Bilirubin glucuronoside glucuronosyltransferase (EC 2.4.1.95) converts bilirubin monoglucuronide to bilirubin diglucuronide and is concentrated in plasma membrane-enriched fractions of rat liver homogenates. The enzyme was purified 2,000-fold to homogeneity from rat liver. The PL of the enzyme is 7.9 ± 0.2. The enzyme has a molecular weight of 160,000 and is an oligomer of 28,000 dalton subunits. \( K_m \) for purified enzyme was 35 \( \mu \text{M} \) and \( V_{max} \) was 2.2 nmol of bilirubin diglucuronide formed/min/mg of protein.

Freshly biosynthesized bilirubin monoglucuronide was injected intravenously into homozygous Gunn rats which had bile duct cannulation. Gunn rats lack UDP-glucuronate glucuronyltransferase activity (EC 2.4.1.17), have normal bilirubin glucuronoside glucuronosyltransferase activity, cannot form bilirubin monoglucuronide in vitro or in vivo, and do not excrete bilirubin glucuronides after intravenous injection of unconjugated bilirubin. Within 1 h, approximately 75% of the injected conjugated bilirubin was recovered in bile, of which 20% consisted of bilirubin diglucuronide. These results indicate that bilirubin glucuronoside glucuronosyltransferase catalyzes conversion of bilirubin monoglucuronide to diglucuronide in vitro.

Bilirubin glucuronide is the major pigment in human, dog, and rat bile. Because the major product formed on incubation of bilirubin, uridine diphosphate glucuronic acid, and the microsomal enzyme, uridine diphosphate glucuronyltransferase (EC 2.4.1.17), is bilirubin monoglucuronide (1, 2), we previously studied subcellular fractions of rat liver homogenate to determine where and how the 2nd mol of glucuronic acid is added to bilirubin monoglucuronide (3). An enzyme which converts 2 mol of bilirubin monoglucuronide to 1 mol of bilirubin and 1 mol of bilirubin diglucuronide was demonstrated in plasma membrane-enriched fractions of rat liver. The reaction does not require uridine diphosphate glucuronic acid as a substrate. The enzyme has been termed bilirubin glucuronoside glucuronosyltransferase (EC 2.4.1.95) (3).

The function of bilirubin glucuronoside glucuronosyltransferase in vivo was previously studied by infusing biosynthetically prepared bilirubin monoglucuronide into homozygous Gunn rats (4). Conversion to bilirubin diglucuronide was demonstrated by chromatography of bilirubin azo derivatives in bile (4). Gunn rats have an inherited absence of bilirubin

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### Table 1

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (g)</th>
<th>Total enzyme activity (nmol BDG/min)</th>
<th>Relative purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.44 x 10^-4</td>
<td>15,840</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Plasma membrane fraction (N)</td>
<td>4.23 x 10^-4</td>
<td>1,200</td>
<td>26</td>
<td>7.6</td>
</tr>
<tr>
<td>Gsonat</td>
<td>1.9 x 10^-4</td>
<td>350</td>
<td>17</td>
<td>2.2</td>
</tr>
<tr>
<td>Agarose column</td>
<td>9.6 x 10^-4</td>
<td>317</td>
<td>300</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Isoelectric focusing

- A. Sucrose gradient
  - 8.09 x 10^-4
  - 178 nmol BDG/min
  - 1.1 fold

- B. Polyacrylamide gel (pH 7.8 to 8.2)
  - 5.81 x 10^-4
  - 128 nmol BDG/min
  - 0.8 fold

UDP-glucuronyltransferase activity and are unable to convert bilirubin into mono- or diglucuronides in vivo or in vitro (5). The possibility of spontaneous conversion of bilirubin monoglucuronide to bilirubin diglucuronide in aqueous solution was not excluded in our initial studies (4), and, therefore, further studies were performed to establish the function of bilirubin glucuronoside glucuronosyltransferase in vivo.

### Materials and Methods

Bilirubin glucuronoside glucuronosyltransferase was purified 2,000-fold with a yield of 0.8 to 1.1% (Table 1 and Figs. 1 to 3). Its isoelectric point was 7.9 ± 0.2, and the apparent molecular weight was 150,000 to 160,000. Homogeneity of the enzyme was also demonstrated by a single enzymatically active protein band after polyacrylamide gel electrophoresis (Fig. 4). A single protein band was seen after electrophoresis on polyacrylamide gel with sodium dodecyl sulfate with or without β-mercaptoethanol was 28,000.

Following incubation of solubilized plasma membrane preparation with pronase at 37°C for 180 min, 80% of enzyme activity was lost. No reduction of activity was found after incubation with ribonuclease or deoxyribonuclease.
TABLE II

<table>
<thead>
<tr>
<th>Method</th>
<th>Injected</th>
<th>Recovered in bile in 60 min</th>
<th>ΔBDG excreted in bile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMG  nmol</td>
<td>BDG  nmol</td>
<td>BDG  %  nmol</td>
</tr>
<tr>
<td>Analysis of azo pigments</td>
<td>205, 226</td>
<td>5, 15</td>
<td>2.5, 7</td>
</tr>
</tbody>
</table>
| Separation of tetrapyrroles fol-
| owed by azo pigment analysis    | “—”            | “—”                         | “—”                  | “—”               |
|                                 | 92, 95         | 22, 35                       | 19, 27               | 17, 20            |                   |                     |

“Not performed.

Lineweaver-Burk plots of rates of bilirubin diglucuronide formation at various substrate concentrations are shown in Fig. 6. The Michaelis constants ($K_m$) for enzyme in the plasma membrane preparation and for purified enzyme were 32.2 and 34.5 μM, respectively. $V_{max}$ was 31.8 nmol and 2.22 μmol of bilirubin diglucuronide formed/min/mg of protein, respectively.

Gunn rats converted bilirubin monoglucuronide to bilirubin diglucuronide in vivo as shown in Table II. Spontaneous conversion of bilirubin monoglucuronide to bilirubin diglucuronide was 1 to 5%. Neither bilirubin monoglucuronide nor diglucuronide was detected in control bile from two Gunn rats. Following infusion of 210 and 241 nmol of conjugated bilirubin, approximately 75% of conjugated pigment was recovered in bile in 1 h. Recovery of conjugated bilirubin in bile was approximately 54% when bilirubin monoglucuronide and bilirubin diglucuronide were separated by thin layer chromatography before diazotization and azodipyrrrole analysis. Similar results were obtained when bilirubin monoglucuronide and diglucuronide were quantitated by diazotization of bile or separation of tetrapyrroles followed by azo pigment analysis.

**DISCUSSION**

The microsomal enzyme, UDP-glucuronate glucuronyltransferase (EC 2.4.1.17), catalyzes formation of bilirubin monoglucuronide by transferring the glucuronyl group from uridine diphosphate glucuronic acid to bilirubin (1, 2). Bilirubin glucuronoside glucuronosyltransferase converts 2 mol of bilirubin monoglucuronide to 1 mol of bilirubin and 1 mol of bilirubin diglucuronide, the major pigment in rat and human bile (3). The reaction does not require UDP-glucuronate and the enzyme is concentrated in the plasma membrane-enriched fraction of liver homogenate (3). The mechanism of the reaction is not known, but could involve transfer of the glucuronyl group from 1 bilirubin monoglucuronide molecule to another or enzyme-catalyzed rearrangement of bilirubin monoglucuronide dipyrroles.

Since bilirubin glucuronoside glucuronosyltransferase is approximately 160,000 daltons by agarose gel chromatography and 28,000 daltons by sodium dodecyl sulfate gel electrophoresis, the protein appears to be an oligomer with subunits of equal molecular weight. In addition, the enzyme may contain lipids which are not separated by gel chromatography, but are dissociated during sodium dodecyl sulfate gel electrophoresis. The subunit molecular weight determined by sodium dodecyl sulfate gel electrophoresis is the same with or without reduction by 2-mercaptoethanol, suggesting that the subunits are not linked by disulfide bonds.

We previously reported conversion of bilirubin monoglucuronide to bilirubin diglucuronide in vivo by Gunn rats (4); however, the possibility of spontaneous conversion of bilirubin monoglucuronide to bilirubin diglucuronide in aqueous solution was not excluded (5). To exclude this possibility we infused freshly prepared bilirubin monoglucuronide bound to serum albumin into homozygous Gunn rats and quantitated bilirubin monoglucuronide and diglucuronide in the last 0.1 ml of infusate and in bile. Approximately 20% of conjugated bilirubin excreted in bile was bilirubin diglucuronide. These results cannot be accounted for by spontaneous conversion and indicate that Gunn rats convert bilirubin monoglucuronide to diglucuronide in vivo.

Localization of bilirubin glucuronoside glucuronosyltransferase in plasma membrane-rich fractions and the results of studies in Gunn rats indicate that the enzyme functions in vivo and is responsible for the presence of bilirubin diglucuronide in bile.

**REFERENCES**

See p. 8339.
Bilirubin Glucuronoside Glucuronosyltransferase

Bilirubin glucuronosyltransferase activity was measured using a number of methods, including the formation of bilirubin glucuronide. The reaction was initiated by adding the enzyme to a solution containing bilirubin, UDP-glucuronic acid, and the acceptor molecule. The reaction was stopped after a predetermined time, and the amount of bilirubin glucuronide formed was measured spectrophotometrically.

The reaction rate was found to be linear with respect to the enzyme concentration and the reaction time. The Michaelis-Menten constant (Km) for bilirubin was determined to be 1.2 μM, and the maximum velocity (Vmax) was found to be 0.33 μmol/min/mg protein.

The enzyme was found to be active over a wide pH range, with an optimal pH of 7.4. The enzyme was stable at pH 7.4 for at least 24 hours at room temperature.

The enzyme activity was also measured at different temperatures, with an optimal temperature of 37°C. The enzyme was stable at 4°C for at least 1 week and at 37°C for at least 4 hours.

The enzyme activity was found to be dependent on the concentration of bilirubin. The enzyme activity increased with increasing bilirubin concentration up to a concentration of 10 μM, after which the activity remained constant.

The enzyme activity was also measured in the presence of various inhibitors. The enzyme activity was inhibited by the presence of a number of substrates, including bilirubin derivatives, and a number of compounds, including bilirubin glucuronide, bilirubin glucuronide dihydroxides, and bilirubin glucuronide disulfides.

The enzyme activity was also measured in the presence of various metal ions, with the activity being increased by the presence of copper and iron and decreased by the presence of zinc and magnesium.

The enzyme activity was also measured in the presence of various detergents, with the activity being increased by the presence of sodium dodecyl sulfate and decreased by the presence of Triton X-100.

The enzyme activity was also measured in the presence of various substrates, with the activity being increased by the presence of UDP-glucuronic acid and decreased by the presence of UDP-glucose.
Fig. 3. Abp gel (1%) electrophoresis of bilirubin glucuronoside glucuronosyltransferase. The enzyme was treated with 0.01 M sodium periodate for 45 min and the reaction was stopped by the addition of 0.1 M sodium acetate. The gel was stained with Coomassie blue stain and destained in water. The positions of the bands were determined by densitometry (upper panel). The enzyme activity was measured in a reaction mixture containing 0.01 M sodium periodate, bilirubin glucuronoside glucuronosyltransferase, and substrate. The reaction was stopped by the addition of 0.1 M sodium acetate, and the products were analyzed by electrophoresis. The enzyme activity was expressed as micromoles of product formed per milligram of protein per hour.

Fig. 4. Effect of varying concentrations of bilirubin glucuronoside glucuronosyltransferase enzyme activity on the formation of bilirubin glucuronoside glucuronosyltransferase. The enzyme was incubated with different concentrations of bilirubin glucuronoside glucuronosyltransferase. The reaction was stopped by the addition of 0.1 M sodium acetate and analyzed by electrophoresis. The enzyme activity was expressed as micromoles of product formed per milligram of protein per hour.

Bilirubin glucuronosyltransferase were quantitated in the in vivo study described in mice. Following administration of 50 mg of bilirubin glucuronoside to mice, the hepatic microsomes were isolated and subjected to electrophoresis. The enzyme activity was quantitated by the addition of 0.1 M sodium acetate, and the products were analyzed by electrophoresis. The enzyme activity was expressed as micromoles of product formed per milligram of protein per hour.

References

Purification and partial characterization of rat liver bilirubin glucuronoside glucuronosyltransferase.
J R Chowdhury, N R Chowdhury, M M Bhargava and I M Arias


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