Substrate specificity and characterization of rat liver *p*-nitrophenol, 3 α -hydroxysteroid and 17 β -hydroxysteroid UDP-glucuronosyltransferases

Charles N. FALANY,* Mitchell D. GREEN,† Elizabeth SWAIN† and Thomas R. TEPHLY††

*McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706, U.S.A., and †The Toxicology Center, Department of Pharmacology, University of Iowa, Iowa City, IA 52242, U.S.A.

Purified preparations of rat liver 17-hydroxysteroid, 3-hydroxyandrogen and *p*-nitrophenol (3-methylcholanthrene-inducible) UDP-glucuronosyltransferases were further characterized as to their substrate specificities, phospholipid-dependency and physical properties. The two steroid UDP-glucuronosyltransferases were shown to exhibit strict stereospecificity with respect to the conjugation of steroids and bile acids. These enzymes have been renamed 17 β -hydroxysteroid and 3 α -hydroxysteroid UDP-glucuronosyltransferase to reflect this specificity for important endogenous substrates. An endogenous substrate has not yet been identified for the *p*-nitrophenol (3-methylcholanthrene-inducible) UDP-glucuronosyltransferase. The steroid UDP-glucuronosyltransferase activities were dependent on phospholipid for maximal catalytic activity. Complete delipidation rendered the UDP-glucuronosyltransferases inactive, and enzymic activity was not restored when phospholipid was added to the reaction mixture. After partial delipidation, phosphatidylcholine was the most efficient phospholipid for restoration of enzymic activity. Partial delipidation also altered the kinetic parameters of the 3 α -hydroxysteroid UDP-glucuronosyltransferase. The three purified UDP-glucuronosyltransferases are separate and distinct proteins, with different amino acid compositions and peptide maps generated by limited proteolysis with *Staphylococcus aureus* V8 proteinase. Some similarity was observed between the amino acid composition and limited proteolytic maps of the steroid UDP-glucuronosyltransferases, suggesting they are more closely related to each other than to the *p*-nitrophenol UDP-glucuronosyltransferase.

INTRODUCTION

Glucuronidation is an important pathway for the metabolism and excretion of both endogenous and exogenous compounds (Dutton, 1980). The catalysts involved in glucuronidation, UDP-glucuronosyltransferase (EC 2.4.1.17), represent a family of isoenzymes responsible for the transfer of the glucuronic acid moiety from UDP-glucuronic acid to the aglycone acceptor compound. The addition of the glucuronide moiety enhances the excretory processes for the aglycone acceptor compounds into either the urine or bile.

Although the heterogeneity of UDP-glucuronosyltransferase activities has been established (Dutton, 1980; Burchell, 1981), the identification and characterization of resolved and purified UDP-glucuronosyltransferase isoenzymes has only been accomplished more recently. Purification of various UDP-glucuronosyltransferase activities have been reported by Gorski & Kasper (1977), Burchell (1977, 1978, 1980), Bock *et al.* (1979), Singh *et al.* (1981b), Mackenzie & Owens (1983) and Matern *et al.* (1982); however, generally not more than one UDP-glucuronosyltransferase activity was clearly resolved from the same tissue preparation. This has limited the determination and characterization of multiple UDP-glucuronosyltransferases present in a given tissue and species. The purification of two UDP-glucuronosyltransferases from the same tissue preparation has been

reported by Tukey & Tephly (1981). These authors purified oestrone and *p*-nitrophenol UDP-glucuronosyltransferases from microsomes (microsomal fractions) of rabbit liver. Although the oestrone and *p*-nitrophenol UDP-glucuronosyltransferase possessed the same subunit M_r value, these enzymes were determined to be separate and different proteins (Tukey & Tephly, 1981).

Subsequently, Falany & Tephly (1983) have reported on the purification of three different UDP-glucuronosyltransferases from liver microsomes of 3-methylcholanthrene-treated female Sprague–Dawley rats. The purified UDP-glucuronosyltransferase isoenzymes were named 3-hydroxyandrogen, 17-hydroxysteroid and 3-methylcholanthrene-inducible *p*-nitrophenol UDP-glucuronosyltransferase, on the basis of their substrate reactivities. The three purified UDP-glucuronosyltransferases displayed different subunit M_r values and different responses to induction with 3-methylcholanthrene. The current paper further characterizes the kinetic and physical properties of these three purified UDP-glucuronosyltransferases. The substrate reactivity of the purified enzymes with both endogenous substrates, such as steroids, and exogenous compounds is more specifically demonstrated, and this has led to the renaming of the purified UDP-glucuronosyltransferases as 17 β -hydroxysteroid UDP-glucuronosyltransferase, 3 α -hydroxysteroid UDP-glucuronosyltransferase and *p*-nitrophenol UDP-glucuronosyltransferase, according to

† To whom reprint requests should be addressed.

their substrate reactivities with important physiological substrates (Bock *et al.*, 1983). Amino acid composition and peptide maps generated by limited proteolysis of the pure UDP-glucuronosyltransferases were compared, and possible relationships between these proteins have been noted. The effect of delipidation and phospholipid reconstitution on the steroid UDP-glucuronosyltransferase activities has also been investigated.

EXPERIMENTAL

Materials

[4-¹⁴C]Oestrone (57 mCi/mmol), [4-¹⁴C]testosterone (50 mCi/mmol), [1,2-³H]etiocolanolone (40–60 Ci/mmol), [1,2-³H]androsterone (40–60 Ci/mmol), [1-¹⁴C]-naphthol (4.3 mCi/mmol), [4-¹⁴C] β -oestradiol (50 mCi/mmol) and UDP-[¹⁴C]glucuronic acid (343 mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. *p*-Nitro[U-¹⁴C]phenol (79 mCi/mmol) was obtained from Amersham, Arlington Heights, IL, U.S.A. L- α -Phosphatidylcholine (egg yolk, type XI-E), lyso-phosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, UDP-glucuronic acid, oestrone, β -oestradiol, *p*-nitrophenol, testosterone, 1-naphthol, lithocholic acid, chenodeoxycholic acid, etiocolanolone, epitestosterone, epiandrosterone, 17 β -hydroxy-5 β -androstane-3-one and 17 α -hydroxyprogesterone were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Phospholipids containing different fatty acid side chains were obtained from Sigma, and individual fatty acids were from Supelco, Bellefonte, PA, U.S.A. Chromatofocusing materials were purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Emulgen 911 was obtained from Kao Atlas, Tokyo, Japan. Bio-Rad dye-reagent protein assay was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were of analytical grade. UDP-hexanolamine-Sepharose 4B affinity resin was prepared as previously described (Tukey & Tephly, 1981).

Assay procedures

p-Nitrophenol, 1-naphthol and steroid glucuronidation assays, which utilized radiolabelled aglycone substrates, were performed as previously described (Falany *et al.*, 1983). Glucuronidation assays with 17 α -hydroxyprogesterone, epitestosterone, epiandrosterone and 17 β -hydroxy-5 α -androstane-3-one as substrates utilized radiolabelled UDP-glucuronic acid and were performed as previously described for 17 β -oestradiol (Falany *et al.*, 1983). Bile acid glucuronidation activity was also determined by using radiolabelled UDP-glucuronic acid as previously described (Kirkpatrick *et al.*, 1984).

All reactions were performed at 37 °C with 5 mM-UDP-glucuronic acid in the presence of 100 μ g of phosphatidylcholine. However, when alternative-substrate-inhibition studies were performed, 10 mM-UDP-glucuronic acid was utilized. The form of inhibition was determined by regression analysis of double-reciprocal plots of velocity versus substrate concentration in the absence and presence of at least two different inhibitor concentrations. All reactions were linear with respect to time and protein concentration. Protein concentrations were determined with the Bio-Rad protein assay originally developed by Bradford (1976).

Separation and purification of 17 β -hydroxysteroid, 3 α -hydroxysteroid and *p*-nitrophenol UDP-glucuronosyltransferase activities

The separation of 17 β -hydroxysteroid (testosterone), 3 α -hydroxysteroid (androsterone) and *p*-nitrophenol UDP-glucuronosyltransferase activities from female Sprague-Dawley rat liver was performed by Chromatofocusing chromatography as previously described by Falany & Tephly (1983), except that a pH 9–7 gradient was generated. The separated fractions of UDP-glucuronosyltransferase activity were then purified by UDP-hexanolamine-Sepharose 4B affinity chromatography (Falany & Tephly, 1983).

Delipidation of 3 α -hydroxysteroid and 17 β -hydroxysteroid UDP-glucuronosyltransferase activities

Powdered hydroxyapatite (Bio-Rad Laboratories) was equilibrated in 20 mM-Tris/HCl buffer, pH 8.0, containing 20% (v/v) glycerol, 0.1 mM-dithiothreitol and 0.05% Emulgen 911. The hydroxyapatite was then packed into glass columns (0.9 cm $\times$ 3.0 cm) and washed with 20 ml of the equilibration buffer. Hydroxyapatite chromatography has been used previously to delipidate UDP-glucuronosyltransferases rapidly and efficiently (Tukey *et al.*, 1979; Singh *et al.*, 1981b; Magdalou *et al.*, 1982).

After Chromatofocusing, the resolved and partially purified fractions containing androsterone and testosterone glucuronidation activity were directly applied to hydroxyapatite columns. The columns were then rinsed with 30 column volumes of 20 mM-Tris/HCl, pH 8.0, containing 20% glycerol, 0.1 mM-dithiothreitol and 0.05% Emulgen 911. The UDP-glucuronosyltransferase activities were eluted from the columns with the same buffer containing 0.5 M-KCl. UDP-glucuronosyltransferase activity in column fractions was determined in both the presence and the absence of added (100 μ g) phosphatidylcholine.

UDP-hexanolamine-Sepharose 4B affinity chromatography could also be used partially to delipidate UDP-glucuronosyltransferase activity (Tukey & Tephly, 1980). Chromatofocusing fractions were applied to the affinity columns, which had been previously equilibrated in 20 mM-Tris/HCl, pH 8.1, containing 20% glycerol, 0.1 mM-dithiothreitol and 0.05% Emulgen 911. After application of the Chromatofocusing fractions, the affinity columns were rinsed with 15–20 column volumes of the same buffer containing 40 mM-KCl but without phosphatidylcholine. The UDP-glucuronosyltransferase activity was then specifically eluted by the addition of UDP-glucuronic acid to the buffer system (Falany & Tephly, 1983).

Various phospholipids were tested for their ability to reconstitute the enzymic activity of partially delipidated steroid UDP-glucuronosyltransferases, as described by Tukey *et al.* (1979). The phospholipids were microdispersed in water by ultrasonication before their addition to the reaction mixture. Enzyme, phospholipid and steroid substrate were preincubated at 37 °C for 5 min before the reaction was initiated by the addition of UDP-glucuronic acid.

Determination of lipid P

Phospholipid was extracted by the method of Bligh & Dyer (1979). Inorganic P was measured as described in

Sigma technical bulletin 670, with modifications described by Bartlett (1959) to increase the sensitivity of the assay. Standards consisted of various amounts of potassium phosphate which were carried through the assay procedure.

Electroelution of UDP-glucuronosyltransferase

Before limited proteolysis or amino acid analysis, preparative electrophoresis of the purified UDP-glucuronosyltransferases were performed in slab gels (14.0 cm × 10.0 cm × 0.15 cm) in 10% acrylamide by the method of Laemmli (1970). Protein bands were detected by staining with 0.1% Coomassie Blue G-250 in propan-2-ol/acetic acid/water (5:1:14, by vol.), adjusted to pH 3.2 with NaOH until the bands were just visible. The gels were then destained with 50% (v/v) methanol. Bands were cut from the gel and electroeluted for 5–6 h at 3 W in 25 mM-Tris/192 mM-glycine/0.1% SDS, pH 8.3, in an ISCO model 1750 sample concentrator. After electroelution, the sample was transferred to dialysis tubing and dialysed against 3 × 4 litres of distilled water to remove buffer components. These procedures were used to delipidate the samples completely and to concentrate them before compositional analysis and peptide mapping.

Amino acid analysis of the UDP-glucuronosyltransferases

The amino acid composition of the purified UDP-glucuronosyltransferases was determined after hydrolysis of the protein in 6 M-HCl containing 0.05% β -mercaptoethanol and 0.05% phenol. Ampoules containing the samples were evacuated and sealed under N₂, and then incubated for 24, 48 and 72 h at 110 °C. Cysteic acid and methionine sulphone were determined by performic acid oxidation and hydrolysis for 24 h at 110 °C in 6 M-HCl containing 0.05% β -mercaptoethanol and 0.05% phenol. By this procedure, tryptophan was not determined. Amino acid analysis was done in a

Beckman 121 M3 Analyser, with norleucine as an internal standard.

Peptide mapping of UDP-glucuronosyltransferases

Limited proteolysis was performed with *Staphylococcus aureus* V8 proteinase (Miles Laboratories) as described by Tukey & Tephly (1981), with the following modifications. After electroelution, each of the purified transferase proteins was placed in 20 mM-Tris/HCl, pH 8.0, containing 20% glycerol, 0.1 mM-dithiothreitol and 0.05% Emulgen 911. *S. aureus* proteinase was added to a final concentration of 0.3 mg/mg of transferase protein, and the mixtures were then incubated for 5 or 30 min at 37 °C. Some samples were incubated at 37 °C for 90 min. These samples were initially incubated for 45 min as described above, then an additional 0.3 mg of *S. aureus* proteinase/mg of transferase protein was added, and the incubation was continued for an additional 45 min. Reactions were terminated by the addition of β -mercaptoethanol and SDS (final concns. 10% and 2% respectively), then heated in a boiling-water bath for 10 min. The samples were then electrophoresed in a SDS/polyacrylamide slab gel (14.0 cm × 16.0 cm × 0.15 cm) (12.5% acrylamide) as described by Laemmli (1970), in a Hoefer Scientific model SE-600 apparatus. Peptide bands were detected by a commercial silver stain procedure (Bio-Rad Laboratories).

RESULTS

Substrate specificity for 17 β -hydroxysteroid, 3 α -hydroxysteroid and *p*-nitrophenol UDP-glucuronosyltransferases

The substrate specificity and inhibitory properties of various steroids for 17 β -hydroxysteroid UDP-glucuronosyltransferase are shown in Table 1. Testosterone has proved to be the best substrate for 17 β -hydroxysteroid UDP-glucuronosyltransferase of all steroids tested so far.

Table 1. Inhibition of 17 β -hydroxysteroid UDP-glucuronosyltransferase (UDPGT) by steroids

The form of inhibition was determined from double-reciprocal plots of initial velocity versus substrate concentration, where testosterone was the variable substrate (5–50 μ M): C, competitive; UC, uncompetitive; NI, no inhibition observed for the compound tested, up to a concentration of 100 μ M. K_{is} was determined from replots of slope versus inhibitor concentration when competitive inhibition of testosterone glucuronidation was observed. Glucuronidation activities for the alternative substrates are expressed as percentages of the rate of testosterone glucuronidation catalysed by purified 17 β -hydroxysteroid UDPGT. Results are means $\pm$ S.D., where appropriate, for the numbers of determinations in parentheses: NA, no glucuronide formation was observed with the indicated compounds.

Compound	Type of inhibition	17 β -Hydroxysteroid UDPGT activity	
		K_{is} (μ M)	Glucuronidation activity (% of that with testosterone)
Testosterone	—	—	100
β -Oestradiol	C	25	33 $\pm$ 4 (4)
Oestrone	NI	—	NA
Epitestosterone	C	16	NA
17 α -Hydroxyprogesterone	C	21	NA
17 β -Hydroxy-5 α -androstan-3-one	C	16	61 (2)
Epiandrosterone	C	31	NA
Etiocholanolone	NI	—	NA
Lithocholic acid	NI	—	NA
Chenodeoxycholic acid	UC	—	NA

17 β -Hydroxy-5 α -androstan-3-one showed the second highest rate of glucuronidation, whereas β -oestradiol displayed the lowest rate. This UDP-glucuronosyltransferase does not conjugate epitestosterone (17 α -hydroxy-testosterone), 17 α -hydroxyprogesterone or epiandrosterone (3 β -hydroxyandrosterone), even though these steroids competitively inhibit testosterone glucuronidation. These results suggest that, in addition to the proper configuration of the hydroxyl group at the 17-position, the structure of the steroid A-ring may also be important in determining the efficiency with which a steroid is conjugated. The K_i for β -oestradiol (25 μ M) determined during the inhibition studies is similar to its apparent K_m as a substrate (23 μ M). Epitestosterone has a K_i (16 μ M) very similar to the apparent K_m for testosterone (14 μ M), even though epitestosterone is not glucuronidated by this UDP-glucuronosyltransferase (Table 1). This enzyme is less specific than the 3 α -hydroxysteroid UDP-glucuronosyltransferase, since it reacts with xenobiotic substrates such as *p*-nitrophenol and 1-naphthol (Falany & Tephly, 1983; Falany *et al.*, 1983). The apparent K_m for *p*-nitrophenol with the 17 β -hydroxysteroid UDP-glucuronosyltransferase is 1.7 mM (Falany & Tephly, 1983), a value 5-fold greater than the K_m reported for *p*-nitrophenol with *p*-nitrophenol UDP-glucuronosyltransferase purified from rat liver (Falany & Tephly, 1983).

3 α -Hydroxysteroid UDP-glucuronosyltransferase conjugates steroids with a 3 α -hydroxy group, such as etiocholanolone or androsterone, or the 3 α -hydroxy position of bile acids, such as lithocholic acid and chenodeoxycholic acid (Falany & Tephly, 1983; Kirkpatrick *et al.*, 1984). It does not glucuronidate the phenolic 3-hydroxy position of oestrogens. Table 2 shows that only those steroids and bile acids having a 3 α -hydroxyl group (androsterone, etiocholanolone, lithocholic acid and chenodeoxycholic acid) are substrates for the 3 α -hydroxysteroid UDP-glucuronosyltransferase. Epiandrosterone, which possesses a 3 β -hydroxy group, is not a substrate. 3 α -Hydroxy substrates competitively

inhibit androsterone glucuronidation activity and have a K_i similar to their apparent K_m as substrates. 3 α -Hydroxysteroid UDP-glucuronosyltransferase is also competitively inhibited by steroids that do not possess a 3 α -hydroxyl group and are not glucuronidated by this enzyme (β -oestradiol, epitestosterone, epiandrosterone and 17 α -hydroxyprogesterone; Table 2). Fig. 1 shows the results of a representative experiment which demonstrates competitive inhibition of androsterone glucuronidation activity by epiandrosterone and by 17 α -hydroxyprogesterone.

Phospholipid-dependence of 3 α -hydroxysteroid and 17 β -hydroxysteroid UDP-glucuronosyltransferases

The phospholipid-dependence of the 3 α -hydroxy- and 17 β -hydroxy-steroid UDP-glucuronosyltransferases was investigated by observing the effect of delipidation and phospholipid reconstitution on enzymic activity. Delipidation of the steroid UDP-glucuronosyltransferase was achieved by either UDP-hexanolamine-Sepharose 4B chromatography (Tukey & Tephly, 1980) or hydroxyapatite chromatography (Tukey *et al.*, 1979; Singh *et al.*, 1981b; Magdalou *et al.*, 1982). UDP-glucuronosyltransferase preparations could be obtained which contained no detectable phospholipid, as indicated by inorganic P determinations, by hydroxyapatite chromatography. These preparations, however, possessed no glucuronidation activity, nor could enzyme activity be restored by the addition of any of the phospholipids tested. When these preparations were analysed by SDS/polyacrylamide-gel electrophoresis, a protein band corresponding to the M_r of the transferase could be observed.

Partially delipidated steroid UDP-glucuronosyltransferase preparations (> 2.5 μ g of inorganic P/mg of protein) were enzymically active, and this glucuronidation activity could be enhanced by the further addition of phospholipid. Fig. 2 shows the enhancement of 17 β -hydroxysteroid and 3 α -hydroxysteroid UDP-glucuronosyltransferase activities by various phospholipids.

Table 2. Inhibition of 3 α -hydroxysteroid UDP-glucuronosyltransferase (UDPGT) by steroids

The form of inhibition was determined from double-reciprocal plots of initial velocity versus substrate concentration, where androsterone was the variable substrate (5.6–56 μ M): C, competitive; UC, uncompetitive; NI, no inhibition observed for the compound tested, up to a concentration of 100 μ M; ND, not determined. K_{is} was determined from replots of slope versus inhibitor concentration when competitive inhibition of androsterone glucuronidation was observed. Glucuronidation activities for the alternative substrates are expressed as percentages of the rate of androsterone glucuronidation catalysed by purified 3 α -hydroxysteroid UDPGT. Results are means $\pm$ S.D., where appropriate, for the numbers of determinations in parentheses: NA, no glucuronide formation was observed with the indicated compounds.

Compound	3 α -Hydroxysteroid UDPGT activity		
	Type of inhibition	K_{is} (μ M)	UDPGT activity (% of that with androsterone)
Androsterone	—	—	100
β -Oestradiol	C	25	NA
Oestrone	NI	—	NA
Epitestosterone	C	ND	NA
17 α -Hydroxyprogesterone	C	33	NA
17 β -Hydroxy-5 α -androstan-3-one	NI	—	NA
Epiandrosterone	C	25	NA
Etiocholanolone	C	5	151 $\pm$ 10 (4)
Lithocholic acid	C	30	17 $\pm$ 1 (3)
Chenodeoxycholic acid	ND	—	3 $\pm$ 1 (3)

Fig. 1. Inhibition of androsterone glucuronidation catalysed by 3α -hydroxysteroid UDP-glucuronosyltransferase

Androsterone glucuronidation was determined with purified 3α -hydroxysteroid UDP-glucuronosyltransferase in the presence of (●) 0 μM -, (□) 25 μM - and (○) 50 μM -epiandrosterone (a) or 17α -hydroxyprogesterone (b). The concentration of UDP-glucuronic acid was 10 mM. Velocity (v) is expressed in nmol of androsterone glucuronidated/min per mg of protein. The concentration of androsterone is expressed in μM . Each point represents the average of triplicate assays.

Phosphatidylcholine gave the greatest increase in glucuronidation activity. This is in agreement with other reports (Gorski & Kasper, 1978; Tukey *et al.*, 1979; Tukey & Tephly, 1980; Singh *et al.*, 1981a,b; Magdalou *et al.*, 1982), where phosphatidylcholine was shown to be the most efficient phospholipid for reconstitution of UDP-glucuronosyltransferase activity in a variety of different purified preparations. 3α -Hydroxysteroid UDP-glucuronosyltransferase was routinely more responsive to phospholipid reconstitution than was 17β -hydroxysteroid UDP-glucuronosyltransferase.

The glucuronidation activity of partially delipidated

Fig. 2. Reconstitution of delipidated 3α -hydroxysteroid and 17β -hydroxysteroid UDP-glucuronosyltransferase activities with various phospholipids

Fractions from the Chromatofocusing column containing 17β -hydroxysteroid and 3α -hydroxysteroid UDP-glucuronosyltransferase activity (36 μg of inorganic P/mg of protein) were applied to 2 ml hydroxyapatite columns and partially delipidated (2.5 μg of inorganic P/per mg of protein) as described in the Experimental section. Testosterone (□) and androsterone (■) glucuronidation activities were determined with the partially delipidated preparations (CONT) and in the presence of 100 μg of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SPH), phosphatidylserine (PS), phosphatidylethanolamine (PE) or phosphatidylinositol (PI) added to the reaction mixture. Reactions were preincubated at 37 °C for 5 min and started by the addition of 5 mM-UDP-glucuronic acid. A representative experiment is presented, and activity is expressed as percentage of delipidated (control) activity and represents the mean of four assays. Control activities were 32 and 30.5 nmol conjugated/min per mg of protein for androsterone and testosterone respectively.

preparations of either 17β -hydroxysteroid or 3α -hydroxysteroid UDP-glucuronosyltransferase was not increased by addition of 100 μg of the fatty acids palmitic acid or oleic acid, nor by phosphatidic acid or choline chloride (results not shown). The reconstitution of delipidated 3α -hydroxysteroid UDP-glucuronosyltransferase activity was assessed after reconstitution with different fatty acid-containing phosphatidylcholines. Phosphatidylcholines containing either myristic acid ($C_{14:0}$) or linoleic acid ($C_{18:2}$) were the most efficient at restoring glucuronidation activity, whereas phosphatidylcholine containing arachidonic acid ($C_{20:4}$) was the least efficient (results not shown). These results are very similar to those observed by Tukey *et al.* (1979) and Tukey & Tephly (1980) during phospholipid reconstitution of delipidated rabbit liver oestrone and *p*-nitrophenol UDP-glucuronosyltransferases.

Effect of delipidation on 3α -hydroxysteroid UDP-glucuronosyltransferase reaction kinetics

The effect of delipidation on the apparent K_m for androsterone and UDP-glucuronic acid with the 3α -

Fig. 3. Effect of delipidation on the kinetics of androsterone glucuronidation by 3α -hydroxysteroid UDP-glucuronosyltransferase

A preparation of 3α -hydroxysteroid UDP-glucuronosyltransferase was divided in half. One half was partially delipidated ($10 \mu g$ of inorganic P/mg of protein) by UDP-hexanolamine affinity chromatography in the absence of added phospholipid. The other half of the enzyme preparation was chromatographed on an identical UDP-hexanolamine affinity column in the presence of added phosphatidylcholine (as described in the Experimental section). The delipidated enzyme was assayed with (○) and without (□) the addition of $100 \mu g$ of phosphatidylcholine in the reaction mixture (a). 3α -Hydroxysteroid UDP-glucuronosyltransferase activity from the control column (●) was also assayed in the usual manner. Androsterone concentrations were 5.625 – $45 \mu M$ and the UDP-glucuronic acid concentration was 10 mM. Velocity is expressed in nmol of androsterone conjugated/min per mg of protein. Each point represents the average of three reactions. Androsterone glucuronidation activity of partially delipidated 3α -hydroxysteroid UDP-glucuronosyltransferase was determined with $56.25 \mu M$ -androsterone and UDP-glucuronic acid concentrations of 0.05 – 1.0 mM. Reactions were performed without (●) or with (○) the addition of $100 \mu g$ of phosphatidylcholine to the reaction mixture (b). Velocity is expressed in nmol of androsterone conjugated/min per mg of protein.

Table 3. Amino acid compositions of *p*-nitrophenol, 17β -hydroxysteroid and 3α -hydroxysteroid UDP-glucuronosyltransferases

Cysteic acid and methionine sulphone were determined after performic acid oxidation. Tryptophan was not determined.

	<i>p</i> -Nitrophenol UDP-glucuronosyltransferase		17β -Hydroxysteroid UDP-glucuronosyltransferase		3α -Hydroxysteroid UDP-glucuronosyltransferase	
	(residues/ subunit)*	(mol %)	(residues/ subunit)†	(mol %)	(residues/ subunit)‡	(mol %)
Cysteic acid	6	1.6	5	1.5	5	1.4
Aspartic acid	50	10.2	43	9.9	43	9.5
Methionine sulphone§	17	3.9	16	4.1	15	3.7
Threonine	22	4.0	20	4.1	19	3.8
Serine	38	6.1	30	5.5	28	4.9
Glutamic acid	52	11.8	39	9.9	38	9.3
Proline§	15	2.6	29	5.8	34	6.5
Glycine	50	5.8	30	3.9	28	3.5
Alanine§	31	4.3	27	4.2	28	4.1
Valine§	20	3.6	27	5.5	25	4.9
Isoleucine§	22	4.4	24	5.4	27	5.9
Leucine§	63	12.7	51	11.6	55	12.0
Tyrosine	16	4.5	16	5.0	17	5.1
Phenylalanine§	27	6.9	26	7.4	31	8.5
Lysine	29	6.5	35	8.9	37	9.0
Histidine	18	4.3	15	4.0	17	4.6
Arginine	25	6.7	11	3.3	12	3.5
Tryptophan	—	—	—	—	—	—
Total	501		444		459	

* Calculated from a subunit M_r of 56000.

† Calculated from a subunit M_r of 50000.

‡ Calculated from a subunit M_r of 52000.

§ Indicates a hydrophobic amino acid.

hydroxysteroid UDP-glucuronosyltransferase was investigated. Fig. 3(a) shows that, in a partially delipidated preparation of 3α -hydroxysteroid UDP-glucuronosyltransferase, the apparent K_m for androsterone increases (5-fold) while the V_{max} decreases. Addition of phosphatidylcholine to the reaction mixture restores the apparent K_m to the value observed in an identical control enzyme preparation which was purified in the presence of phospholipid (see the Experimental section). However, the delipidation procedure resulted in a loss of total enzyme activity recovered even when phospholipid is present in the reaction mixture. Fig 3(b) demonstrates that partial delipidation of 3α -hydroxysteroid UDP-glucuronosyltransferase results in an increase in the apparent K_m for UDP-glucuronic acid, but no apparent change in the V_{max} . These results strongly suggest that the presence or absence of phospholipid in the buffers used during affinity chromatography may markedly affect the kinetics and recovery of UDP-glucuronosyltransferase activities.

Physical characterization of *p*-nitrophenol, 3α -hydroxysteroid and 17β -hydroxysteroid UDP-glucuronosyltransferases

Purified *p*-nitrophenol, 3α -hydroxysteroid and 17β -hydroxysteroid UDP-glucuronosyltransferases display subunit M_r values of 56000, 52000 and 50000 respectively, after SDS/polyacrylamide-gel electrophoresis (Falany & Tephly, 1983). To compare the physical characteristics of the purified transferase enzymes, the amino acid composition was determined as well as the pattern of peptide fragments generated by proteolysis with *S. aureus* proteinase.

The amino acid compositions of the three purified UDP-glucuronosyltransferases are shown in Table 3. Even though the purified 3α -hydroxysteroid and 17β -hydroxysteroid UDP-glucuronosyltransferase enzymes display different subunit M_r values, the mol % values of the individual amino acids in the two purified enzymes are very similar, and no substantial differences were observed. The mol % values of hydrophobic amino acids in the 17β -hydroxysteroid and 3α -hydroxysteroid UDP-glucuronosyltransferases are 44% and 46% respectively. The high mol % of hydrophobic amino acid is probably due to the fact that UDP-glucuronosyltransferase are lipid-dependent enzymes. Both of the purified steroid UDP-glucuronosyltransferase, however, show a relatively different amino acid composition as compared with the purified rat liver *p*-nitrophenol (3-methylcholanthrene-inducible) UDP-glucuronosyltransferase.

The *p*-nitrophenol UDP-glucuronosyltransferase contains a different mol % of lysine, glutamic acid, glycine and valine as compared with the steroid isoenzymes. The amount of proline in the *p*-nitrophenol UDP-glucuronosyltransferase is approx. 50% of that in the steroid UDP-glucuronosyltransferases, and the mol % of arginine is considerably higher in the *p*-nitrophenol UDP-glucuronosyltransferase. The high content of arginine may contribute to the higher pI observed for the *p*-nitrophenol UDP-glucuronosyltransferase (Falany & Tephly, 1983). The mol % of hydrophobic amino acids in the *p*-nitrophenol UDP-glucuronosyltransferase is 38%. This value is somewhat lower than observed for the steroid UDP-glucuronosyltransferases.

Peptide fragments were generated by limited proteolytic digestion of the *p*-nitrophenol, 3α -hydroxysteroid and

Fig. 4. Limited proteolysis of *p*-nitrophenol, 3α -hydroxysteroid and 17β -hydroxysteroid UDP-glucuronosyltransferase with *Staph. aureus* proteinase

Digestions were performed as described in the Experimental section. Bands representing *Staph. aureus* proteinase are indicated in lane A. Lanes B, C and D contain 3 μ g of *p*-nitrophenol UDP-glucuronosyltransferase after incubation with the proteinase for 5, 30 and 90 min respectively. Lanes E, F and G contain proteolytic digests of 3 μ g of 3α -hydroxysteroid UDP-glucuronosyltransferase, and lanes H, I and J contain proteolytic digests of 17β -hydroxysteroid UDP-glucuronosyltransferase, obtained after incubation for 5, 30 and 90 min respectively.

17β -hydroxysteroid UDP-glucuronosyltransferases with *S. aureus* proteinase. The peptide maps for the three proteins were distinctly different, as expected, since the proteins have different M_r values (Fig. 4). Peptide fragments generated from the 3α -hydroxysteroid and 17β -hydroxysteroid UDP-glucuronosyltransferases were different from those obtained from the *p*-nitrophenol UDP-glucuronosyltransferase. Some similarities in low- M_r peptide fragments were observed between the 3α -hydroxysteroid and 17β -hydroxysteroid UDP-glucuronosyltransferases after prolonged digestions. These results suggest a high degree of structural similarity between these two proteins.

DISCUSSION

The substrate specificity of rat liver 17β -hydroxysteroid and 3α -hydroxysteroid UDP-glucuronosyltransferases has been further defined and shown to be very specific for the site on steroids with which they react. In accordance with the suggestion of naming UDP-glucuronosyltrans-

ferases after important endogenous substrates when possible (Bock *et al.*, 1983), the enzymes have been renamed 17 β -hydroxysteroid and 3 α -hydroxysteroid UDP-glucuronosyltransferases.

17 β -Hydroxysteroid UDP-glucuronosyltransferase conjugates the 17 β -hydroxy position, but not the 17 α -hydroxy position or the 3-hydroxy phenolic position, of steroids. The 3 α -hydroxysteroid UDP-glucuronosyltransferase specifically glucuronidates the 3 α -hydroxy position of steroids and bile acids, but not the 3 β -hydroxy or 24-carboxyl positions of steroids and bile acids or the phenolic 3-hydroxy position of oestrogens. These results suggest that both UDP-glucuronosyltransferases possess a steroid-binding site, but glucuronidation of the steroid in the binding site depends on position and orientation of the hydroxyl groups to the plane of the steroid ring structure. The orientation of the hydrogens present on the steroid A-ring also affects the role of conjugation, since etiocholanolone (3 α -OH, 5 β -H) is glucuronidated more rapidly and has a lower apparent K_m than androsterone (3 α -OH, 5 α -H). An oestrone UDP-glucuronosyltransferase activity reactive with the phenolic 3-hydroxy position of oestrone and β -oestradiol, but not the 17 β -hydroxy of β -oestradiol, has also been separated from the majority of 17 β -hydroxysteroid and 3 α -hydroxysteroid UDP-glucuronosyltransferase activities during Chromatofocusing of rat liver microsomes (Falany *et al.*, 1983). These results indicate that there are at least three different UDP-glucuronosyltransferase(s) present in rat liver that are highly specific for the glucuronidation of different positions of the steroid ring system. Oestrone UDP-glucuronosyltransferase purified from rabbit liver microsomes has also been shown to be specific for the conjugation of the phenolic 3-hydroxy position of β -oestradiol (Falany *et al.*, 1983). Preparations of UDP-glucuronosyltransferase which contain activity towards chenodeoxycholate and testosterone (Matern *et al.*, 1982), testosterone, morphine and oestrone (Bock *et al.*, 1979), or testosterone and androsterone (Mackenzie & Owens, 1983), probably contain more than one form of UDP-glucuronosyltransferase.

Although we have demonstrated a narrow substrate specificity of steroid UDP-glucuronosyltransferases for steroidal compounds, these enzymes exhibit much less specificity for xenobiotics such as *p*-nitrophenol, 1-naphthol or 4-methylumbelliferone (Falany & Tephly, 1983; Falany *et al.*, 1983). The use of *p*-nitrophenol as a general indicator of 'UDP-glucuronosyltransferase activity', as it is commonly used, does not accurately reflect either total UDP-glucuronosyltransferase activity or the amount of *p*-nitrophenol UDP-glucuronosyltransferase isoenzymes in untreated rats. A major portion of activity on *p*-nitrophenol in untreated rat liver is catalysed by the 17 β -hydroxysteroid UDP-glucuronosyltransferase (Falany & Tephly, 1983). Only in 3-methylcholanthrene-pretreated rats is a *p*-nitrophenol UDP-glucuronosyltransferase present in substantial amounts to account for a major portion of *p*-nitrophenol glucuronidation. Although *p*-nitrophenol and α -naphthol are conjugated by at least two different enzymes in rat liver, only the *p*-nitrophenol UDP-glucuronosyltransferase has been shown to catalyse the glucuronidation of 4-methylumbelliferone (Falany & Tephly, 1983). These results suggest that 4-methylumbelliferone would be the preferred substrate for the determination of *p*-nitrophenol UDP-glucuronosyltransferase in rats. These results also

indicate that, during purification procedures for a given UDP-glucuronosyltransferase isoenzyme, a combination of endogenous and exogenous substrate should be used to monitor enzyme activity.

Extensive delipidation of the 3 α -hydroxysteroid UDP-glucuronosyltransferase resulted in complete inactivation of glucuronidation activity, whereas partial delipidation produced significant changes in the kinetic parameters of the enzyme. The kinetic changes are probably due to changes in the tertiary conformation of the enzyme, with subsequent effects on substrate binding. Singh *et al.* (1981a) have reported that delipidation does not affect the tertiary conformation of guinea-pig liver *p*-nitrophenol UDP-glucuronosyltransferase. These results suggest that purification procedures such as UDP-hexanolamine affinity chromatography in the absence of phospholipid may lead to a change in enzyme activity and a decrease in the recovery of enzymically active protein, or may lead to a loss of some forms of UDP-glucuronosyltransferase.

Hochman *et al.* (1981) have demonstrated that reconstitution of UDP-glucuronosyltransferase activities purified from pig liver with lysophosphatidylcholines containing different fatty acid moieties affects the V_{max} of the enzyme, the binding constants of substrates and the fraction of energy available, 'from binding of UDP-glucuronic acid with the enzyme, for breaking the UDP-pyrophosphate-glucuronic acid bond. Hochman *et al.* (1981) used these observations to support the conformation-constraint hypothesis of the regulation of UDP-glucuronosyltransferase activity. Our results showed that phosphatidylcholines containing specific fatty acid moieties (such as myristic acid or linoleic acid) were more effective than others (such as arachidonic acid) in reconstitution of partially delipidated 3 α -hydroxysteroid UDP-glucuronosyltransferase activity. These results are similar to those obtained by Tukey *et al.* (1979) and Tukey & Tephly (1980), using purified rabbit UDP-glucuronosyltransferase. However, it seems unlikely that, *in vivo*, UDP-glucuronosyltransferases are specifically associated with certain phosphatidylcholines. Tukey *et al.* (1979) have shown that, during resolution and delipidation of rabbit liver oestrone and *p*-nitrophenol UDP-glucuronosyltransferase activities, the types of fatty acids (phospholipid not specified) associated with the transferase activities were heterogeneous, suggesting that the transferases were not associated with specific fatty acid-containing phospholipids *in vivo*. The most likely functions of phospholipid are to maintain the UDP-glucuronosyltransferase in an active configuration and to anchor the transferase in the membrane.

Although the 3 α -hydroxysteroid and 17 β -hydroxysteroid UDP-glucuronosyltransferases are different proteins, these two transferases behaved very similarly through several chromatographic procedures. The separation of these transferases was not possible by ion-exchange-chromatographic procedures which are known to separate other UDP-glucuronosyltransferase (Tukey & Tephly, 1981; Tukey *et al.*, 1982). However, separation was accomplished, as previously described (Falany & Tephly, 1983), by column isoelectric focusing (Chromatofocusing). The similarities observed in the behaviour of the 17 β -hydroxysteroid and 3 α -hydroxysteroid UDP-glucuronosyltransferase activities during the chromatographic procedures are most probably due to the similar physical properties of the two proteins. The

two steroid UDP-glucuronosyltransferases have very similar amino acid compositions and the mol % of hydrophobic amino acids. In addition, the peptide maps generated by proteolytic digestion of the 17 β -hydroxysteroid and 3 α -hydroxysteroid UDP-glucuronosyltransferases also showed a number of similar peptides after prolonged digestion, even though the protein subunit M_r values are different. These results suggest that the two steroid UDP-glucuronosyltransferases are closely related and may show a great deal of homology, even though their substrate specificities are distinctly different. Indeed, preliminary results with antibodies raised to the 3 α -hydroxysteroid and 17 β -hydroxysteroid UDP-glucuronosyltransferases indicates substantial cross-reactivity. Mackenzie & Owens (1984) have reported that a fraction of M_r -52000 rat liver UDP-glucuronosyltransferase protein is post-translationally cleaved by dog pancreatic microsomes to produce a M_r -50000 protein. It may be possible that the 17 β -hydroxysteroid UDP-glucuronosyltransferase is synthesized as a M_r -52000 protein and then cleaved to a M_r -50000 protein by removal of a peptide fragment, such as hydrophobic leader sequence.

The *p*-nitrophenol UDP-glucuronosyltransferase appears less closely related to the two steroid transferases. This isoenzyme does not glucuronidate steroids, it is inducible by 3-methylcholanthrene, and it has a different amino acid composition and a distinctly different peptide map. These observations suggest the possibility that families of related UDP-glucuronosyltransferases exist, as have been described for other drug-metabolizing enzymes. A better appreciation of the relationship between these enzymes requires the knowledge of their protein structure and genomic organization.

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