The Hepatic Microsomal Formation of Bilirubin Diglucuronide*

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Although the formation of bilirubin monoglucuronide by hepatic microsomes has been easily demonstrable, that of bilirubin diglucuronide, the principal conjugate of bile, has been more difficult. Therefore, an examination of the uridine diphosphate glucuronate-dependent microsomal formation of these two conjugates has been made utilizing a high performance liquid chromatographic method which quantitates the isomeric forms of the products. Initial studies indicated that at high starting bilirubin concentrations, only bilirubin monogluconide was formed; whereas at lower concentrations (≤ 15 μM), bilirubin diglucuronide could be formed efficiently, but only under very specific conditions. Untreated microsomes and microsomes treated with Triton X-100 did not differ; each formed monoglucuronide efficiently, yet formed diglucuronide poorly. Digitonin or UDP-N-acetylglucosamine pretreatment, in contrast, was found to facilitate bilirubin diglucuronide formation, the former much more than the latter. The activity of mannose 6-phosphatase, an enzyme located on the inner surface of the microsomal vesicles, did not correlate well with the bilirubin diglucuronide formation. Time course studies with digitonin and UDP-N-acetylglucosamine indicated a precursor-product relation between bilirubin monoglucuronide and bilirubin diglucuronide, and product isomer composition studies indicated that the bilirubin tetrapyrroles were stable (no random dipyrrole exchange had occurred). Temperature studies with the digitonin-treated preparation demonstrated an increase in monoglucuronide-forming activity over the 0–25 °C range, whereas diglucuronide formation increased dramatically over the range from 25 to 35 °C. The results indicate that microsomal diglucuronide-forming activity differs characteristically from monoglucuronide-forming activity, and that it is intensely sensitive to the manipulation of its microsomal membrane environment.

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The hepatic UDP-glucuronyltransferases (EC 2.4.1.17) consist of a series of microsomal membrane-bound enzymes (1, 2) whose properties are modulated by their immediate environment (3–10). In its reactions, this group of enzymes catalyzes the transfer of D-glucuronic acid from UDP-D-glucuronic acid to the substrate with the inversion of the C-1 atom of the sugar to yield the β-glucuronide (1). One of the known endogenous substrates of this system is bilirubin, the end product of heme catabolism (11). The major polar compound of bilirubin detected in the bile of humans, dogs, rats, or cats is not bilirubin monoglucuronide but rather bilirubin diglucuronide (12–16). The bilirubin glucuronidation mechanism is thus more complex than that for most other substrates. It would appear that either the microsomal glucuronotransferase system has the capacity to add 2 mol of glucuronide successively to 1 mol of bilirubin or that two successive enzyme reactions, potentially located in different sites, are involved in the formation of bilirubin diglucuronide. Earlier studies appeared to support the latter concept. It was demonstrated by Jansen et al. (17) and by Chowdhury and colleagues (18) that hepatic preparations enriched in plasma membranes and bile canalicular converted 2 mol of bilirubin monoglucuronide to 1 mol of bilirubin diglucuronide and 1 mol of bilirubin by what appeared to be a transglucuronidation reaction, in the absence of uridine diphosphate glucurionate.

The idea that the second glucuronidation occurs at the cell membrane or canalicular level in vivo has recently been challenged, however. It has been demonstrated by ourselves (19) and by Blanckaert et al. (20) that digitonin-treated hepatic microsomal preparations have the capacity to form bilirubin diglucuronide in the presence of uridine diphosphate glucuronate, if physiological levels of bilirubin (5–15 μM) are utilized in the assay system. Moreover, it was also possible to demonstrate that at the higher concentrations of bilirubin (160–340 μM) utilized in earlier studies, bilirubin monoglucuronide was the only product formed (21–23). Systematic in vitro studies indicated that the relative proportions of bilirubin monoglucuronide and bilirubin diglucuronide formed by these microsomal preparations depended on the concentration of bilirubin utilized in the assay system (19), the proportion of bilirubin diglucuronide formed decreasing with increasing bilirubin concentration. Sieg and colleagues (24) recently reinvestigated the formation of bilirubin diglucuronide by plasma membranes from rat liver. They demonstrated that the conversion of bilirubin monoglucuronide to bilirubin diglucuronide by these preparations was independent of the amount of membrane present, that it was not prevented by heat denaturation, that it was characterized by the formation of the nonphysiological IIIα and XIIIα isomers in proportions indicating random dipyrrole exchange, and that it was prevented by small proportions of the free radical scavenger ascorbic acid. They concluded that this proposed second site reaction was a nonenzymic in vitro phenomenon, and that it does not represent a major in vivo metabolic pathway.

These findings reinforce the need to further characterize the UDP-glucuronate-dependent microsomal mechanism for the formation of both the mono- and diconjugates of bilirubin, since it now appears likely that this mechanism is responsible for the in vivo production of bilirubin diglucuronide. The
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Mechanism Underlying the Addition of the Second Glucuronide to Bilirubin

The addition of the second glucuronide to bilirubin has been particularly difficult to unmask. Studies carried out with other hepatic UDP-glucuronosyltransferase systems indicate that the action of these membrane-associated enzymes can usually be enhanced if one can modify the structural organization of the membrane in an appropriate fashion (3-10).

The focus of the present work is to characterize in detail the microsomal systems catalyzing the formation of bilirubin diglucuronide. As part of this, the effects of a group of agents expected to affect membrane organization have been explored. The data are presented below.

EXPERIMENTAL PROCEDURES

Preparation and Treatment of Hepatic Microsomal Fractions

Male Sprague-Dawley rats in the fed state were utilized in this study. Under light anesthesia, the abdomen was opened, the portal vein cannulated, and the liver perfused with 20 ml of medium containing 0.25M sucrose and 1mM EDTA. The liver was then removed and homogenized, and the microsomal fraction was isolated by differential centrifugation (22). The microsomal pellet was resuspended in the isolation medium and used in the enzyme assay procedures. The activity of the fraction was examined both in the untreated state and after the treatments outlined below (in the case of the other glucuronosyltransférases, these approaches have usually been found to "activate" the preparation).

Isolated microsomes were resuspended in isolation media containing varying concentrations of digitonin (0.05-0.9 mg/mg of microsomal protein) and at 4°C for 60 min. Triton X-100—The microsomal pellet was resuspended in isolation media containing various final concentrations of Triton X-100 (0.125, 0.25, 0.50, and 0.625%, v/v) and left at 4°C for 1 h. In some experiments, Triton X-100 was removed from the suspension by adding Bio-Beads SM-2 (styrényldivinylbenzene copolymer, Bio-Rad). The preparation of the beads and the procedure followed was as described by Holloway and Katz (29). The samples were mixed for 2 h in the dark at 4°C. The supernatant was then removed from the beads and centrifuged at 105,000 G for 1 h. Aliquots of the supernatant were then analyzed for UDP-glucuronate-dependent glucuronosyltransferase activity with bilirubin as substrate.

UDP-N-acetylglucosamine—Microsomal pellets were suspended in isolation media (10 mg of microsomal protein/ml) which contained varying concentrations of UDP-N-acetylglucosamine (0.5-4.4 mg/mg of microsomal protein).

The UDP-glucuronate-dependent Glucuronosyltransferase Assay

At zero time, the reaction mixture contained untreated or perturbed microsomes (1.3 mg of microsomal protein/ml), bilirubin (5.7-20 mM), 0.05 M triethanolamine buffer, pH 7.8, 5.7 mM UDP-glucuronate, and 8.3 mM MgCl₂ (this provides divalent cation substantially in excess of the 1 mM EDTA in the microsomal isolation medium). The bilirubin was dissolved in 0.05N NaOH, and appropriate aliquots were added to triethanolamine buffer, pH 7.8. In some of the experiments, glucuronol-1,4-lactone (90 mM), an inhibitor of β-glucuronidase, was added to the assay medium. The incubation times and the temperature at which the reactions were carried out were varied and are therefore specified in each table and in the legends of each figure. The reaction was stopped by immersing the samples in dry ice and acetone, and chloroform containing 10 mM tetraethyiammonium chloride was used to extract the bilirubin and its conjugates from the reaction mixture (19). The samples were mixed on a Vortex mixer and centrifuged. The supernatant was then analyzed for UDP-glucuronate-dependent glucuronosyltransferase activity with bilirubin as substrate.

RESULTS

The Effect of Varying the Concentrations and the Duration of the Pretreatment with the Microsome-perturbing Agents on the Total Bilirubin Glucuronosyltransferase Activity—In a first set of experiments, the microsomal preparations were pretreated with varying concentrations of Triton X-100, digitonin, and UDP-N-acetylglucosamine for different periods of time and the formation of conjugates at the bilirubin concentration of 12.5 μM was measured. Varying the concentration of Triton X-100 from 0.025 to 0.25% (v/v) did not markedly affect the total activity of the system (that is, the conversion of bilirubin to conjugated species, over the incubation period), and removal of the Triton X-100 from the preparation prior to the reaction with bilirubin did not alter either the activity or the pattern of products formed. Exposure of the microsomal pellets to varying concentrations of Triton X-100 for periods longer than 60 min produced no enhancement of the activity of the enzyme system, nor were the relative proportions of the products altered. Varying the concentration of the digitonin from 0.05 to 1.0 mg/ml of microsomal protein did not markedly affect the total amount of bilirubin conjugated. Similar studies were carried out utilizing UDP-N-acetylglucosamine (0.5-4.4 mg/ml of microsomal protein), and again no significant differences were noted in the total amount of bilirubin converted. Therefore, in the next experiment, the following concentrations of perturbing agents were used: Triton X-100, 0.1% (v/v); UDP-N-acetylglucosamine, 0.5 mg/ml.

Analysis of Bile Pigments

A recently developed and validated high performance liquid chromatographic assay (29) was utilized to measure the levels of bilirubin and its conjugates in the extracts. The procedure offers the advantages of being both direct and relatively quick. The tetrahydroproline products themselves are detected and quantitated (this contrasts with classical diazo methodology, in which diazotization is carried out under reaction conditions designed to distinguish bilirubin from its conjugates and in which the resulting azodipyrroles are then identified and measured) (24), and the procedure is completed in a fraction of an hour rather than the 2 days needed for thin layer chromatographic separation of the somewhat labile bilirubin tetrapyrroles (30).

The chromatographic procedure is, briefly, as follows. The assays were carried out on a Hewlett-Packard 1084 high performance liquid chromatograph with a variable wavelength detector. Separation of the bile pigments was achieved with an oven temperature at 57°C using a Hewlett-Packard reverse phase RP-18 column in series, each 200 mm in length and 4.6 mm in diameter, with a particle size of 10 μm. The variable wavelength detection was set at 440 nm. The bile pigments were separated by the system in the following manner. The flow rate of the mobile phase, which consisted of 6 mM heptane-sulfonic acid in 0.1 M phosphate buffer, pH 4.8 (solvent A), and cetoditritile (solvent B), was maintained at 2.0 ml/min for 22 min and then abruptly changed to 3.5 ml/min. At zero time, the proportion of solvent A was arranged to be 75% (v/v) and that of solvent B to be 25% (v/v). During the first 20 min of the run, solvent B was increased linearly from 25% (v/v) to 45% (v/v) and then in the next 7 min, from 45% (v/v) to 80% (v/v) of the mobile phase, following which this ratio of solvents was maintained for the next 7 min, that being the time required to complete the assay. The standard utilized to calibrate the assay was as follows. Bilirubin obtained from Fisher (Montreal) was used both for calibration and assay procedures. After proofing by thin layer chromatography (30) that it was not contaminated with other bile pigments, it was used without further purification. Human, dog, and rat bile were used as sources for the conjugates of bilirubin; these were purified as outlined previously (19).

Mannose 6-Phosphatase

Activity (27) was determined in the microsomal preparations treated with various agents using mannose 6-phosphate as substrate. The reaction medium contained 0.01 M mannose 6-phosphate, 1 mM EDTA, 13 mM histidine, 50 mM Tris-HCl buffer, pH 6.5, and varying concentrations of microsomal protein (100-500 μg) in a total volume of 0.5 ml. Samples were incubated at 37°C for 10 or 30 min. The reaction was stopped by adding 2.5 ml of ice-cold 8% trichloroacetic acid solution, and, following centrifugation of the precipitate, inorganic phosphate was determined on the supernatant (28). Results are expressed as nanomoles of P; formed min⁻¹ mg⁻¹.
mg of microsomal protein; and digitonin, 0.35 mg/mg of microsomal protein.

The Effect of Initial Bilirubin Concentrations on the Total Bilirubin Glucurononyltransferase Activity of Microsomal Preparations Treated with Different Perturbing Agents—The total activity of the bilirubin glucurononyltransferase systems in microsomal preparations treated with various agents known to alter microsomal membranes is presented in Fig. 1. The microsomal preparations were treated with digitonin (0.35 mg of digitonin/mg of microsomal protein), UDP-N-acetylglucosamine (0.5 mg/mg of microsomal protein), or Triton X-100 (0.1%). The concentration of bilirubin used varied from 5.7 to 30 μM. At the lowest concentration of bilirubin used (5.7 μM), no significant differences were detected in the rate of bilirubin conversion to polar compounds by the microsomal preparations treated with the various agents, and, as the concentration of bilirubin was increased, the activity of the UDP-glucuronyltransferase system increased in all cases, at relatively the same rate. A peak in glucuronide formation occurred. The results of a set of studies at various levels of initial bilirubin concentration at which bilirubin diglucuronide was formed relatively more efficiently.

The Effect of Initial Bilirubin Concentrations on the Conjugates Formed—Experiments were carried out to define the level of initial bilirubin concentration at which bilirubin diglucuronide formation occurred. The results of a set of studies in which microsomes were pretreated with digitonin (0.35 mg/mg of microsomal protein) are illustrated in the top of Fig. 2. Bilirubin monoglucuronide is the major product formed by rat hepatic microsomal preparations at high concentrations of bilirubin (50–60 μM). As the initial concentration of bilirubin is lowered, however, the proportion of bilirubin monoglucuronide decreases and that of bilirubin diglucuronide increases until, at the lowest initial concentration, the latter becomes the major product. At an initial bilirubin concentration of 12.5 μM, approximately equal amounts of bilirubin diglucuronide and bilirubin monoglucuronide were formed. This level of bilirubin was hereafter used in the assay system unless stated otherwise; it was felt that, with this initial concentration, either stimulation or inhibition of the mechanism resulting in the formation of bilirubin diglucuronide would be evident. The conjugate data correspond in form to those previously found by use of thin layer chromatographic analysis of the reaction products (19, 20).

The effect of the initial bilirubin concentration on the microsomal bilirubin-conjugating activity. Top, assays were carried out in a microsomal preparation pretreated with digitonin (0.35 mg/mg of microsomal protein); incubations were carried out for 30 min at 37 °C. The filled triangles represent the percentage of which was bilirubin conjugate in the sample at 30 min which was bilirubin diglucuronide. Middle, the conversion of bilirubin to conjugates at 30 min by microsomes pretreated with digitonin. The total conjugates are represented by the filled squares; bilirubin monoglucuronide, by filled triangles; and bilirubin diglucuronide, by filled circles. Bottom, the conversion of bilirubin to conjugates at 30 min by microsomes pretreated with UDP-N-acetylglucosamine. The symbols are as in the middle of the figure.

![Fig. 1. Changes in total conjugate formed at 30 min as a function of initial bilirubin concentrations.](http://www.jbc.org/)

![Fig. 2. The effect of the initial bilirubin concentration on the microsomal bilirubin-conjugating activity. Top, assays were carried out in a microsomal preparation pretreated with digitonin (0.35 mg/mg of microsomal protein); incubations were carried out for 30 min at 37 °C. The filled triangles represent the percentage of which was bilirubin conjugate in the sample at 30 min which was bilirubin diglucuronide. Middle, the conversion of bilirubin to conjugates at 30 min by microsomes pretreated with digitonin. The total conjugates are represented by the filled squares; bilirubin monoglucuronide, by filled triangles; and bilirubin diglucuronide, by filled circles. Bottom, the conversion of bilirubin to conjugates at 30 min by microsomes pretreated with UDP-N-acetylglucosamine. The symbols are as in the middle of the figure.)
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Forming activity, in contrast, continued to increase over the whole of the concentration range explored.

Similar studies were carried out with microsomes treated with UDP-N-acetylglucosamine (0.5 mg/mg of microsomal protein) or Triton X-100. The data obtained by use of microsomes treated with UDP-N-acetylglucosamine are presented in the bottom of Fig. 2. An inhibiting effect of increasing concentrations of substrate on bilirubin diglucuronide formation is apparent. The total activity increased markedly (4.3-13.8 nmol of bilirubin converted per mg of microsomal protein) as the substrate levels increased from 5 to 25 μM. At low substrate concentrations (5 μM), bilirubin diglucuronide accounted for 28% of the total products formed. As the substrate level was increased, however, the total amount of bilirubin diglucuronide formed slowly decreased, and that of bilirubin monoglucuronide rapidly increased. When microsomes were treated with Triton X-100, the major product detected was bilirubin monoglucuronide; this response was not affected by the concentration of substrate.

A summary of the relative proportions of bilirubin monoglucuronide and bilirubin diglucuronide formed when the initial bilirubin concentration was 12.5 μM and the microsomes were treated by various agents is presented in Table I. Bilirubin monoglucuronide was the major conjugate formed when the assay was conducted on untreated microsomal preparations. It accounted for approximately 96% of the bilirubin conjugated (4% was converted to bilirubin diglucuronide). Triton X-100 did not alter this pattern. In contrast, after treatment of the microsomes with UDP-N-acetylglucosamine (0.5 mg/mg of microsomal protein), 25% of conjugate formed was bilirubin diglucuronide; and in the presence of digitonin (0.35 mg/mg of microsomal protein), 50% of the conjugate was bilirubin diglucuronide. These two substances selectively activated the mechanism underlying the addition of the second glucuronide to the bilirubin molecule.

The Effect of Varying the Concentrations of the Microsomal Perturbing Agents on the Conjugates Formed—As stated before, varying the concentration of digitonin from 0.05 to 1.0 mg/mg of microsomal protein did not markedly affect the total amount of bilirubin conjugated. On the other hand, the proportions of bilirubin monoglucuronide and diglucuronide formed were affected markedly (see Fig. 3). At digitonin levels of 0.05 mg/mg of microsomal protein, less than 5% of the total conjugates formed were accounted for by the formation of bilirubin diglucuronide. As the concentration of digitonin was increased, the proportion of the bilirubin converted to bilirubin diglucuronide increased until, at digitonin levels of 0.3-0.4 mg/mg of microsomal protein, 50% of the conjugates formed were accounted for by bilirubin diglucuronide. Increasing the concentration of digitonin even more suppressed the formation of bilirubin diglucuronide. At digitonin levels of 0.9 mg/mg of microsomal protein, diglucuronide accounted for only approximately one third of the bilirubin conjugates.

Treatment of the microsomal preparations with varying concentrations of digitonin for periods longer than 60 min did not markedly alter the observed pattern of response. A range of concentrations of added UDP-N-acetylglucosamine was also explored. Varying the concentration of this compound from 0.5 to 4.4 mg/mg of microsomal protein did not affect either the total conversion of bilirubin to conjugated bilirubin or the pattern of conjugates formed. Therefore, in subsequent experiments, 0.5 mg of UDP-N-acetylglucosamine/mg of microsomal protein was utilized. With Triton X-100, no set of concentrations or conditions (presence or removal) was found which would promote more than the minimal diglucuronide formation recorded above.

The effect of perturbing the microsomal membranes with both Triton X-100 and digitonin is summarized in Table II. The total glucuronide-forming activity was the same in microsomal preparations treated with Triton X-100 or digitonin, or with both agents. However, the nature of the products formed differed. As noted before, bilirubin monoglucuronide was the major product formed when the microsomes were treated with Triton X-100, while bilirubin diglucuronide accounted for 49% of the product formed when microsomes were treated with digitonin. When the microsomes were treated with both agents, the formation of bilirubin diglucuronide was abolished.

The Latency of Mannose 6-Phosphatase Activity—The effect of the various agents on the leakiness of vesicles formed by the microsomal membranes was assessed by measuring the activity of mannose 6-phosphatase, a membrane-bound enzyme considered to be located on the cisternal side of the microsomal membrane. The mannose 6-phosphatase activity of the microsomes changed with the various treatments (Fig. 4). The untreated and Triton X-100-treated microsomes exhibited the same low level of activity, and UDP-N-acetylglucosamine-treated microsomes, a minor increase in activity. The digitonin-treated microsomes showed a marked increase in activity, indicating that the enzyme was now more accessible to substrate, and this accessibility was maintained when the microsomes were pretreated with Triton X-100 or UDP-

![Graph](http://www.jbc.org/)

**TABLE I**

*Effect of various forms of pretreatment on the proportion of bilirubin diglucuronide formed by hepatic microsomes*

The initial bilirubin concentration was 12.5 μM. The values given are means ± S.D.

| Treatment of microsomal preparations | Conjugates* | Bilirubin monoglucuronide | Bilirubin diglucuronide | No. of experiments |
|--------------------------------------|------------|--------------------------|------------------------|-------------------|
| Untreated                            |            | 96.0 ± 6.2               | 4.0 ± 1.6              | 3                 |
| Triton X-100                         |            | 93.8 ± 8.5               | 6.2 ± 2.8              | 7                 |
| 0.125%                               |            | 97.1 ± 9.4               | 2.9 ± 2.2              | 7                 |
| 0.250%                               |            | 74.9 ± 11.0              | 25.1 ± 5.9             | 6                 |
| UDP-N-acetylglucosamine (0.5 mg/mg microsomal protein) | | 49.9 ± 5.5 | 50.1 ± 5.9 | 12 |

*The conjugate formed is expressed in each case as a percentage of the total bilirubin converted. Standard deviations are given.*

**Fig. 3.** The variation in the final proportions of bilirubin and its conjugates with change in digitonin concentration. In each assay, the initial bilirubin concentration was 12.5 μM, the duration of the incubation was 30 minutes, and the temperature of the incubation was 37 °C. The open squares represent the remaining unconjugated bilirubin; the filled squares, bilirubin monoglucuronide; and the filled circles, bilirubin diglucuronide.
TABLE II

Effect of Triton X-100 and digitonin on the nature of the products formed by the microsomal UDP-glucuronyltransferase system

| Treatment | Conjugates formed* |
|-----------|--------------------|
|           | Bilirubin diglucuronide | Bilirubin monoglucuronide |
| Triton X-100 (0.1% v/v) | 6.2 ± 1.0 | 93.8 ± 1.0 |
| Digitonin (0.35 mg/mg microsomal protein) | 50.5 ± 3.6 | 49.4 ± 3.8 |
| Triton X-100 (0.1% v/v) and digitonin (0.35 mg/mg microsomal protein) | 5.8 ± 0.5 | 94.3 ± 0.5 |

*Mean values ± S.D. are given. Four preparations were tested in each case.

Fig. 4. Bar graph illustrating the rate of release of phosphate by the mannose 6-phosphatase activity of the various microsomal preparations.

N-acetylglucosamine, as well as digitonin. For the microsomes treated with single agents, there is a kind of parallelism between the mannose 6-phosphatase activity and the diglucuronide-forming activity. This breaks down, however, when the combination digitonin/Triton X-100 pretreatment is utilized. The mannose 6-phosphatase continues to be accessible to its substrate, whereas the microsomal diglucuronide-forming activity is completely suppressed.

The Time Course of the Formation of Bilirubin Monoglucuronide and Bilirubin Diglucuronide—In our next series of experiments, the kinetic course of the formation of the conjugates of bilirubin was characterized. These experiments were carried out with hepatic microsomal preparations treated either with 0.35 mg of digitonin or 0.5 mg of UDP-N-acetylglucosamine/mg of microsomal protein. The amounts of bilirubin converted to bilirubin monoglucuronide and bilirubin diglucuronide were determined at 2- or 5-min intervals during a 30-min incubation. The results of these time course experiments are presented in Figure 5.

As illustrated in the top of Fig. 5, when the microsomal preparations were treated with digitonin, there was a very rapid formation of the conjugates of bilirubin. Over the first 10 min, 80% of the bilirubin disappeared, with a correspondingly rapid appearance of bilirubin monoglucuronide. Bilirubin diglucuronide appearance was delayed; at 10 min, over 70% of the total bile pigment was still bilirubin monoglucuronide. The rate of formation of bilirubin diglucuronide did not become substantial until the succeeding time interval, between 10 and 20 min, when a large increment in bilirubin diglucuronide concentration was observed. With this, a marked decrease of bilirubin monoglucuronide concentration occurred. During this time period, there was no significant change in the concentration of unconjugated bilirubin. At 30 min, bilirubin diglucuronide accounted for approximately 55% of the total bile pigments, bilirubin monoglucuronide 30%, and bilirubin 15%. The forms of the concentration versus time curves indicate that the reaction mode is sequential; bilirubin monoglucuronide is formed as a first step, and this then serves as the substrate for the second glucuronidation reaction.

Similar time course experiments were carried out with microsomal preparations activated with UDP-N-acetylgluco-
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The changes found in the proportions of bilirubin and its conjugates with time are illustrated in the bottom of Fig. 5. Following UDP-N-acetylglucosamine pretreatment, the rate of conversion of bilirubin to its conjugates was less than that found with digitonin pretreatment, and the amount of bilirubin converted to the bilirubin diglucuronide at 30 min was also decreased. Although the rates were lower, the pattern of formation of the conjugates was generally similar to that noted following digitonin treatment. Thirty-eight per cent of the bilirubin was converted in the first 10 min, thereafter the rate of conversion decreased, and by 30 min, only 70% of bilirubin had been converted. In the first 10 min, bilirubin monoglucuronide accounted for 90% of the conjugates; thereafter the amount of bilirubin diglucuronide increased fairly quickly, while that of bilirubin monoglucuronide increased only slowly. At the end of the incubation period, bilirubin diglucuronide accounted for 25%, bilirubin monoglucuronide for 45%, and bilirubin for 30% of the total bile pigments. The time course curves indicate that, in this instance, a larger conversion of bilirubin diglucuronide would have been achieved if the incubation had been carried on for a longer period. The form of the concentration-time course curves obtained with this preparation again indicate that bilirubin monoglucuronide is the precursor of bilirubin diglucuronide.

Experiments were carried out in which microsomal preparations were treated with both digitonin (0.35 mg/mg of microsomal protein) and UDP-N-acetylglucosamine (0.5 mg/mg of microsomal protein). The rate of formation of the conjugates was compared. The time courses and magnitudes of conjugate formation (initial bilirubin concentration, 15 μM; temperature, 37 °C) corresponded virtually exactly to that of digitonin alone. The digitonin effect completely superseded that of the N-acetylglucosamine. No additive effects were obtained.

In these experiments, the presence of glucurono-1,4-lactone made no difference to the compositions of the final product conjugate mixtures. There was no evidence of any substantial β-glucuronidase activity in the microsomal preparations.

The high performance liquid chromatographic analysis (29) produces, as part of the run, data on the isomeric composition of bilirubin and its conjugates (the XIIIa, IXα, and IIIa isomers). The preponderant of the starting material was, in each case, the IXα isomer. The proportion of the IXα isomer in the conjugates matched that in the added bilirubin (that is, the proportion of the material which was IXα did not change during conjugate formation). The lack of change in the IXα content indicates that dipyrrole exchange can be effectively excluded (24) as part of the reaction mechanism underlying the in vitro microsomal glucuronidation processes. In a dipyrrole exchange reaction, the product distribution is, in terms of the XIIIa, IXα, and IIIα isomers, 1:2:1; the proportion of the IXα isomer in the mixture then necessarily decreases, so long as its initial proportion of the total is substantially above 0.5, the value corresponding to total randomization.

The Effects of Temperature on the Relative Amounts of Bilirubin Monoglucuronide and Bilirubin Diglucuronide Formed by the Microsomal Glucuronyltransferase System—A series of experiments were carried out to determine the effect of temperature on the rate of formation of the conjugates of bilirubin. The data are presented in Fig. 6. With an initial bilirubin concentration of 12.5 μM, 35% of the bilirubin was converted to bilirubin monoglucuronide at 10 °C; the proportion rose to 80% at 30 °C. The change in the IXα content indicates that dipyrrole exchange did not occur until the incubation temperature exceeded 20 °C. The formation of bilirubin diglucuronide at the higher temperature appears to indicate an underlying steric change with temperature in either the enzyme or the membrane, or more likely both.

DISCUSSION

These studies indicate that the formation of bilirubin diglucuronide by rat hepatic microsomal preparations is governed by both the concentration of bilirubin in the preparation and the structural state of the microsomal membranes. The time course studies indicate that the glucuronidation reactions take place in two steps in vitro, with bilirubin monoglucuronide serving as substrate for the formation of bilirubin diglucuronide. It is not yet known if more than one uridine diphosphate MOSMICRAOS that the formation of bilirubin diglucuronide is a random process.

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The Effects of Temperature on the Relative Amounts of Bilirubin Monoglucuronide and Bilirubin Diglucuronide Formed by the Microsomal Glucuronyltransferase System—A series of experiments were carried out to determine the effect of temperature on the rate of formation of the conjugates of bilirubin. The data are presented in Fig. 6. With an initial bilirubin concentration of 12.5 μM, 35% of the bilirubin was converted to bilirubin monoglucuronide at 10 °C; the proportion rose to 80% at 30 °C. The change in the IXα content indicates that dipyrrole exchange did not occur until the incubation temperature exceeded 20 °C. The formation of bilirubin diglucuronide at the higher temperature appears to indicate an underlying steric change with temperature in either the enzyme or the membrane, or more likely both.
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glucuronyltransferase is involved in the microsomal glucuronidation of bilirubin, or if a single membrane-bound enzyme regulates the addition of two glucuronides by virtue of changes in its conformational structure. The uridine diphosphate glucuronyltransferase reactions convert a wide variety of compounds into biologically inactive products. The chemical diversity of the compounds capable of being glucuronidated raises the question of the functional molecular basis for the heterogeneity of the system. Studies on differential induction (31, 32) and subcellular diversity of activity (33), as well as developmental studies (34), clearly demonstrate that the substrates of UDP-glucuronate-dependent glucuronyltransferase reactions fall into a series of distinct groups. Several investigators have attempted to elucidate this complex system further by purifying enzymic moieties with glucuronyltransferase activity from hepatic microsomal preparations (35–38). These studies indicate that there are various forms of UDP-glucuronyltransferase with different substrate affinities. As pointed out by Axelrod et al. in 1958 (39), all of the substrates possess a nucleophilic functional group and one enzyme could catalyze a nucleophilic substitution reaction between all acceptor compounds and UDP-glucuronic acid. Although three distinct forms of microsomal UDP-glucuronate-dependent glucuronyltransferase appear to have been isolated, it is difficult to associate from the reconstituted systems whether these are distinct or are isoenzymes or parts thereof. Their functional differences, such as substrate specificity, could simply reflect the properties of the membranes regulating their activity (4, 39). A microsomal glucuronyltransferase which exhibits a high affinity for bilirubin but shows little activity with p-nitrophenol or estrone has recently been shown to be partially purified (40). In a reconstituted system, the partially purified enzyme is found to convert bilirubin to bilirubin monoglucuronide but not to bilirubin diglucuronide (in the presence of UDP-glucuronate). The inability of this in vitro preparation to form bilirubin diglucuronide may simply indicate that the diglucuronide-forming enzyme has a separate identity or that in the reconstituted system the required structural environment was not recreated.

A survey of the literature indicates that factors regulating the activity of membrane-bound enzymes are very complex (5, 41) and that the functional capacities of these enzymes will usually vary, depending on the assay conditions under which the membrane-bound enzyme is investigated (19, 20, 42, 43). The data obtained from our experiments substantiate these findings. Thus, although the total activity of the UDP-glucuronate-dependent glucuronyltransferase system as measured by the conversion of bilirubin to total conjugates of bilirubin in the presence of UDP-glucuronate was more accessible to the UDP-glucuronate. The activity associated with these membrane vesicles was not augmented, the access of mannose 6-phosphate to the intramembranous mannose 6-phosphatase was not increased. The only product formed in substantial proportion was bilirubin monoglucuronide. Now consider those perturbations which enhance the formation of bilirubin diglucuronide. The most effective of these, treatment of the microsomal preparation with digitonin, has been found to remove cholesterol from the membrane. At the concentrations of digitonin found optimal for the formation of bilirubin diglucuronide, the membrane was more permeable to mannose 6-phosphate. This response suggests that the second enzymic activity may be on the cisternal side of the microsomal membrane. Thus, it would appear that treatment of the membrane with digitonin makes the active sites of the diglucuronide-forming enzyme more accessible but at the same time preserves the linkage of the enzyme to its milieu in the membrane in such a fashion as to preserve its activity. When Triton X-100 is present as well as digitonin, although the increased mannose 6-phosphatase activity is preserved, the diglucuronide-forming activity no longer persists. The reasons for this are not clear. The Triton X-100 may be inhibiting a cisternally located glucuronidation activity, or it may be changing the local milieu of such an enzyme by virtue of its removal of the phospholipid from the membrane; or, alternately, the diglucuronide-forming activity may be lost on the cisternal side of the membrane at all, but may simply undergo conformational changes, in a very sensitive fashion, in response to local changes in the membrane. The effect of UDP-N-acetylglucosamine on membrane structure is not known, but it has been inferred that the basis of UDP-N-acetylglucosamine-induced activation in other systems is an enhancement of the affinity of the enzyme for UDP-glucuronic acid (5). It is of interest to note that after the addition of both these agents to the microsomal preparation, the digitonin effect was dominant and was not modified. The qualitative nature of their actions on the enzyme may be similar. Both may simply make the active site on the enzyme more accessible to the UDP-glucuronate.

The mannose 6-phosphatase activity determinations have provided a new set of directions for exploration. They have, unfortunately, not provided crisp clear answers concerning the location and state of the diglucuronide-forming activity in the microsomal membrane.

Many investigators have noted that the activity of membrane-bound enzymes is governed in part by the structural state of the phospholipid environment. The glucuronidation of other nonpolar compounds by microsomal glucuronyltrans-
ferase systems has been shown to be regulated in such a fashion. Zakim and Vessey (8), for instance, have shown that the activity of UDP-glucuronyltransferase with p-nitrophenol as substrate is modulated in parallel with change in the physical state of the membrane phospholipid, as measured by a spin-labeled molecular probe. Arrhenius-type plots revealed a discontinuity in enzyme activity in the range of 13-16 °C. It was proposed that a phase change in the phospholipid of the membrane had occurred and that this led to a modification of the properties of this enzyme system. The change was shown to be associated with both an alteration in the specificity of substrate binding at the UDP-glucuronic acid site and a change in the response of the enzyme to allosteric activators. Below 13 °C, UDP-glucose, UDP-mannose, and UDP-xylene all inhibited this UDP-glucuronyltransferase but not at 37 °C. A similar pattern was also observed with UDP-N-acetylgalactosamine which had no effect below 13 °C but activated the p-nitrophenol glucuronyltransferase at temperatures above 16 °C (5). Our experiments also indicated that the activity and functions of the second step of the uridine di-phosphate glucuronyltransferase system with bilirubin mono-glucuronide as a substrate are markedly dependent on temperature. Bilirubin diglucuronide was formed in appreciable amounts only at temperatures above 25 °C, and its rate of formation then increased with temperature. The formation of bilirubin monoglucuronide from bilirubin exhibited a qualitatively different change in behavior over this range. This activity (as reflected by the amount of unconverted bilirubin remaining at the end of the period of incubation), rather than increasing, was constant over this temperature interval.

These studies in aggregate appeared to indicate that the function of UDP-glucuronyltransferase in vitro, with bilirubin monoglucuronide as substrate (the activity underlying the addition of the second glucuronide), is regulated in a particular way by the physical state of the membrane. Perturbations of the membrane by treatments which disrupt the structure of the membrane do not alter the capability to produce bilirubin monoglucuronide, nor do they enhance the formation of bilirubin diglucuronide. The uncovering of the diglucuronide-forming activity appears to require a less radical alteration, involving some preservation of the structure of the membrane but at the same time making the active site of the enzyme more accessible. The results of the temperature-dependent studies indicate that the immediate environment of the enzyme system must have a regulatory effect on this activity. Although thermal induced phase transitions are not expected to play a part in living systems which maintain a constant temperature, the results of our studies demonstrate that a specific structural state of the microsomal membrane, which is determined by the temperature, is integral to the formation of bilirubin diglucuronide.

It is clear that the present studies provide an unequivocal demonstration of the capability of liver microsomal preparations to form bilirubin diglucuronide in vitro. They show that there is a sequential reaction mechanism, bilirubin monoglucuronide formed during a first step, providing the substrate for diglucuronide formation; they provide some insight into the approaches needed to uncover the second glucuronidation activity; and they indicate that only when the integrity of the membrane is relatively maintained will bilirubin diglucuronide be formed in vitro. It is likely, in view of the findings of Sieg et al. (24), reviewed in the Introduction, that this microsomal mechanism represents the major in vivo mechanism for bilirubin diglucuronide formation in the liver.

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