Isolation and Characterization of Rat Liver Microsomal UDP-glucuronosyltransferase Activity toward Chenodeoxycholic Acid and Testosterone as a Single Form of Enzyme*

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Microsomal UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone has been isolated from rat liver and appears to be homogeneous in sodium dodecyl sulfate gel electrophoresis and polyacrylamide gradient gel electrophoresis. The conjugating activities toward chenodeoxycholic acid and testosterone co-purified and showed identical mobilities in disc gel electrophoresis, indicating that chenodeoxycholic acid and testosterone are glucuronidated by a single form of enzyme. UDP-glucuronosyltransferase activities toward estrone, bilirubin, 4-nitrophenol, and morphine did not co-purify with the activity toward chenodeoxycholic acid and testosterone and were not detectable in the pure enzyme in the presence or absence of phospholipids. In addition to glucuronic acid conjugation, the enzyme is able to catalyze galacturonic acid conjugation of chenodeoxycholic acid and testosterone. The enzyme has a subunit $M_r \approx 54,000$ and in the presence of the stabilizing detergent chenodeoxycholic acid, it appears to exist as an aggregate with an apparent $M_r = 318,000$ as estimated by gel filtration and 316,000 by polyacrylamide gradient gel electrophoresis.

Materials—Egg yolk $L$-$\alpha$-phosphatidylcholine, egg yolk $L$-$\alpha$-phosphatidylethanolamine, egg yolk $L$-$\alpha$-lysophosphatidylcholine, $\beta$-glucuronidase from bovine liver, UDP-galacturonic acid, UDP-xylene, and CDP-glucose were obtained from Sigma (München, Germany). UDP-galactose, UDP-glucose, and UDP-N-acetylgalactosamine were obtained from Boehringer (Mannheim, Germany). 3-$\alpha$-Hydroxysteroid dehydrogenase (Sterogent 3-Pho) was obtained from Nyegaard (Oslo, Norway). Octyl-$\beta$-D-glucopyranoside and Zwittergent 3-12 were purchased from Calbiochem (Giessen, Germany). Polyethylene glycol 6000 was from Serva (Heidelberg, Germany). Bio-Gel A-1.5m (100-200 mesh) and Bio-Beads SM 2 were obtained from Bio-Rad Laboratories (München, Germany). DEAE-Sepharose Cl-4B, protein standards for molecular weight determination, and polyacrylamide gradient slab gels, PA-A 4/30, were obtained from Pharmacia (Freiburg, Germany). Silica Gel 60 plates were from Merck (Darmstadt, Germany). [N-methyl-$^14$C]morphine hydrochloride was from Amersham/Buchler (Braunschweig, Germany); [34-$^3$H]cholanic acid, [4-$^14$C]estrone, and [4-$^14$C]estradiol were purchased from NEN Chemicals (Dreieich, Germany). UDP-hexanolamine-Sepharose 4B (4-$^14$C) was purchased from Pharmacia. Sources of other chemicals were the same as described in previous papers (5, 19).

Enzyme Assays—UDP-glucuronosyltransferase activities toward

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Dedicated to Professor Dr. Helmut Holzer on the occasion of his 60th birthday.
4-nitrophenol (29), morphine (21), bilirubin (22), estrone (23), testosterone (23), and chenoxycholic acid (5) were assayed according to the method of Bradford (25), using bovine serum albumin as standard. Prior to protein determination, chenoxycholic acid was removed from samples as described by Makino and Spivall (26) with the modification that the sample was diluted with an equal volume of 0.2 M sodium hydroxide containing 1.8% (w/v) NaCl. Treatment with Amberlite XAD-2 was performed in a batch procedure with shaking for 60 min. The recovery of protein in the neutralized supernatant was 96% as determined with bovine serum albumin as internal standard.

Analytical Methods—Protein concentrations were determined by the method of Segrest and Jackson (27). Human transferrin was used as the standard. Quantitative evaluation of results was performed as described (28).

For the determination of phospholipids in enzyme preparations, the method as described by Singh et al. (29) was used with the following modifications. After removal of detergents by treatment with Bio-Beads SM-2 (29) and extraction of phospholipids with chloroform/methanol (29) the organic solvent phase containing phospholipids was evaporated to dryness and assayed for lipid phosphorus as described by Bartlett (30).

To characterize the product of the reaction of chenoxycholic acid and UDP-galacturonic acid, the pure enzyme was incubated for 2 h at 37 °C in a reaction mixture containing 9 mM chenoxycholic acid, 2 mM UDP-galacturonic acid and 0.1 M sodium acetate, pH 5, for 1 h. The reaction mixture was then applied to the column as described by Frohling et al. (17).

For the determination of phospholipids in enzyme preparations, the method of Bradford (25), using bovine serum albumin as standard, was performed as described (28).

Centrifugation was performed at 100,000 g for at least 1 month. A common microsomal fraction was prepared from the livers of three rats by fractional centrifugation as previously described (34). The microsomal sediment was washed once with 0.15 M KCl and suspended in 0.1 M potassium phosphate, pH 7.4, containing 1 mM dithioerythritol.

Purification of UDP-galactosyltransferase Activity toward Chenoxycholic Acid and Testosterone—If not stated otherwise, all steps were carried out at 4 °C. Standard buffer was 20 mM Tris/acetate, pH 7.4, containing 20% (v/v) glycerol, 1 mM dithioerythritol.

Step 1: Solubilization of Rat Liver Microsomes—The microsomal suspension having a protein concentration of 17 mg/ml was solubilized by the addition of an equal volume of 0.1 M potassium phosphate, pH 7.4, containing 20% (v/v) glycerol, 1 mM dithioerythritol, 3% (w/v) octyl-β-D-glucopyranoside. After stirring for 10 min, the mixture was centrifuged at 100,000 g for 60 min.

Step 2: Polyethylene Glycol Fractionation—A 50% (w/v) polyethylene glycol solution in standard buffer was added to the clear supernatant of step 1 to make a final concentration of 3% (w/v). After stirring for 15 min, the suspension was centrifuged for 15 min at 40,000 g. The precipitate was discarded. To the supernatant, 50% (w/v) polyethylene glycol was added to a final concentration of 6.5% (w/v). After the suspension was stirred for 15 min, it was centrifuged for 15 min at 40,000 g. The precipitate was dissolved in the standard buffer containing 0.2% (v/v) of the zwitterionic detergent Zwittergent 3-12. Dialysis was performed against two 1-liter changes of the same buffer.

Step 3: DEAE-Sepharose CI 4B Chromatography—The dialyzed turbid protein fraction was solubilized by the addition of 1% (w/v) Zwittergent 3-12 in standard buffer to a final concentration of 0.8% (w/v) Zwittergent 3-12. After 10 min, the mixture was centrifuged for 90 min at 100,000 g. The clear supernatant was applied to a DEAE-Sepharose CI 4B column (2.6 × 24 cm) equilibrated and eluted with standard buffer containing 0.2% (w/v) Zwittergent 3-12 (flow rate, 40 ml/h). Two peaks of UDP-galactosyltransferase activity toward chenoxycholic acid and testosterone were eluted from the column (see Fig. 1). The first activity peak (peak I) appeared with the flow-through volume. The second activity peak (peak II) was found in the protein fraction which was eluted with UDP-galactosyltransferase activity toward chenoxycholic acid, but not with UDP-galactosyltransferase activity toward testosterone. No additional enzyme activity toward chenoxycholic acid and testosterone was eluted when a linear gradient from 0 to 0.5 M NaCl in standard buffer containing 0.2% (w/v) Zwittergent 3-12 was applied to the column.

Step 4: UDP-hexanolamine-Sepharose 4B Chromatography—The fractions containing UDP-galactosyltransferase activity toward chenoxycholic acid and testosterone as obtained from peak I of step 3 were diluted by an equal volume of standard buffer containing 10 mM MgCl₂ and applied to a UDP-hexanolamine-Sepharose 4B column (1.6 × 4.2 cm) equilibrated with standard buffer containing 0.2% (w/v) Zwittergent 3-12, 5 mM MgCl₂ (flow rate, 6 ml/h). After washing with 80 ml of equilibration buffer, UDP-galactosyltransferase activity toward chenoxycholic acid and testosterone was eluted with equilibration buffer containing 2 mM chenoxycholic acid, if not otherwise stated (see Fig. 2).

Step 5: Bio-Gel A-1.5m Chromatography—The active fractions of step 4 were applied to a Bio-Gel A-1.5m column (1.6 × 100 cm) equilibrated and eluted with standard buffer containing 0.05% (w/v) Zwittergent 3-12, 2 mM chenoxycholic acid (flow rate, 5.4 ml/h). The fractions containing UDP-galactosyltransferase activity toward chenoxycholic acid and testosterone were pooled and stored at −20 °C. Under these conditions, the enzyme is stable for at least 1 month.

Results

Isolation of UDP-galactosyltransferase Activity toward Chenoxycholic Acid and Testosterone—The results of the purification procedure are summarized in Table I. The puri-
Purification of UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone from solubilized rat liver microsomes

<table>
<thead>
<tr>
<th>Step</th>
<th>Volumes</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Ratio showing specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>With chenodeoxycholic acid</td>
<td>With testosterone</td>
<td>(mg/ml)</td>
</tr>
<tr>
<td>1. Solubilized microsomes</td>
<td>72</td>
<td>555</td>
<td>3.3</td>
<td>18.2</td>
<td>0.18</td>
</tr>
<tr>
<td>2. Polyethylene glycol fractionation</td>
<td>17.5</td>
<td>262</td>
<td>4.6</td>
<td>27.2</td>
<td>0.17</td>
</tr>
<tr>
<td>3. DEAE-Sepharose CI-8B</td>
<td>36</td>
<td>6</td>
<td>35.8</td>
<td>201</td>
<td>0.18</td>
</tr>
<tr>
<td>4. UDP-hexanolamine-Sepharose 4B</td>
<td>7</td>
<td>0.4</td>
<td>140.8</td>
<td>848</td>
<td>0.17</td>
</tr>
<tr>
<td>5. Bio-Gel A-1.5m</td>
<td>12</td>
<td>0.2</td>
<td>198.3</td>
<td>983</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* With chenodeoxycholic acid as substrate.

* Data are given for peak I of DEAE-Sepharose chromatography (see Fig. 1). The specific activity is expressed as units/mg of protein. See "Experimental Procedures" for definition of units and experimental details.
than about 0.1 mM (see Fig. 5), suggesting that above this concentration chenodeoxycholic acid may decrease the affinity of the enzyme to the ligand UDP-hexanolamine. The elution of enzyme activity from the affinity column was, however, performed with 2 mM chenodeoxycholic acid, since the bile acid exerted a stabilizing effect on enzyme activity at concentrations between 2 and 5 mM.

The last step of the purification procedure was passage through a Bio-Gel A-1.5m column. This step was necessary to remove traces of contaminating proteins. The enzyme activity toward chenodeoxycholic acid and testosterone emerged as a single symmetrical peak which paralleled the main protein peak.

As shown in Fig. 2, the protein prepared in this manner yielded a single band after disc gel electrophoresis in the presence of sodium dodecyl sulfate and polyacrylamide gradient slab gel electrophoresis.

**Molecular Weight**—The molecular weight of the purified UDP-glucuronosyltransferase was calculated by Bio-Gel A-1.5m gel filtration, by polyacrylamide gradient slab gel electrophoresis, and by sodium dodecyl sulfate and polyacrylamide gel electrophoresis. Gel filtration of the purified material and a comparison with proteins of known molecular weight are shown in Fig. 3. From these results, an apparent $M_w = 318,000$ can be interpolated. From determination of the mobilities of the purified enzyme and various reference proteins in polyacrylamide gradient slab gel electrophoresis according to the method of Anderson et al. (35), the apparent molecular weight of the enzyme was calculated to be 316,000 (Fig. 2, b and c). Since no phospholipid could be detected when 200 µg of the purified enzyme were estimated for lipid phosphorus, whereas the protein fraction of peak 1 of DEAE-Sephacore chromatography yielded a phospholipid content of 0.02 mg/mg of protein, the phospholipid content of the pure enzyme was calculated from the lower limit of detectable phosphorus to be less than 3 mol of phospholipid/mol of enzyme. Therefore, traces of phospholipids which might be associated with the purified enzyme can be neglected for calculation of the molecular weight of the enzyme. The detergents, in particular chenodeoxycholic acid, which had to be added to buffers used for molecular weight determinations because of the stabilizing effect on the enzyme might influence the molecular size and shape of proteins. The reference proteins, however, showed the same elution volumes in gel filtration and the same mobilities in polyacrylamide gradient slab gel electrophoresis in the presence and absence of the detergents used. Studies on the interaction of chenodeoxycholic acid with the purified UDP-glucuronosyltransferase are now in progress. These studies may not only give information on the influence of the bile acid on the molecular weight of the enzyme but may also serve as a model system for biological membranes in which the amphiphilic species is primarily phospholipid since chenodeoxycholic acid was able to replace phospholipids in maintaining enzyme activity (see below).

The subunit molecular weight was determined by sodium dodecyl sulfate gel electrophoresis to be approximately 54,000. About the same value of subunit molecular weight was obtained with or without reduction by 2-mercaptoethanol, suggesting that the purified enzyme appears to be an aggregate with subunits that are not linked to disulfide bridges.

**Substrate Specificity**—The pooled fractions of the purification procedure containing UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone were assayed for conjugating activities toward estrone, bilirubin, 4-nitrophenol, and morphine. The results are shown in Table II. UDP-glucuronosyltransferase activities toward estrone, bilirubin, 4-nitrophenol, and morphine were present in the 3–6.5% polyethylene glycol fraction in addition to the enzyme.

![Fig. 2. Electrophoresis of pure UDP-glucuronosyltransferase and protein standards in polyacrylamide gels. a, disc gel electrophoresis of pure enzyme in the presence of sodium dodecyl sulfate (protein applied, 10 µg). b and c, polyacrylamide gradient slab gel electrophoresis. b, a mixture of hog thyroid thyroglobulin ($M_w = 669,000$), horse spleen ferritin ($M_w = 440,000$), beef liver catalase ($M_w = 232,000$), beef heart lactate dehydrogenase ($M_w = 140,000$), and bovine serum albumin ($M_w = 67,000$), 2.5 µg of each; c, 3 µg of pure UDP-glucuronosyltransferase. Wire denotes the position of bromophenol blue as the tracking dye.]

![Fig. 3. Molecular weight determination of pure UDP-glucuronosyltransferase by Bio-Gel A-1.5m gel filtration. The column (1.6 x 100 cm) was run as described under "Experimental Procedures." $V_e$ was estimated with dextran blue; calibration was obtained with 200 µg of pure UDP-glucuronosyltransferase, 2 mg of ferritin, and 10 mg of all other proteins. The sample volume applied was 2 ml. The elution positions were determined by the absorbance at 280 nm for the reference proteins and for UDP-glucuronosyltransferase by estimation of conjugating activity toward chenodeoxycholic acid and testosterone (see "Experimental Procedures"). 1, rabbit muscle aldolase; 2, bovine liver catalase; 3, horse spleen ferritin; 4, bovine thyroid thyroglobulin.](http://www.jbc.org/)
significant effect on enzyme activities toward all aglycone substrates. In these concentrations, the bile acid did not have a detectable effect on enzyme activities toward all aglycone substrates tested. When enzyme preparations after UDP-hexanolamine-Sepharose 4B chromatography containing chenodeoxycholic acid were assayed for enzyme activities, the concentration of the bile acid did not exceed 0.05 mM in the assay mixtures for testosterone and 0.2 mM in the assay mixtures for estrone, bilirubin, 4-nitrophenol, and morphine. In these concentrations, the bile acid did not have a significant effect on enzyme activities toward all aglycone substrates tested. Enzyme activity is expressed as units/mg of protein. ND, not detectable.

activities toward chenodeoxycholic acid and testosterone. However, after DEAE-Sepharose chromatography, activities toward bilirubin, 4-nitrophenol, and morphine were not detectable in peak I and showed a decrease in specific activity in peak II compared to the values obtained in the polyethylene glycol fraction, whereas activities toward chenodeoxycholic acid, testosterone, and estrone were present in peak I and peak II of DEAE-Sepharose chromatography. However, only the activities toward chenodeoxycholic acid and testosterone exhibited co-purification, whereas the specific activity of UDP-glucuronosyltransferase with estrone as substrate decreased. When the protein fraction of peak I from the DEAE-Sepharose chromatography was subjected to affinity chromatography on UDP-hexanolamine-Sepharose 4B, the pooled fractions of the chenodeoxycholic acid eluate (see "Experimental Procedures") exhibited only conjugating activity toward chenodeoxycholic acid and testosterone whereas UDP-glucuronosyltransferase activities toward the other aglycone substrates listed in Table II were not detectable in the presence of sodium dodecyl sulfate. The amount of protein applied to the gel was 8 µg. For activity assay, two unstained gels with the protein load of 8 µg were sliced into 2-mm slices. Each slice was homogenized in 0.2 ml of standard buffer (see "Experimental Procedures") containing 0.05% Zwittergent 3-12 and assayed for UDP-glucuronosyltransferase activity toward chenodeoxycholic acid (○) and testosterone (○) as described under "Experimental Procedures."

The sugar nucleotide substrate specificity of the purified UDP-glucuronosyltransferase is shown in Table III. In addition to glucuronic acid conjugation of chenodeoxycholic acid and testosterone, the enzyme was able to catalyze galacturonic acid conjugation of both substrates. No conjugating-enzyme activity was detected in the presence of the other sugar nucleotides listed in Table III. When enzyme activity toward chenodeoxycholic acid and testosterone was measured in the presence of UDP-glucuronic acid and the sugar nucleotides listed in Table III, UDP-galacturonic acid, UDP-xylose, UDP-glucose, UDP-galactose, and UDP-N-acetylgalactosamine showed inhibition of chenodeoxycholic acid and testosterone glucuronidation whereas CDP-glucose had no significant effect on the glucuronidation of both substrates. In the presence of a mixture of UDP-galacturonic acid (9 mM) and UDP-glucuronic acid (3 mM), the reaction rate with chenodeoxycholic acid was 78% and with testosterone 68% of the sum of reaction rates when both co-substrates were incubated separately, which is an indication that UDP-glucuronic acid and UDP-galacturonic acid are co-substrates of the same enzyme.

TABLE II

<table>
<thead>
<tr>
<th>Aglycone substrate</th>
<th>Enzyme activity in purification steps</th>
<th>Polyethylene glycol fraction</th>
<th>DEAE-Sepharose Cl-4B</th>
<th>UDP-hexanolamine-Sepharose 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peak I</td>
<td>Peak II</td>
<td></td>
</tr>
<tr>
<td>Cheno-deoxycholic acid</td>
<td>4.6</td>
<td>35.8</td>
<td>20.6</td>
<td>140.8</td>
</tr>
<tr>
<td>Testosterone</td>
<td>27.2</td>
<td>201</td>
<td>121</td>
<td>848</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.7</td>
<td>0.1</td>
<td>0.2</td>
<td>ND</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>1.3</td>
<td>ND</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>16.4</td>
<td>ND</td>
<td>4.3</td>
<td>ND</td>
</tr>
<tr>
<td>Morphine</td>
<td>34.7</td>
<td>ND</td>
<td>19.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

Fig. 4. Disc gel electrophoresis of pure UDP-glucuronosyltransferase in the absence of sodium dodecyl sulfate. For details of electrophoresis, see "Experimental Procedures." The amount of protein applied to the gel was 8 µg. For activity assay, two unstained gels with the protein load of 8 µg were sliced into 2-mm slices. Each slice was homogenized in 0.2 ml of standard buffer (see "Experimental Procedures") containing 0.05% Zwittergent 3-12 and assayed for UDP-glucuronosyltransferase activity toward chenodeoxycholic acid (○) and testosterone (○) as described under "Experimental Procedures."

TABLE III

<table>
<thead>
<tr>
<th>Sugar nucleotide</th>
<th>Conjugation of</th>
<th>Effect of sugar nucleotides on glucuronidation of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cheno-deoxycholic acid</td>
<td>Testosterone</td>
</tr>
<tr>
<td></td>
<td>units/mg of protein</td>
<td>% of control</td>
</tr>
<tr>
<td>UDP-glucuronic acid, 3 mM</td>
<td>112</td>
<td>920</td>
</tr>
<tr>
<td>UDP-glucuronic acid, 9 mM</td>
<td>160</td>
<td>989</td>
</tr>
<tr>
<td>UDP-galacturonic acid, 9 mM</td>
<td>15</td>
<td>196</td>
</tr>
<tr>
<td>UDP-xylene, 9 mM</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UDP-glucose, 9 mM</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UDP-galactose, 9 mM</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UDP-N-acetylgalactosamine, 9 mM</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CDP-glucose, 9 mM</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Control, enzyme activity in the presence of 3 mM UDP-glucuronic acid without the addition of other sugar nucleotides. The effect of sugar nucleotides on bile acid and testosterone glucuronidation was estimated in the presence of 3 mM UDP-glucuronic acid. For the characterization of chenodeoxycholic acid galacturonide, assays of enzyme activity and definition of units, see "Experimental Procedures."

* ND, not detectable.
If the conjugation of chenodeoxycholic acid and testosterone with glucuronic acid and galacturonic acid had been due to catalysis by two different enzymes, a UDP-glucuronosyltransferase and a UDP-galacturonosyltransferase, the reaction rate in the presence of both co-substrates should have been the sum of separate velocities according to the mixed substrate phenomenon (36). The studies on the sugar nucleotide substrate specificity of the purified UDP-glucuronosyltransferase suggest that the affinity of the sugar nucleotide to the enzyme depends not only on the nature of the nucleotide moiety but also on the nature of the sugar moiety.

**Kinetic Studies**—Double reciprocal plots of the initial rates of enzyme activity as a function of varying concentrations of chenodeoxycholic acid at several fixed concentrations of UDP-glucuronic acid yielded substrate inhibition curves as shown in Fig. 5. The apparent $K_m$ for chenodeoxycholic acid was calculated from the intersection of the extrapolated lines (Fig. 5) to be 0.054 mM. A similar substrate inhibition kinetics was obtained for chenodeoxycholic acid when microsomal suspensions instead of the purified enzyme were used as enzyme source. With testosterone as the variable substrate, no substrate inhibition kinetics was obtained, and the data when plotted as shown in Fig. 6 yielded straight lines. The apparent $K_m$ for testosterone was calculated from the intersection of the straight lines to be 0.063 mM. The inhibition of enzyme activity observed at concentrations of chenodeoxycholic acid greater than about 0.1 mM (Fig. 5) might be due to the detergent effect of the bile acid. The kinetic studies indicate that according to Cleland (37) a sequential rather than a ping-pong mechanism applies to UDP-glucuronosyltransferase-catalyzed synthesis of chenodeoxycholic acid glucuronide and testosterone glucuronide. More information about the reaction mechanism is expected when product inhibition studies become available. A sequential-type of reaction mechanism has also been proposed for the enzymatic glucuronidation of estrone (38) and bilirubin (39).

**Other Properties**—Measurement of carbohydrate with the periodic acid-Schiff-staining procedure for glycoproteins (27) yielded less than 0.4 mol of hexose equivalents/mol of enzyme.

Since the pure UDP-glucuronosyltransferase did not appear to be associated with phospholipids, the effect of phospholipids on enzyme activity was investigated. No significant effect on chenodeoxycholic acid glucuronidation or testosterone glucuronidation was observed when dispersions of egg yolk L-a-phosphatidylcholine, L-a-lysophosphatidylcholine, or L-a-phosphatidylethanolamine were added in a lipid-to-protein ratio of 1:1, 2:1, or 5:1 (w/w) to the purified enzyme containing 2 mM chenodeoxycholic acid. The lack of dependency of enzyme activity on phospholipid could be due to the presence of chenodeoxycholic acid acting as phospholipid substitute since after removal of this detergent by treatment with Bio-Beads SM-2 (40) enzyme activity was lost by about 70%. Reconstitution of enzyme activity toward chenodeoxycholic acid and testosterone to about the value obtained before removal of the detergent was observed with L-a-phosphatidylcholine (lipid/protein = 4:1, w/w), L-a-lysophosphatidylcholine (lipid/protein = 1:1, w/w), or L-a-phosphatidylethanolamine (lipid/protein = 2:1, w/w). These results suggest that, in the absence of the stabilizing detergent chenodeoxycholic acid, the purified UDP-glucuronosyltransferase is a phospholipid-requiring enzyme. Studies of other authors on UDP-glucuronosyltransferase activities toward bilirubin (13, 41), estrone (11, 42), or 4-nitrophenol (29, 42) showed that these enzymes were nearly completely inactive in the dephospholipid form and efficient reactivation was achieved by the addition of phospholipids.

**DISCUSSION**

The present paper describes the isolation and characterization of UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone from rat liver microsomes. The following results show that the bile acid UDP-glucuronosyltransferase is identical with testosterone UDP-glucuronosyltransferase. (a) The ratio of chenodeoxycholic acid glucuronidation to testosterone glucuronidation was constant throughout the purification (Table I). (b) The isolated enzyme showed the same sugar nucleotide substrate specificity toward chenodeoxycholic acid and testosterone, catalyzing glucuronic acid and galacturonic acid conjugation of both aglycone substrates (Table III), whereas the UDP-glucuronosyltransferase activity toward 4-nitrophenol isolated by Burchell did not
react with UDP-galacturonic acid as co-substrate (10). (c) In disc gel electrophoresis in the presence of sodium dodecyl sulfate and polyacrylamide gradient gel electrophoresis, the purified protein exhibited a single band (Fig. 2). (d) In disc gel electrophoresis in the absence of sodium dodecyl sulfate, the purified enzyme yielded two main Coomassie blue-stained bands which coincided with the activities toward testosterone and chenodeoxycholic acid in extracted gel slices (Fig. 4). Since in polyacrylamide gradient gel electrophoresis (Fig. 2c) and gel filtration (Fig. 3) the purified enzyme exhibited size homogeneity, the bands observed in disc gel electrophoresis in the absence of sodium dodecyl sulfate (Fig. 4) are probably due to charge heterogeneity which was also observed for the purified UDP-glucuronosyltransferase activities toward 4-nitrophenol (8) and estrone (11). These enzymes (8, 11) exhibited a single band in sodium dodecyl sulfate gel electrophoresis and two peaks of transferase activity after polyacrylamide gel isoelectric focusing corresponding to two different isoelectric points of the purified enzymes.

The kinetic studies on the UDP-glucuronosyltransferase-catalyzed synthesis of chenodeoxycholic acid glucuronide and testosterone glucuronide as shown in Figs. 5 and 6 yielded a pattern of lines intersecting to the left of the vertical axis when data were graphed as double reciprocal plots. According to Cleland (37), initial velocity studies may be used to gather information on whether an enzymatic reaction has either a sequential or ping pong mechanism. The intersecting patterns observed in the present kinetic studies (Figs. 5 and 6) are characteristic of a sequential reaction in which all substrates must add to the enzyme before any products are released (37). The ping-pong mechanism may therefore be eliminated since its initial velocity pattern consists of parallel lines, when graphed as a double reciprocal plot (37).

The isolated enzyme described in the present paper catalyzes the glucuronidation of chenodeoxycholic acid and testosterone. Conjugating activities toward estrone, bilirubin, 4-nitrophenol, and morphine were not co-purified with the activity toward chenodeoxycholic acid and testosterone and were not detectable in the purified enzyme (Table II). Lack of activity toward estrone, bilirubin, 4-nitrophenol, and morphine of the purified enzyme could be assumed to be due to lipid depletion of the enzyme, since it has been described that UDP-glucuronosyltransferase activities toward bilirubin (13, 41), 4-nitrophenol (29, 42), estrone (11, 42), and testosterone (43) were almost completely inactive in the lipid-depleted form. However, although the pure enzyme described in the present paper was obtained in a phospholipid-depleted form, it was active toward chenodeoxycholic acid and testosterone in the presence as well as in the absence of phospholipids when 2 mM chenodeoxycholic acid was added to enzyme preparations, suggesting that this detergent might act as phospholipid substitute. After removal of the stabilizing detergent, enzyme activity toward chenodeoxycholic acid and testosterone could be reconstituted to the value observed in the presence of the detergent by the addition of phospholipids. Furthermore, activities toward estrone, bilirubin, 4-nitrophenol, and morphine were not detectable in the purified enzyme, even if enzyme preparations in the presence of chenodeoxycholic acid or after removal of chenodeoxycholic acid were supplemented with phosphatidylcholine, whereas other authors described efficient reconstitution of conjugating activities of delipidated enzymes toward estrone (11, 42), bilirubin (13, 41), and 4-nitrophenol (29, 42) with phosphatidylcholine. These results suggest that lack of activity toward bilirubin, estrone, 4-nitrophenol, and morphine of the purified enzyme cannot be explained by lipid depletion of the enzyme, but might be due to the existence of different substrate specific forms of UDP-glucuronosyltransferases.

UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone was resolved into two separate peaks on DEAE-Sepharose (Fig. 1); each peak contained conjugating activity toward both aglycone substrates. The following results show that the conjugating activity toward chenodeoxycholic acid and testosterone present in peak II after DEAE-Sepharose chromatography appears to be identical with the enzyme present in peak I of the DEAE-Sepharose chromatography. (a) UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone can be shifted from the protein fraction of peak II to the protein fraction of peak I by increasing the concentration of the solubilizing agent (Fig. 1), suggesting that UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone may tend to form aggregates with components of peak II (Table II). (b) After purification of the protein fraction of peak II by affinity chromatography and gel filtration as described for peak I (see "Experimental Procedures"), the final enzyme preparation exhibited the same characteristics as the enzyme obtained from peak I in regard to aglycone substrate specificity, sugar nucleotide substrate specificity, and mobilities in sodium dodecyl sulfate gel electrophoresis, polyacrylamide gradient slab gel electrophoresis, and disc gel electrophoresis in the absence of sodium dodecyl sulfate.

The separation of the UDP-glucuronosyltransferase activity toward testosterone from that toward 4-nitrophenol by DEAE-Sepharose chromatography and the lack of conjugating activity toward 4-nitrophenol of the pure enzyme as described in the present paper (Table II) are in contrast to findings of Weatherill and Burchell who reported that purified UDP-glucuronosyltransferase activity toward 4-nitrophenol exhibited activity toward testosterone (43). Although the ratio of 4-nitrophenol glucuronidation to testosterone glucuronidation calculated from the purification data given by Weatherill and Burchell (43) was not constant throughout the purification, these authors proposed that testosterone and 4-nitrophenol may be glucuronidated by a single form of UDP-glucuronosyltransferase since the enzyme preparation yielded a single band in sodium dodecyl sulfate gel electrophoresis (43). However, different forms of UDP-glucuronosyltransferases might not be separated by sodium dodecyl sulfate gel electrophoresis since the subunit molecular weights of purified rat or rabbit liver UDP-glucuronosyltransferases appear to be very similar, with values of 56,000 determined for the activity toward morphee (7), 54,900 (7), 57,000 (9-11), or 59,000 (8) for the activity toward 4-nitrophenol, 57,000 for the activity toward estrone (11), and 54,000 for the enzyme described in the present paper. Therefore, the purified UDP-glucuronosyltransferase activity toward 4-nitrophenol described by Weatherill and Burchell (43) may be contaminated with testosterone UDP-glucuronosyltransferase.

In the present paper, it is shown that activity of a purified UDP-glucuronosyltransferase can be recovered from extracted gel slices after disc gel electrophoresis in the absence of sodium dodecyl sulfate. Analysis of the purified protein by this method was used to establish the existence of a single form of UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone that does not glucuronidate morpheine, 4-nitrophenol, bilirubin, and estrone, even if the preparation is supplemented with phospholipids.

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