs (40, 41)], the amino acid sequence of *p*-nitrophenol UDPGT is quite different from those of the two hydroxysteroid UDPGTs (42). This may explain why the anti-*p*-nitrophenol UDPGT demonstrates greater specificity in immunoblotting experiments than do the antibodies to 3α- and 17β-hydroxysteroid UDPGTs.

References

1. Dutton, G. J. *Glucuronidation of Drugs and Other Compounds.* CRC Press, Inc., Boca Raton, FL (1980).
2. Bock, K. W., B. Burchell, G. J. Dutton, O. Hänninen, G. J. Mulder, I. S. Owens, G. Siest, and T. R. Tephly. UDP-glucuronosyltransferase activities. Guidelines for consistent interim terminology and assay conditions. *Biochem. Pharmacol.* **32**:953–955 (1983).
3. Falany, C. N., and T. R. Tephly. Separation, purification and characterization of three isoenzymes of UDP-glucuronyltransferase from rat liver microsomes. *Arch. Biochem. Biophys.* **227**:248–258 (1983).
4. von Meyerinck, L., B. L. Coffman, M. D. Green, R. B. Kirkpatrick, A. Schmoldt, and T. R. Tephly. Separation, purification, and characterization of digitoxigenin-monodigitoxoside UDP-glucuronosyltransferase activity. *Drug Metab. Dispos.* **13**:700–704 (1985).
5. Burchell, B. Substrate specificity and properties of uridine diphosphate

glucuronyltransferase purified to apparent homogeneity from phenobarbital-treated rat liver. *Biochem. J.* **173**:749–757 (1978).
6. Falany, C. N., M. D. Green, E. Swain, and T. R. Tephly. Substrate specificity and characterization of rat liver *p*-nitrophenol, 3α-hydroxysteroid and 17β-hydroxysteroid UDP-glucuronosyltransferases. *Biochem. J.* **238**:65–73 (1986).
7. Bock, K. W., D. Josting, W. Lilienblum, and H. Pfeil. Purification of rat liver microsomal UDP-glucuronyltransferase. Separation of two enzyme forms inducible by 3-methylcholanthrene or phenobarbital. *Eur. J. Biochem.* **98**:19–26 (1979).
8. Owens, I. S., and P. I. Mackenzie. A comparison of the isoelectric points of mouse liver UDP-glucuronosyltransferase enzymes conjugating the twelve benzo[*a*]pyrene phenols. *Biochem. Biophys. Res. Commun.* **109**:1075–1082 (1982).
9. Mackenzie, P. I., M. M. Joffe, P. J. Munson, and I. S. Owens. Separation of different UDP-glucuronosyltransferase activities according to charge heterogeneity by chromatofocusing using mouse liver microsomes. *Biochem. Pharmacol.* **34**:737–746 (1985).
10. Puig, J. F., and T. R. Tephly. Isolation and purification of rat liver morphine UDP-glucuronosyltransferase. *Mol. Pharmacol.* **30**:558–565 (1986).
11. Roy Chowdhury, J., N. Roy Chowdhury, C. N. Falany, T. R. Tephly, and I. M. Arias. Isolation and characterization of multiple forms of rat liver UDP-glucuronate glucuronosyltransferase. *Biochem. J.* **233**:827–837 (1986).
12. Falany, C. N., J. Roy Chowdhury, N. Roy Chowdhury, and T. R. Tephly. Steroid 3- and 17-OH UDP-glucuronosyltransferase activities in rat and rabbit liver microsomes. *Drug Metab. Dispos.* **11**:426–432 (1983).
13. Kirkpatrick, R. B., C. N. Falany, and T. R. Tephly. Glucuronidation of bile acids by rat liver 3-OH androgen UDP-glucuronyltransferase. *J. Biol. Chem.* **259**:6176–6180 (1984).
14. Burchell, B., and N. Blanckaert. Bilirubin mono- and di-glucuronide formation by purified rat liver microsomal bilirubin UDP-glucuronyltransferase. *Biochem. J.* **223**:461–465 (1984).
15. Roy Chowdhury, N., I. M. Arias, M. Lederstein, and J. Roy Chowdhury. Substrates and products of purified rat liver bilirubin UDP-glucuronosyltransferase. *Hepatology* **6**:123–128 (1986).
16. Matsui, M., and M. Hakozaki. Discontinuous variation in hepatic uridine diphosphate glucuronosyltransferase toward androsterone in Wistar rats. A regulatory factor for *in vivo* metabolism of androsterone. *Biochem. Pharmacol.* **28**:411–415 (1979).
17. Matsui, M., and H. K. Watanabe. Classification and genetic expression of Wistar rats with high and low hepatic microsomal UDP-glucuronosyltransferase activity towards androsterone. *Biochem. J.* **202**:171–174 (1982).
18. Green, M. D., C. N. Falany, R. B. Kirkpatrick, and T. R. Tephly. Strain differences in purified rat hepatic 3α-hydroxysteroid UDP-glucuronosyltransferase. *Biochem. J.* **230**:403–409 (1985).
19. Matsui, M., and F. Nagai. Genetic deficiency of androsterone UDP-glucuronosyltransferase activity in Wistar rats is due to the loss of enzyme protein. *Biochem. J.* **234**:139–144 (1986).
20. Green, M. D., and T. R. Tephly. *N*-Glucuronidation of carcinogenic aromatic amines catalyzed by rat hepatic microsomal preparations and purified rat liver uridine 5′-diphosphate-glucuronosyltransferases. *Cancer Res.* **47**:2028–2031 (1987).
21. Conway, J. G., F. C. Kauffman, S. Ji, and R. G. Thurman. Rates of sulfation and glucuronidation of 7-hydroxycoumarin in periportal and pericentral regions of the liver lobule. *Mol. Pharmacol.* **22**:509–516 (1982).
22. Pang, K. S., H. Koster, I. C. M. Halsema, E. Scholtens, G. J. Mulder, and R. N. Stillwell. Normal and retrograde perfusion to probe the zonal distribution of sulfation and glucuronidation activities of harmol in the perfused rat liver preparation. *J. Pharmacol. Exp. Ther.* **224**:647–653 (1983).
23. El Mouelhi, M., and F. C. Kauffman. Sublobular distribution of transferases and hydrolases associated with glucuronide, sulfate and glutathione conjugation in human liver. *Hepatology* **6**:450–456 (1986).
24. Ullrich, D., G. Fischer, N. Katz, and K. W. Bock. Intralobular distribution of UDP-glucuronosyltransferase in livers from untreated, 3-methylcholanthrene- and phenobarbital-treated rats. *Chem.-Biol. Interact.* **48**:181–190 (1984).
25. Hurn, B. A. L., and S. M. Chantler. Production of reagent antibodies. *Methods Enzymol.* **70**:104–142 (1980).
26. Mackenzie, P. I., L. M. Hjelmeland, and I. S. Owens. Purification and immunochemical characterization of a low-pI form of UDP-glucuronosyltransferase from mouse liver. *Arch. Biochem. Biophys.* **231**:487–497 (1984).
27. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
28. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
29. Tukey, R. H., R. E. Billings, and T. R. Tephly. Separation of oestrone UDP-glucuronyltransferase and *p*-nitrophenol UDP-glucuronyltransferase activities. *Biochem. J.* **171**:659–663 (1978).
30. Frei, J., E. Schmid, and H. Bischmeier. UDP-glucuronyltransferase, in *Methoden der Enzymatischen Analyze* (H. U. Bergmeyer, ed.). Verlag Chemie, Weinheim, 763–768 (1974).
31. Hsu, S. M., L. Raine, and H. Fanger. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29**:577–580 (1981).
32. Baron, J., J. M. Voigt, T. T. Kawabata, and J. A. Redick. Immunohistochemistry, in *Regulation of Hepatic Metabolism* (R. G. Thurman, F. C. Kauffman, and K. Jungermann, eds.). Plenum Publishing Corp., New York, 87–118 (1986).
33. Baron, J., and T. T. Kawabata. Intratissue distribution of activating and detoxicating enzymes, in *Biological Basis of Detoxication* (J. Caldwell and W. B. Jakoby, eds.). Academic Press, New York, 105–135 (1983).
34. Baron, J., T. T. Kawabata, S. A. Knapp, J. M. Voigt, J. A. Redick, W. B. Jakoby, and F. P. Guengerich. Intrahepatic distribution of xenobiotic-metabolizing enzymes, in *Foreign Compound Metabolism* (J. Caldwell and G. D. Paulson, eds.). Taylor & Francis, London, 17–36 (1984).
35. Wolf, C. R., E. Moll, T. Friedberg, F. Oesch, A. Buchmann, W. D. Kuhlmann, and H. W. Kunz. Characterization, localization and regulation of a novel phenobarbital-inducible form of cytochrome P_{450}, compared with three further P_{450}-isoenzymes, NADPH P_{450}-reductase, glutathione transferases and microsomal epoxide hydrolase. *Carcinogenesis* **5**:993–1001 (1984).
36. Thurman, R. G., F. C. Kauffman, and J. Baron. Biotransformation and zonal toxicity, in *Regulation of Hepatic Metabolism* (R. G. Thurman, F. C. Kauffman, and K. Jungermann, eds.). Plenum Publishing Corp., New York, 321–382 (1986).
37. Roy Chowdhury, J., P. M. Novikoff, N. Roy Chowdhury, and A. B. Novikoff. Distribution of UDP-glucuronosyltransferase in rat tissue. *Proc. Natl. Acad. Sci. USA* **82**:2990–2994 (1985).
38. Baron, J., J. A. Redick, and F. P. Guengerich. An immunohistochemical study on the localizations and distributions of phenobarbital- and 3-methylcholanthrene-inducible cytochromes P-450 within the livers of untreated rats. *J. Biol. Chem.* **256**:5931–5937 (1981).
39. Redick, J. A., W. B. Jakoby, and J. Baron. Immunohistochemical localization of glutathione *S*-transferases in livers of untreated rats. *J. Biol. Chem.* **257**:15200–15203 (1982).
40. Jackson, M. R., and B. Burchell. The full-length coding of rat liver androsterone UDP-glucuronyltransferase cDNA and comparison with other members of this gene family. *Nucleic Acids Res.* **14**:779–795 (1986).
41. Harding, D., S. M. Wilson, M. R. Jackson, B. Burchell, M. D. Green, and T. R. Tephly. Nucleotide and deduced amino acid sequence of rat liver 17β-hydroxysteroid UDP-glucuronosyltransferase. *Nucleic Acids Res.* **15**:3936 (1987).
42. Iyanagi, T., M. Haniu, K. Sogawa, Y. Fujii-Kuriyama, S. Watanabe, J. E. Shively, and K. F. Anan. Cloning and characterization of cDNA encoding 3-methylcholanthrene-inducible rat mRNA for UDP-glucuronosyltransferase. *J. Biol. Chem.* **261**:15607–15614 (1986).

Send reprint requests to: Dr. Jeffrey Baron, Department of Pharmacology, The University of Iowa, Bowen Science Building, Iowa City, IA 52242.