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Isolation and Purification of Two Human Liver UDP-Glucuronosyltransferases

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SUMMARY

Two UDP-glucuronosyltransferases (EC 2.4.1.17) were purified from human liver microsomes. Human liver microsomes were solubilized with Emulgen 911 and the UDP-glucuronosyltransferases were separated and purified by chromatofocusing and UDP-hexanolamine Sepharose 4B affinity chromatography. One isoenzyme eluted with an apparent pI of 7.4, displayed a subunit molecular weight of 53,000 after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and catalyzed the glucuronidation of *p*-nitrophenol, 4-methylumbelliferone, α-naphthylamine, and estriol, but not that of 4-aminobiphenyl. A second isoenzyme eluted with an apparent pI of 6.2, displayed a subunit molecular weight of 54,000 after SDS-PAGE, and catalyzed the glucuronidation of *p*-nitrophenol, 4-methylumbelliferone, α-naphthylamine, and 4-aminobiphenyl, but not that of estriol. Neither of the purified human liver UDP-glucuronosyltransferases employed estrone, β-estradiol, testosterone, androsterone, or 5α-androstane-3α,17β-diol as substrate. These enzymes displayed apparent K_m values in the same order of magnitude for a given substrate. In general, high concentrations of phosphatidylcholine were required for reconstitution of maximal glucuronidation activity. This report documents the existence of multiple UDP-glucuronosyltransferases in human liver.

Glucuronidation represents one of the most important forms of biological transformations of endogenous and exogenous chemicals in mammalian metabolism (1). The process involves a transfer of glucuronic acid from UDPGA to a wide variety of substrates of endogenous origin, such as bilirubin and steroids, as well as many xenobiotics of diverse chemical structure (1). An increase in the polarity of the compound occurs which facilitates its excretion from the body by the renal and biliary systems. For this reason, it is tempting to consider glucuronidation as a detoxication reaction. However, there are important exceptions to this generalization. Steroid D-ring glucuronides such as testosterone-17β-glucuronide and estradiol 17β-glucuronide are active cholestatic agents in rats (2, 3). Some *N*-*O*-glucuronides (such as *N*-hydroxy-2-acetylaminofluorene glucuronide and *N*-hydroxyphenacetin glucuronide) may possess biological activity and may bind covalently to certain tissue macromolecules (4–6).

UDPGTs (EC 2.4.1.17) are a family of membrane-bound enzymes of the endoplasmic reticulum which catalyze the glucuronide conjugation reaction. The existence of multiple forms of UDPGT has been established in laboratory animals. There are species differences in the capacity to glucuronidate (1), different developmental patterns of glucuronidation toward different substrates (7–10), different induction patterns for different substrates by chronic administration of various chemicals (7, 11, 12), and strain differences in animals in their ability to glucuronidate (13–16). Gunn rats are a mutant strain of Wistar rats which lack the ability to glucuronidate bilirubin and serve as an animal model for the human hereditary disorder, Crigler-Najjar syndrome (13). A large percentage of Wistar rats have a defect in androsterone glucuronidation (14, 15).

The separation, purification, and characterization of two different forms of UDPGT from rabbit liver microsomes catalyzing estrone and *p*-nitrophenol glucuronidations have been demonstrated (17). The separation, purification, and characterization of five different isoenzymes of UDPGT from rat liver microsomes (18–20) have been shown by our laboratory. Other laboratories, e.g., Bock *et al.* (21), Weatherill and Burchell (22), and Hochman and Zakim (23), have also reported on the separation of various UDPGTs. However, such information is lacking in regard to human UDPGT. This report documents the separation and purification of two isoenzymes from human liver.

Materials and Methods

Liver samples. Human liver samples were obtained from organ donors as part of an organ transplant program. Livers were perfused briefly (flushed) with ice-cold Collins solution, dissected, and frozen in

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ABBREVIATIONS: UDPGA, UDP-glucuronic acid; UDPGT, UDP-glucuronosyltransferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pI, isoelectric point.

dry ice. The livers were stored at −70° until used for the preparation of microsomes. The sources and characteristics of liver samples are shown in Table 1.

Chemicals. [4-^{14}C]Estrone (57 mCi/mmol), [4-^{14}C]testosterone (50 mCi/mmol), [4-^{14}C]-β-estradiol (50 mCi/mmol), [1,2-^{3}H]androsterone (40–60 Ci/mmol), [9,11-^{3}H]-5α-androstane-3α,17β-diol (60 Ci/mmol), and [2,4,6,7-^{3}H]estriol (94 Ci/mmol) were purchased from New England Nuclear, Boston, MA. [2,6-^{14}C]-*p*-Nitrophenol (30 mCi/mmol) was obtained from ICN Radiochemicals, Irvine, CA. L-α-Phosphatidylcholine (egg yolk type XI-E), UDPGA, androsterone, 5α-androstane-3α,17β-diol, estriol, β-estradiol, estrone, testosterone, 4-methylumbelliferone, α-naphthylamine, 4-aminobiphenyl, *p*-nitrophenol, Coomassie Blue reagent, molecular weight standards, and human albumin were purchased from Sigma Chemical Co., St. Louis, MO. Chromatofocusing materials were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Emulgen 911 was a gift from Kao Atlas Ltd., Tokyo, Japan. Bio-Rad dye reagent for protein assay and SDS-PAGE material (SDS, acrylamide, bisacrylamide, glycine, Tris buffer, etc.) were purchased from Bio-Rad Laboratories, Richmond, CA. All other chemicals were of analytical grade. UDP-hexanolamine Sepharose 4B affinity resin was prepared in this laboratory as previously described (17).

Enzyme assays. Androsterone, 5α-androstane-3α,17β-diol, estriol, β-estradiol, estrone, and testosterone glucuronidation was measured by the method of Tukey *et al.* (24) with final substrate concentrations of 56 μM, 56 μM, 56 μM, 37.5 μM, 37.5 μM, and 50 μM, respectively. *p*-Nitrophenol glucuronidation was assayed as previously described (24) with a final *p*-nitrophenol concentration of 3 mM. 4-Methylumbelliferone glucuronidation was assayed by the method of Frei *et al.* (25), with 2 mM 4-methylumbelliferone. α-Naphthylamine and 4-aminobiphenyl glucuronidation was assayed by the method of Lilienblum and Bock (26) with final substrate concentrations of 2 mM, except for the 4-aminobiphenyl assay which was performed at pH 6.4 and, after the reaction was stopped, the pH was raised to 7.4. When solubilized microsomal preparations were studied 0.8 mg of Emulgen 911 was used per mg of microsomal protein in the equilibrium buffer system used in purification procedures. This provided for activation of microsomes to rates similar to those described previously (26). All reactions were carried out in the presence of 5 mM UDPGA except for chromatographic column fractions where 2 mM UDPGA was utilized. All reactions contained 100 mM Tris-HCl (bis-Tris-HCl in the case of 4-aminobiphenyl) and 20 mM $MgCl_2$ with the appropriate pH. Reactions were carried out at 37° in a shaking water bath for 10 min in the presence of the appropriate amount of phosphatidylcholine. All reactions were linear with respect to time and protein concentration. Protein concentration was determined using the Bio-Rad protein assay originally described by Bradford (27) using human albumin as a standard.

TABLE 1
Characteristics of human liver samples

Sample	Sex	Age	Cause of death	Drugs
		years		
1	Female	44	Cerebrovascular accident	Digoxin Furosemide Alcoholic
2	Male	25	Motorcycle accident	
3	Female	5	Car accident	
4	Female	65	Cerebrovascular accident	Mannitol Dopamine Dexamethasone Methylprednisolone Vasopressin Hydralazine Ampicillin
5	Male	13	Car accident	
6	Male	24	Crushing chest injury	Mannitol Dopamine Dexamethasone Cephalasporin (one dose)

Preparation of microsomes. Microsomes were prepared as previously described (18) and were used within 3 months after preparation with no apparent loss of glucuronidation activity.

Separation and purification of human UDPGT activities. The frozen microsomal pellets were thawed, the KCl overlay was removed, and a suspension of microsomal protein (20 mg/ml) was prepared by resuspension in 25 mM Tris-acetate buffer, pH 8.1. All buffers used in purification procedures contained 20% (v/v) glycerol, 0.3 mM dithiothreitol, and 0.05% (w/v) Emulgen 911. The microsomal suspension was solubilized with 0.8 mg of Emulgen 911/mg of protein, stirred for 30 min at 4°, and centrifuged at 100,000 × *g* for 30 min. About 85% of the UDPGT activity toward phenolic substrates and total protein was recovered in the supernatant fluid. Generally, 90–110 mg of solubilized microsomal protein were applied to a chromatofocusing PBE 94 column (1 × 40 cm) pre-equilibrated in 25 mM Tris-acetate buffer, pH 8.1. The column was then washed with 2 bed volumes of equilibration buffer. Enzyme activity was then eluted from the column with polybuffer 96-acetate, pH 6.0, to generate the pH gradient. Three peaks of enzymatic activity were eluted after the void volume. The first had an apparent pI of 7.70, the second had an apparent pI of 7.4, and the third possessed an apparent pI of 6.2. A pH gradient of 8.0–7.0 was found to be a superior procedure for isolation of the pI 7.4 enzyme and was employed for the following step where the pI 7.4 protein was isolated.

After the chromatofocusing procedure, fractions with enzymatic activity eluting at about pH 7.4 were divided into two parts, an alkaline and an acidic half. Fractions with enzymatic activity on the alkaline side were pooled and $MgCl_2$ was added to achieve 5 mM concentration. The pool (100 ml) was applied to a 10-ml UDP-hexanolamine Sepharose 4B affinity column pre-equilibrated with 25 mM Tris-acetate, pH 8.1. The column was then washed with 15 bed volumes of 25 mM KCl in the same buffer containing 100 μg of phosphatidylcholine/ml. The enzyme activity was eluted with 8 mM UDPGA in the same buffer.

After the pH 8.0–6.0 chromatofocusing procedure, fractions with enzymatic activity eluting at about pH 6.2 were pooled and $MgCl_2$ was added to achieve a 5 mM concentration. The pool (80 ml) was applied to a 14-ml UDP-hexanolamine Sepharose 4B affinity column pre-equilibrated with 25 mM bis-Tris-acetate buffer, pH 6.5 (when this procedure was performed at pH 8.1, most UDPGT activity and protein did not bind to the affinity resin). The column was then washed with 15 bed volumes of 30 mM KCl in the same bis-Tris buffer, pH 6.5, containing 100 μg of phosphalidylcholine/ml. The enzyme was eluted with 3 mM UDPGA in the same buffer.

Gel electrophoresis. PAGE was performed in the presence of SDS in a Hoefer Scientific model 600 apparatus by the method of Laemmli (28), as previously described (18). Proteins were visualized with Coomassie Blue G-250 stain (18).

pH Effect on glucuronidation by the purified human UDPGTs. The pH was varied using Tris-HCl when studying the pH effect on the glucuronidation of *p*-nitrophenol, 4-methylumbelliferone, and α-naphthylamine, and bis-Tris-HCl was used when studying the pH effect on 4-aminobiphenyl glucuronidation. The concentration of buffers was 100 mM; UDPGA 5 mM; *p*-nitrophenol 3 mM; α-naphthylamine, 4-methylumbelliferone, and 4-aminobiphenyl 2 mM each. Phosphatidylcholine was added in a concentration of 100 μg, 200 μg, 200 μg, and 300 μg/ml reaction for *p*-nitrophenol, 4-methylumbelliferone, 4-aminobiphenyl, and α-naphthylamine, respectively. The reaction contained 2 μg of purified enzyme. When the experiment was performed with microsomal UDPGT, 20 μg of solubilized microsomal protein without added phosphatidylcholine were used.

Effect of phosphatidylcholine on glucuronidation by purified human liver UDPGTs. The concentration of phosphatidylcholine per ml reaction was varied and 2 μg of affinity purified enzyme were

used. The control reaction had no added phosphatidylcholine but it contained at least 10 μg of phosphatidylcholine/ml reaction. (The enzyme was eluted from the affinity column in the presence of 100 μg of phosphatidylcholine/ml of buffer and 100 μl of enzyme preparation were used/ml of reaction.) Substrates concentration were 5, 3, 2, 2, and 2 mM for UDPGA, *p*-nitrophenol, 4-methylumbelliferone, 4-aminobiphenyl, and α-naphthylamine, respectively. All reactions were incubated at 37° for 10 min.

Measurement of the kinetic parameters of purified human UDPGTs. Apparent K_m values were determined. The aglycone concentration was varied in the presence of 10 mM UDPGA. Phosphatidylcholine was added as follows: 100 μg, 200 μg, 200 μg, and 300 μg/ml reaction for *p*-nitrophenol, 4-methylumbelliferone, 4-aminobiphenyl, and α-napthylamine, respectively. Each reaction tube contained 2 μg of purified enzyme per ml and the reaction mixture was incubated for 10 min at 37°. When the apparent K_m for UDPGA was measured, the concentration of UDPGA was varied in the presence of 3 mM *p*-nitrophenol and 2 mM each of 4-methylumbelliferone, α-naphthylamine, and 4-aminobiphenyl.

Results

Glucuronidation rates of various substrates in solubilized human liver microsomes. Rates of metabolism for 10 different substrates that are known to be glucuronidated are shown in Table 2. Rates obtained in solubilized and activated microsomes were comparable in 5 of the 6 liver samples examined and agree with rates reported previously (26). Interestingly, one of the livers (liver 4) possessed activity that was considerably higher toward almost all substrates studied.

Isolation and purification of human liver UDPGTs. Chromatofocusing was used as an initial procedure for the separation and purification of UDPGTs. A pH gradient of 8.0–6.0 was utilized and results are shown in Fig. 1. Three peaks of UDPGT activity were eluted after the void volume. The first peak elutes with an apparent pI of 7.7 and possesses activity with *p*-nitrophenol, 4-methylumbelliferone, α-naphthylamine, and 4-aminobiphenyl. The second peak elutes with an apparent pI of 7.4 and has activity with *p*-nitrophenol, 4-methylumbelliferone, and α-naphthylamine but not with 4-aminobiphenyl. The third peak elutes with an apparent pI of 6.2 and catalyzes *p*-nitrophenol, 4-methylumbelliferone, α-naphthylamine, and 4-aminobiphenyl glucuronide formation. *p*-Nitrophenol glucuronidation activity always followed that of 4-methylumbelliferone. A pH 8.0–pH 7.0 gradient was found to be preferable to a pH 8.0–pH 6.0 gradient for the purification of the pI 7.4 protein. This procedure was used for all subsequent studies involving the pI 7.4 enzyme. Recovery of most enzyme activities after chromatofocusing averaged about 25%.

TABLE 2

Glucuronidation rates of various substrates by solubilized human liver microsomes[a]

Substrate	Liver sample					
	1	2	3	4	5	6
p-Nitrophenol	32	55	40	120	25	38
4-Methylumbelliferone	NP[b]	45	34	117	21	30
α-Naphthylamine	NP	10	10	26	4.4	10
4-Aminobiphenyl	NP	4.5	7.1	18	3.8	7.2
Androsterone	1.8	2.3	2	4.0	1.3	1.4
5α-Androstane-3α,17β-diol	NP	NP	2.2	5.6	NP	1.4
Testosterone	0.4	0.4	ND	ND	0.25	0.3
Estrone	ND[c]	ND	ND	0.1	ND	ND
β-Estradiol	NP	NP	0.6	0.8	NP	0.5
Estriol	NP	NP	9.5	10	NP	2.9

[a] Glucuronidation rates are in nmol conjugated/min/mg of protein. These values are the average of 3–11 determinations. The variability about these numbers is about 10%.

[b] NP, not performed.

[c] ND, not detectable [for limits see Puig and Tephly (20)].

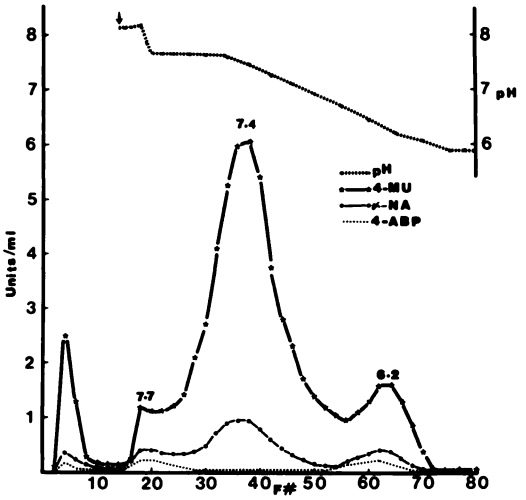

Fig. 1. Chromatofocusing of human liver microsomes. One hundred mg of solubilized microsomal protein were applied to a 1 × 40 cm chromatofocusing column. The column was washed with 2 bed volumes of equilibration buffer. The column was then eluted with polybuffer 96-acetate to generate a pH 8–6 gradient. Fraction number (*F#*) is shown on the *abscissa*, each fraction equals 5 ml. Enzymatic activity is shown on the *ordinate*. One unit of activity represents 1 nmol of glucuronide formed/min. *Numbers* over the peaks indicate the pH of elution (apparent pI). *4-MU*, 4-methylumbelliferone; α-NA, α-naphthylamine; 4-ABP, 4-aminobiphenyl.

Affinity chromatography on UDP-hexanolamine Sepharose 4B was used to further purify human liver UDPGTs. The enzyme eluting with an apparent pI of 7.4 was further purified by affinity chromatography as detailed under Materials and Methods. The elution profile for 4-methylumbelliferone and α-naphthylamine glucuronidation is shown in Fig. 2. This enzyme also catalyzed the glucuronidation of *p*-nitrophenol and estriol but not 4-aminobiphenyl, testosterone, 17β-estradiol, estrone, androsterone, or 5α-androstane-3α,17β-diol. The recovery of enzyme for the affinity procedure averaged about 40%. SDS-PAGE of this preparation showed an apparently homogeneous protein displaying a subunit molecular weight of 53,000 (Fig. 3).

Enzyme activity eluting with an apparent pI of 6.2 was further purified by affinity chromatography as detailed under Materials and Methods. The elution profile for 4-methylumbelliferone, α-naphthylamine, and 4-aminobiphenyl UDPGT is shown in Fig. 4. This enzyme also catalyzed the glucuronidation of *p*-nitrophenol but not testosterone, 17β-estradiol, estrone, estriol, androsterone, or 5α-androstane-3α,17β-diol. SDS-PAGE of this preparation showed a homogeneous protein displaying a subunit molecular weight of 54,000 (Fig. 5).

Effect of pH on glucuronidation by purified human UDPGTs. The pH optima for glucuronidation of various substrates by human liver microsomes and by purified human liver UDPGTs were studied. There was no difference in the pH optimum for a given substrate between microsomes and the purified enzyme preparations (Table 3).

Effect of phosphatidylcholine on the activity of puri-

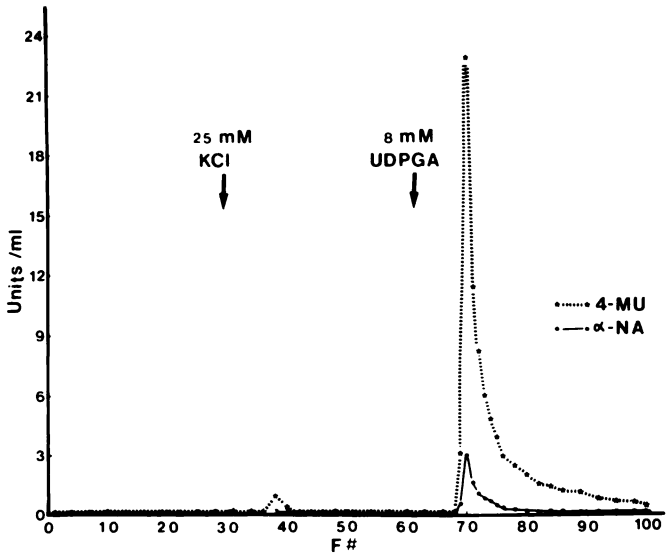

Fig. 2. Affinity chromatography on UDP-hexanolamine Sepharose 4B of chromatofocusing fractions eluting at pH 7.4. The sample was loaded slowly as described under Materials and Methods. The column was then washed with 15 bed volumes of equilibration buffer, pH 8.1, containing 25 mM KCl and 100 μg of phosphatidylcholine/ml. The enzyme activity was eluted with 8 mM UDPGA. Fraction number (*F#*) is shown on the *abscissa* and glucuronidation activity is shown on the *ordinate*. Abbreviations are defined in the legend to Fig. 1.

fied human UDPGTs. The effect of varied concentrations of phosphatidylcholine on the activity of affinity purified human UDPGTs was performed as explained under Materials and Methods. The minimum added concentrations of phosphatidylcholine to give maximal glucuronidation rates were 200, 200, and 300 μg/ml using *p*-nitrophenol, 4-methylumbelliferone, and α-naphthylamine as substrates, respectively, with the pI 7.4 UDPGT (Fig. 6). Concentrations of 75, 200, 100, and 300 μg/ml using *p*-nitrophenol, 4-methylumbelliferone, 4-aminobiphenyl, and α-naphthylamine as substrates, respectively, with the pI 6.2 UDPGT were found to be optimal (Fig. 7). With this enzyme, 4-aminobiphenyl glucuronidation was maintained at a maximum rate between 100 and 300 μg of phosphatidylcholine/ml of reaction, but at higher phospholipid concentrations, glucuronidation rates were decreased.

Substrate specificities of purified human liver UDPGTs. The purified human liver UDPGTs were tested with different substrates of both endogenous origin (estrogens and androgens) as well as with xenobiotics (Table 4). The key difference found for these proteins is that the pI 7.4 enzyme reacts with estriol but not with 4-aminobiphenyl. The pI 6.2 enzyme reacts with 4-aminobiphenyl but not with estriol.

Kinetic parameters for some substrates reacting with purified human liver UDPGTs. The apparent K_m values for different substrates reacting with purified human liver UDPGT were determined (Table 5). The apparent K_m values for a given substrate were in the same order of magnitude with both purified human UDPGTs.

Discussion

A considerable body of information is now available on UDPGT multiplicity in laboratory animals (17–23, 29), but no direct data on the multiplicity of human UDPGT exist. This report demonstrates a method for the resolution of two human

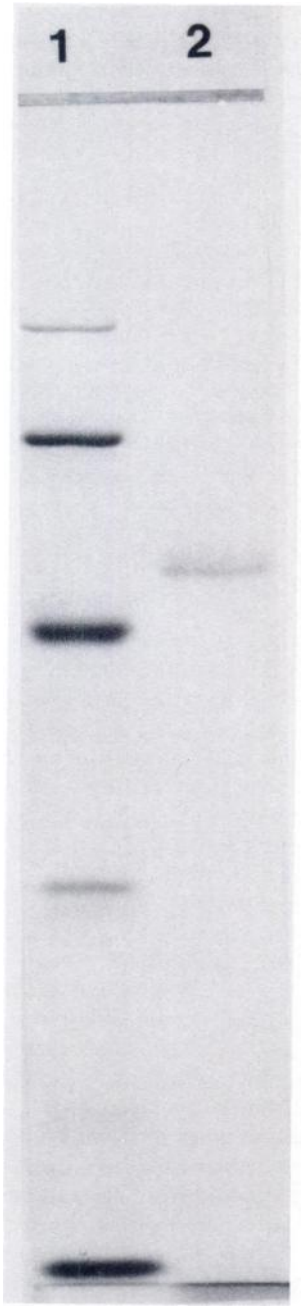

Fig. 3. SDS-PAGE of the pI 7.4 chromatofocusing fraction after affinity chromatography and commercial molecular weight standards. *Lane 1* contains the molecular weight standards of 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400, and *lane 2* contains the affinity purified UDPGT. Migration is from *top* to *bottom*. Proteins were visualized with Coomassie Blue G-250 stain.

liver UDPGTs. Six liver samples of different ages and sexes were studied. These livers were found to be similar in the pattern of glucuronidation except for one (liver sample 4) which had higher microsomal rates of glucuronidation than the others. The reason for this is largely unknown. However, this patient was known to be taking multiple medications during the course of hospitalization, including dexamethasone. It is known that the development of the late fetal group of UDPGT is associated with a surge of glucocorticoids during late pregnancy, and glucocorticoids have been reported to induce early development of the late fetal cluster of UDPGTs when given to rats during

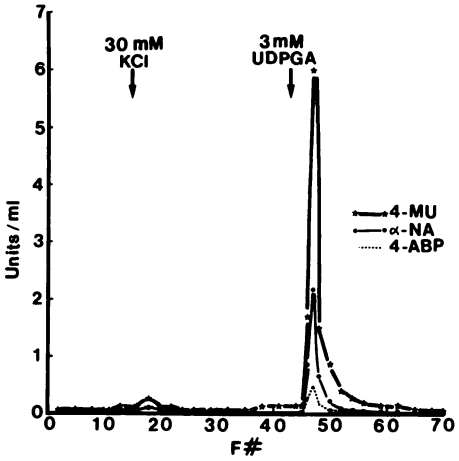

Fig. 4. Affinity chromatography on UDP-hexanolamine Sepharose 4B of chromatofocusing fractions eluting at pH 6.2. The sample was loaded slowly as described under Materials and Methods. The column was then washed with 15 bed volumes of equilibration buffer, pH 6.5, containing 30 mM KCl and 100 μg of phosphatidylcholine/ml. The enzyme activity was then eluted with 3 mM UDPGA in the same buffer. Fraction number (*F#*) is shown on the *abscissa* and glucuronidation activity is shown on the *ordinate*. Abbreviations are defined in the legend to Fig. 1.

pregnancy (7). Dexamethasone has been reported to increase UDPGT activity toward bilirubin, estradiol, and testosterone in fetal liver obtained from rhesus monkeys during late gestation (30). Prednisone treatment reduced the serum bilirubin level in a patient with Crigler-Najjar syndrome (31). Therefore, the individual receiving dexamethasone may have had induced enzyme activity. Digitoxigenin monodigitoxoside UDPGT activity has recently been reported to be increased in the livers of two patients who had received dexamethasone, phenobarbital, and diphenylhydantoin (32).

Low rates of glucuronidation for various steroid hormones in human liver microsomes were observed. This is in agreement with previous reports (33) which showed that glucuronidation might not be the major pathway of conjugation for estrone and β-estradiol in human liver slices. Others have reported (34) that estriol 16α-glucuronide is the major estriol metabolite during late pregnancy. We have observed that estriol had the highest rate of glucuronidation among the steroid hormones tested in human liver microsomal preparation. Estriol glucuronide formed by human liver microsomes comigrated with authentic estriol-16α-glucuronide on thin layer chromatography (data not shown). Testosterone is metabolized by peripheral tissues to dihydrotestosterone which is then converted to androstanediol which is excreted as the 3α-diol glucuronide (35). It was also reported (36) that neither the 3α-diol nor its glucuronide was secreted by the liver or gut in either young or elderly men. In short, glucuronidation of steroids may be more active in tissues other than the liver in humans.

Using chromatofocusing and affinity chromatography procedures, we could resolve and purify two separate forms of UDPGT from human liver. These forms have apparent pI values of 7.4 and 6.2 and have different monomeric molecular weights. One (pI 7.4) has a subunit molecular weight of 53,000 and another (pI 6.2) has a subunit molecular weight of 54,000. Both of these forms reacted with *p*-nitrophenol, 4-methylumbelliferone, and α-naphthylamine, but the pI 7.4 form reacted with estriol but not with 4-aminobiphenyl. The pI 6.2 form reacted with 4-aminobiphenyl but not with estriol. Neither of

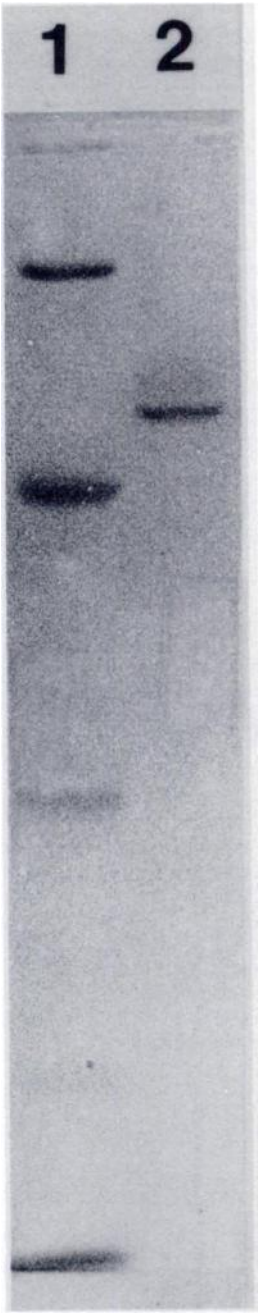

Fig. 5. SDS-PAGE of the pI 6.2 chromatofocusing fraction after affinity chromatography and commercial molecular weight standards. *Lane 1* contains commercial molecular weight standards with minimum molecular weights the same as those shown in Fig. 3. *Lane 2* contained the affinity purified UDPGT. Migration is from *top* to *bottom*. Proteins were visualized with Coomassie Blue G-250 stain.

these enzymes reacted with estrone, β-estradiol, testosterone, androsterone, or 5α-androstane-3α,17β-diol.

A human liver enzyme with a pI of 7.7 was also isolated. A major band on SDS-PAGE was observed at a subunit molecular weight of about 53,000. Peptide mapping using *Staphylococcus aureus* protease showed that the pI 7.7 and pI 7.4 proteins at 53,000 Da had identical peptide maps. The glucuronidation activity of 4-aminobiphenyl observed with the pI 7.7 fractions is probably due to the presence of a second enzyme protein in the pI 7.7 fraction that cannot be visualized by the Coomassie Blue G-250 stain. Indeed, by silver staining, we have evidence

TABLE 3

Effect of pH on glucuronidation of various substrates by human liver microsomes and purified human liver UDPGTs

Substrate	pH Optima		
	Solubilized microsomes	UDPGT[a] (pI 7.4)	UDPGT (pI 6.2)
4-Methylumbelliferone	7.3	7.3	7.3
p-Nitrophenol	7.3	7.3	7.3
α-Naphthylamine	7.75	7.75	7.75
4-Aminobiphenyl	6.4		6.4

[a] UDPGT represents purified human liver UDP-glucuronosyltransferase.

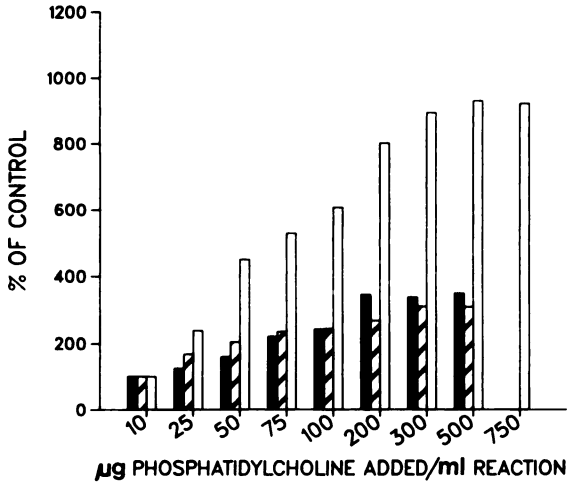

Fig. 6. Phosphatidylcholine requirement for reconstitution of glucuronidation activity by the affinity purified UDPGT eluting with a pI of 7.4. The control had at least 10 µg of phosphatidylcholine/ml reaction. ■, *p*-nitrophenol glucuronidation activity; □, α-naphthylamine glucuronidation activity; ▨, 4-methylumbelliferone glucuronidation activity. The 100% control glucuronidation rates were 55, 90, and 2.0 units/mg of protein for 4-methylumbelliferone, *p*-nitrophenol, and α-naphthylamine, respectively. These results are representative of experiments performed with enzyme purified from liver sample 3.

of another protein present in the pI 7.7 fractions (data not shown).

The pH optima for glucuronidation of various substrates by these isoforms were similar to that observed using purified rat UDPGTs except for 4-aminobiphenyl glucuronidation. The glucuronidation of 4-aminobiphenyl displayed an unusual pH optimum of about 6.4. This is low and atypical of UDPGT reactions and unlike the pH optimum for 4-aminobiphenyl glucuronidation in rats,[1] which is about pH 7.4.

UDPGTs require phospholipids for reconstitution of maximal glucuronidation activity (1, 37, 38). Interestingly, we found that different substrates require different concentrations of phosphatidylcholine for reconstitution of maximal glucuronidation activity. α-Naphthylamine required the highest concentration of phosphatidylcholine for maximal glucuronidation by the two purified human liver UDPGTs and the maximal activity was 6–8 times higher than that of control. Other substrates (*p*-nitrophenol, 4-methylumbelliferone, and 4-aminobiphenyl) required less phosphatidylcholine and the maximal activity was only 2–3 times higher than that of control. Generally speaking, lipid-soluble substrates (α-naphthylamine) required more phosphalidylcholine than did water-soluble substrates (*p*-nitrophenol). The interaction of phospholipids with microsomal

[1] M. D. Green and T. R. Tephly, unpublished results.

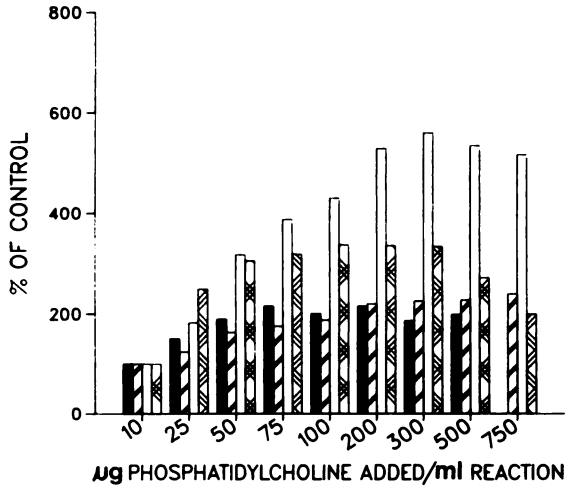

Fig. 7. Phosphatidylcholine requirements for reconstitution of glucuronidation activity by the affinity purified UDPGT eluting with a pI of 6.2. The control had at least 10 µg of phosphatidylcholine/ml reaction. ■, *p*-nitrophenol glucuronidation activity; □, α-naphthylamine glucuronidation activity; ▨, 4-methylumbelliferone glucuronidation activity. The *cross-hatched bars* represent 4-aminobiphenyl glucuronidation activity. The 100% control glucuronidation rates were 385, 438, 20, and 15 units/mg for 4-methylumbelliferone, *p*-nitrophenol, α-naphthylamine, and 4-aminobiphenyl, respectively. These results are representative of experiments performed with enzyme purified from liver sample 4.

TABLE 4

Substrate specificities of purified human liver UDPGTs

These results are representative of those found in liver sample 4.

Substrate	Specific activity (units/mg of protein)[a]	
	UDPGT (pI 7.4)	UDPGT (pI 6.2)
4-Methylumbelliferone	512	1055
p-Nitrophenol	520	1300
α-Naphthylamine	77	117
4-Aminobiphenyl	ND[b]	45
Estriol	13	ND
Estrone	ND	ND
17β-Estradiol	ND	ND
Testosterone	ND	ND
5α-Androstane-3α,17β-diol	ND	ND
Androsterone	ND	ND

[a] One unit of activity equals 1 nmol of substrate conjugated/min.
[b] ND, no detectable activity.

TABLE 5

Apparent K_m values for substrates reacting with purified human liver UDPGTs

Substrate	K_m^{app} (mM)[a]	
	UDPGT (pI 7.4)	UDPGT (pI 6.2)
4-Methylumbelliferone	0.17 ± 0.03	0.23 ± 0.05
p-Nitrophenol	1.0 ± 0.15	0.82 ± 0.05
α-Naphthylamine	0.93 ± 0.19	1.02 ± 0.17
4-Aminobiphenyl		0.53 ± 0.07
UDPGA[b]	0.04–0.11	0.1–0.17

[a] Apparent K_m was determined from the double reciprocal plots (1/*v* vs. 1/*s*) in the presence of 10 mM UDPGA. Values are given as mean ± standard deviation of three separate experiments with liver number 4.
[b] K_m for UDPGA was tested once with all aglycones listed.

UDPGT has been studied (37, 38). Nonpolar substrates are highly soluble in the hydrophobic environment of membrane-bound enzymes, and lipids may be an ideal phase for concentrating these substrates in close proximity to the enzyme. This enables the substrates to reach the active site without leaving the plane of the membrane. This hypothesis was tested using estrone glucuronidation activity catalyzed by guinea pig liver microsomes (37). It was found that membrane-associated estrone, and not estrone in solution, was the preferred substrate for UDPGT. This finding could explain why α-naphthylamine required high amounts of phosphatidylcholine for reconstitution of maximal glucuronidation activity and why α-naphthylamine glucuronidation was stimulated more than the other substrates. Another hypothesis which explains the regulation of UDPGT activity by phospholipids is that phospholipids interact with the enzyme and affect conformation in a way that produces a catalytically active form (38). Delipidation of purified *p*-nitrophenol UDPGT from guinea pig liver microsomes was associated with loss of activity and α-helix content (as studied by circular dichroism spectroscopy) (38). Reconstitution with phosphatidylcholine restored the enzyme to a much more helical, high reactivity conformation (38). If different substrates need different enzyme conformation for proper alignment at the active site, then they may need different amounts of phosphatidylcholine. This hypothesis could explain why different substrates needed different amounts of phosphatidylcholine for reconstitution of maximal glucuronidation activity by the purified human liver UDPGTs.

The purified human liver UDPGTs possess features which are different from purified rat liver UDPGTs. Human UDPGTs have apparent pI values of 7.4 and 6.2 which are lower than the pI values of rat UDPGTs reported from this laboratory (18, 19), which ranged from 7.8 to 10.0. There were also differences in the pI values between species, even for enzymes which catalyzed the glucuronidation of a particular substrate. For example, *p*-nitrophenol was glucuronidated by both purified human liver UDPGTs which had pI values of 7.4 and 6.2, whereas in rat liver microsomes *p*-nitrophenol was glucuronidated by two UDPGTs which had pI values of 9.0 and 8.5. These differences in pI values may reflect different physical properties. Purified human liver UDPGTs also have functional differences from purified rat liver UDPGTs. Human enzymes did not catalyze the glucuronidation of androgenic steroids such as androsterone and testosterone. These substrates were glucuronidated by two separate rat liver UDPGTs (18). The purified human UDPGTs did not glucuronidate estrone and 17β-estradiol, but the human UDPGT which displayed a pI value of 7.4 did glucuronidate estriol. None of the purified rat liver UDPGTs catalyzed glucuronidation of estriol. 4-Aminobiphenyl was glucuronidated by the human UDPGT which possessed a pI value of 6.2 but which did not glucuronidate androsterone. In the rat liver, 4-aminobiphenyl was glucuronidated by the 3α-hydroxy steroid (androsterone) UDPGT (39). These findings indicate the existence of both physical and functional differences between purified human and rat liver UDPGTs.

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