Mechanism and Subcellular Site of Bilirubin Diglucuronide Formation in Rat Liver*

(Received for publication, October 18, 1983)

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Two different subcellular sites and mechanisms have been proposed for the hepatic conversion of bilirubin monoglucuronide to bilirubin diglucuronide: a microsomal system requiring UDP-glucuronate and a UDP-glucuronate-independent transglucuronidation or dismutase reaction localized at the bile canalicular plasma membrane. To further define these, canaliculal plasma membranes were highly purified from rat liver, and the capacity of these to form bilirubin diglucuronide was compared with that of simultaneously isolated hepatic microsomes. The canaliculal liver plasma membranes were 48-116-fold enriched over homogenate in various canaliculal marker enzyme activities; microsomal contamination was <10% based on the NADPH-cytochrome c reductase activity. No evidence of any conversion of highly purified bilirubin IXa monoglucuronide to bilirubin diglucuronide was found with canaliculal liver plasma membranes either in the absence or presence of UDP-glucuronate. In contrast, digitonin-treated microsomes isolated under similar conditions converted 31% of added bilirubin monoglucuronide (9.4–17.1 μM) into bilirubin diglucuronide in 30 min, the reaction being dependent on UDP-glucuronate. When bilirubin (12.5 μM) was added to the microsomes, 42.3% was converted to bilirubin monoglucuronide and 40.9% to bilirubin diglucuronide in 30 min. These data establish that the bile endoplasmic reticulum and not the canaliculal liver plasma membranes forms bilirubin diglucuronide from bilirubin monoglucuronide and that the reaction requires UDP-glucuronate.

Bilirubin diglucuronide is the major bile pigment detected in bile (1-7). Its formation from bilirubin, the end product of heme catabolism, occurs within the liver and involves the addition of glucuronic acid to both propionic acid side chains of bilirubin IXa (8). In vitro, these glucuronidations of bilirubin appear to occur as two sequential steps; proposed mechanisms are summarized in Table I. First, bilirubin monoglucuronide is formed, with UDP-glucuronate as the glucouronyl donor, by a microsomal glucuronyltransferase (UDP-glucuronyltransferase, EC 2.4.1.17) (8). Second, bilirubin monoglucuronide is converted to bilirubin diglucuronide, but the enzymic process involved and the hepatocellular site of this second reaction remain uncertain. Some investigators have demonstrated that bilirubin diglucuronide is formed in the microsomes by a UDP-glucuronate-requiring transferase (9-12), whereas others have detected the formation of bilirubin diglucuronide from bilirubin monoglucuronide in hepatic plasma membranes enriched in bile canaliculi (13, 14). This latter reaction requires that 2 mol of bilirubin monoglucuronide-are converted to 1 mol of bilirubin diglucuronide and 1 mol of bilirubin in the absence of UDP-glucuronate. Chowdhury et al. (15) have partially purified from hepatic plasma membranes an enzyme that appears to catalyze this dismutation. Interpretation of the experimental observations has, however, been questioned since it has been reported recently that the formation of bilirubin diglucuronide from bilirubin monoglucuronide by bile canaliculal enriched liver plasma membranes in vitro may result from nonenzymic dipyrrole exchange. This latter reaction forms the nonphysiological symmetrical XIIIa and IIIa isomers, which are not expected with the originally postulated dismutase reaction (16). Hence, the role of the bile canaliculal plasma membrane in the formation of bilirubin diglucuronide remains controversial. In the present study, we compare the capacity of highly purified canaliculal liver plasma membranes and simultaneously isolated microsomes to convert bilirubin monoglucuronide to bilirubin diglucuronide, utilizing a recently developed high performance liquid chromatographic assay that characterizes the isomeric forms of the products (17). The studies provide no evidence for an enzymic formation of bilirubin diglucuronide by canaliculal liver plasma membranes and establish that UDP-glucuronate is an absolute prerequisite for bilirubin diglucuronide synthesis by hepatic microsomes. This work has previously been presented in preliminary form (18).

EXPERIMENTAL PROCEDURES

Animals—Male Wistar or Sprague-Dawley rats, weighing 200–250 g, were used in this study. The animals had free access to water, were fed Purina Rodent Chow ad libitum, and were housed in a constant temperature-humidity environment with alternated 12-h light and dark cycles. The animals were routinely killed between 7:30 and 8:30 a.m.

Isolation of Canaliculal Liver Plasma Membranes—Highly purified canaliculal liver plasma membranes were isolated from rat liver by a newly developed procedure involving rate zonal flotation procedure and high speed centrifugations through discontinuous sucrose gradients (19). All isolation steps were done at 0–4 °C. 100–110 g of liver tissue were routinely obtained from 10–12 normal fed rats. 10-g portions of liver were minced, washed three times in 80 ml of cold 1 mm NaHCO3, and homogenized in a loose Dounce homogenizer (Type...
Hepatic endoplasmic reticulum

![UDP-glucuronate + bilirubin monoglucuronide](image)

**TABLE I**

| Reaction | In vivo | In vitro scrambling |
|----------|---------|--------------------|
| Bilirubin | UDP-glucuronate | glucuronyltransferase |
| Bilirubin monoglucuronide | UDP-glucuronate | glucuronyltransferase |
| Hepatic bile canalicular plasma membranes | glucuronosidase | glucuronosyltransferase |
| Bilirubin monoglucuronide | glucuronosidase | glucuronosyltransferase |
| Bilirubin diglucuronide | bilirubin diglucuronide + bilirubin monoglucuronide |

A) by seven up and down strokes. The homogenate was diluted to 2000 ml with cold 1 mM NaHCO₃, filtered twice through two layers of cheesecloth (80 grade), and centrifuged at 1,500 × g for 15 min. The crude nuclear pellet was resuspended in 5.5 volumes of 56% sucrose (w/w) and stirred for 15 min to disrupt membrane aggregates. The homogenate was then centrifuged dynamically (9,000 rpm) loaded onto a 100-ml cushion of 56% (w/w) sucrose with a variable speed Sorvall pump into a zonal rotor TZ-28 (Sorvall) and overlaid by 400 ml of 44% sucrose (w/w) and 200 ml of 36.5% sucrose (w/w), respectively. Finally, the rotor was filled to its total volume capacity (1,550 ml) with 0.25 M sucrose. The completed discontinuous sucrose gradient system was centrifuged at 20,000 rpm for 120 min. After slow deceleration to a complete stop, 70 15-ml fractions were collected from the bottom of the rotor. The fractions containing the bulk of plasma membrane fragments (the 44/36.5% sucrose interface) were combined and diluted with NaHCO₃ (1 mM) to 1,000 ml, and the material was sedimented at 7,000 rpm for 60 min, and finally resuspended in 0.25 M sucrose and tightly homogenized (Type B glass-glass Dounce homogenizer) by 50 up and down strokes to dissociate canalicular from basolateral liver plasma membranes.

Canalicular liver plasma membranes were separated from the basolateral plasma membrane fragments by centrifugation of the "mixed plasma membrane" fraction through 31% (w/w) sucrose at 40,000 rpm (9,000 × g) for 3 h in a Beckman SW 41 rotor, and the vesiculated canalicular liver plasma membranes, recovered from the top of the 31% sucrose layer, were diluted in 0.25 M sucrose, sedimented at 105,000 × g for 60 min, and finally resuspended in 0.25 M sucrose.

**Isolation of Microsomes**—Two procedures were used for the isolation of microsomes from rat liver. As a routine source for glucuronoyltransferase activity, microsomal (i.e. endoplasmic reticulum) membranes were isolated by differential centrifugation from a 20% homogenate prepared from approximately 10 g of liver in isotonic 0.25 M sucrose, 1 mM EDTA (microsomes A). Since the isolation of canalicular liver plasma membranes was started in hypotonic NaHCO₃ (see above), in some experiments both canalicular liver plasma membranes and microsomes were isolated in 1 mM NaHCO₃ from the same homogenate. In these experiments, the initial 1,500 × g supernatant was made isotonic (0.25 M) with sucrose and then tightly rehomogenized by 10 up and down strokes in a Type B Dounce homogenizer. Mitochondrial membranes were sedimented at 7,000 × g for 15 min, and microsomes were prepared from the corresponding postmitochondrial supernatant by centrifugation at 105,000 × g for 60 min (microsomes B). These microsomes B were either resuspended in 0.25 M sucrose or 1 mM NaHCO₃. Isolated membrane subfractions were stored in liquid nitrogen (−70°C) for up to 4 weeks before use.

**Analytical Methods**—The purity of the canalicular liver plasma membrane fraction was determined by use of marker enzyme assays: succinate- and NADH-cytochrome c reductase for mitochondria (21), NADPH-cytochrome c reductase for microsomes (21), acid phosphatase for lysosomes (22), and galactosyltransferase for Golgi membranes (23). The plasma membrane markers Mg²⁺-ATPase and ouabain-sensitive (Na⁺/K⁺)-ATPase activities were determined by a coupled kinetic assay as modified by Scharschmidt et al. (24). Alkaline phosphatase was assayed using p-nitrophenyl phosphate as substrate (25). The method of Goldberg and Rutenberg (26) was used for determination of low-sodium phosphatase activity. γ-Glutamyl transpeptidase and alkaline phosphodiesterase I activities were measured according to Orlovski and Meister (27) and Razell (28), respectively. The adenylyl cyclase activity in the presence and absence of glucagon (10⁻¹⁰ M final concentration) was measured by the method of Stewart et al. (29). Protein was determined according to Lowry et al. (30) with bovine serum albumin as a standard.

**Source of Bilirubin and Bilirubin Monoglucuronide**—Commercial bilirubin was obtained from British Drug House. The purity of the bilirubin was checked by high pressure liquid chromatography as outlined below. Bilirubin monoglucuronide is not available from commercial sources. To obtain it, one has the choice of either isolating it from bile or synthesizing it from commercial bilirubin with a liver microsomal preparation. The nature of the product and the nature of the experiment will dictate to some extent which source is more suitable for a given study.

In the systems under investigation, bilirubin diglucuronide can potentially be formed from bilirubin monoglucuronide either by enzymatic activity or by nonenzymic dipyrole exchange, as outlined in Table 1 and Fig. 1. If enzymic glucuronidation occurs by either the UDP-glucuronate-dependent transferase or by dismutation (the reaction postulated at the canalicular level), the isomeric composition of the products will be identical to that of the substrate (in vivo, bilirubin and its products are almost completely in the IXa form; in vitro, the starting isomeric composition of the bilirubin will vary depending on its commercial source but will not be expected to change during these reactions). In contrast, if there is nonenzymic dipyrole exchange in vitro in our reaction mixture, cleavage of bilirubin monoglucuronide will occur randomly at either side of the central methylene bridge, with random recombination of the resultant dipyroles. The reaction products arising from this will be a mixture of bilirubin, bilirubin monoglucuronide, and bilirubin diglucuronide in the ratio 1:2:1, and the isomeric composition of the products arising from the IXa isomer will be in each case XIIa:IXa:IIIa in the ratio 1:2:1. Since bilirubin monoglucuronide has two IXa isomers (with the glucuronide conjugated to the propionic acid group attached to either C-8 or C-12 of the tetrapyrrole skeleton), there will be four species, in the 1:1:1:1 equivalence, resulting from this exchange. With the progress of scrambling, the composition of the reaction mixture will approach its random equilibrium state, both in terms of species and isomeric forms. Since the contribution of dipyrole exchange to any reaction is revealed by changes in bilirubin isomeric composition, it was mandatory to be able to ascertain the isomeric composition of the original bilirubin monoglucuronide and of its reaction products to determine the mechanism of any conjugation observed. The high performance liquid chromatographic procedure which we use for our analyses (which is described later) provided us with this capability (17). It permitted us to detect and quantitate the isomeric forms of...
The bilirubin monoglucuronide purified in this fashion was used for further studies only if it was composed predominantly of IXα isomers. Expressed as a percentage of the total bilirubin monoglucuronide present, the average isomeric composition of seven such preparations (mean ± S.D.) was: XIα, 3.8 ± 0.1%; the IXα-C-8 isomer, 54.7 ± 2.2%; the IXα-C-12 isomer, 39.8 ± 0.1%; and IIα, 1.7 ± 0.1%.

Bilirubin monoglucuronide was also prepared biosynthetically by use of a liver microsomal preparation solubilized with Triton X-100 in the fashion described by Jansen et al. (13) for their original distamycin studies. Its composition was quite different. The isomeric composition of the source bilirubin was: XIα, 6.8 ± 2.6%; IXα, 78.4 ± 3.6%; and IIα, 14.8 ± 1.8% (mean ± S.D. of eight determinations).

The bilirubin monoglucuronide preparation, extracted with ethanol acetate as originally described by Jansen et al. (13), was contaminated with varying but usually small proportions of bilirubin diglucuronide. The average isomeric composition of the bilirubin monoglucuronide produced was: XIα, 12.9%; the IXα-C-8 isomer, 42.5%; the IXα-C-12 isomer, 26.6%; and IIα, 18.1%. The increase in the relative amounts of XIα and IIα isomers and the decrease in the total of the IXα isomers indicated that random isomerization was occurring when the synthesis and extraction of bilirubin monoglucuronide were being carried out in this way. If scrambling had not occurred, the bilirubin monoglucuronide would have had the same isomeric composition as the parent bilirubin from which the bilirubin monoglucuronide was being prepared.

The isomeric compositions of the bilirubin monoglucuronide prepared from bile and that prepared biosynthetically by the method of Jansen et al. (13) were thus dramatically different. It appeared appropriate to utilize the preparation isolated from bile for most of the bilirubin monoglucuronide studies since this bilirubin monoglucuronide preparation has an almost exclusively IXα composition (corresponding to the in vivo situation) and, with it, scrambling effects, when they occur, are immediately evident.

**Glucuronidation of Bilirubin**—The reaction mixture contained at zero time: microsomes at a concentration 1.3 mg of microsomal protein/ml (either untreated or preincubated for 60 min at 4 °C with digitonin, 0.35 mg of microsomal protein (12)), bilirubin (12-15 μM), 0.05 M triethanolamine buffer, pH 7.8, 2.8 mM UDP-glucuronic, 8 mM MgCl₂, and, in some experiments, 1 mM glucaro-1,4-lactone. Incubations were carried out for 30 min at 37 °C in the dark, and the reaction was stopped by placing the reaction vessels on dry ice. The samples were then stored at −20 °C overnight.

In initial experiments, albumin was added to the bilirubin stock solution. As demonstrated in Table II, albumin at very low concentrations (~0.02 mg/ml) had no effect on the rate of glucuronidation of bilirubin or the nature of the products formed. With increase of the albumin concentration to 0.2 or 1.0 mg/ml, although the monoglucuronidation of bilirubin was not affected, the formation of bilirubin diglucuronide was markedly decreased. Under these conditions, bilirubin monoglucuronide was the dominant product formed. When the concentration of albumin was further increased to 2.0 mg/ml, the glucuronidation of bilirubin was almost completely inhibited; bilirubin monoglucuronide was the only product detected in significant amounts. The inhibition of the glucuronidation of bilirubin by high concentrations of albumin has also been noted by others (32). Therefore, albumin was omitted from all subsequent reaction mixtures.

**Glucuronidation of Bilirubin Monoglucuronide**—When digitonin-treated microsomes were utilized, the reaction was carried out at 37 °C.
described above except that bilirubin monoglucuronide (9.4-17.1 μM) was used as substrate.

When canalicular liver plasma membranes were utilized as a potential source of enzyme, the reactions were carried out in the following manner. 100-500 μg of membrane protein were preincubated in 0.9 ml of a 0.1 M KH₂PO₄ buffer, pH 6.4, containing 10 mM glucose-1,4-lactone at 25 °C for 1 h. Bilirubin monoglucuronide (9.4-17.1 μM) in 0.1 M Tris-HCl buffer, pH 7.8, was then added (final pH 6.6, that reported optimal for the dismutase reaction (13)), and the reaction mixture was further incubated at 37 °C for 10 min. The reaction was stopped by placing the reaction vessel in dry ice. The samples were stored at −20 °C overnight.

**Extraction of Bile Pigments from Reaction Mixture**—Bilirubin and the conjugates of bilirubin were extracted quantitatively in chloroform containing 10 mM tetraheptylammonium chloride. The mixtures were vortexed and centrifuged, and the chloroform phases were removed, pooled, and taken to dryness under N₂. The pigments were then dissolved in chloroform/acetonitrile (3:5, v/v). The concentrations of bilirubin and its conjugates were determined by a recently developed high performance liquid chromatographic technique (17). All procedures were carried out under subdued light.

**Analysis of Bile Pigments**—The chromatographic procedure used to quantitate bilirubin and its conjugates was, briefly, as follows. The assays were carried out on a Hewlett-Packard 1084 high performance liquid chromatograph with a variable wavelength detector set at 440 nm. Separation of the bile pigments was achieved with an oven temperature at 37 °C using two Hewlett-Packard reverse phase RP-18 columns in series, each 200 mm in length and 4.6 mm in diameter, with a particle size of 10 μm. The bile pigments and their isomers were separated by carrying out the following procedure (17). The flow rate of the mobile phase, which consisted of 5 mM heptanesulfonic acid in 0.1 M acetate buffer, pH 4.8 (solvent A), and acetonitrile (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 75% (v/v) and that of solvent B was 25% (v/v). During the first 20 min of the run, solvent B was increased linearly from 25% (v/v) to 40% (v/v) and then, in the next 7 min, from 45% (v/v) to 80% (v/v). The flow rate 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 isomers of bilirubin and its conjugates as well as the species themselves were separated by this procedure.

**RESULTS**

**Characteristics of Isolated Canalicular Liver Plasma Membranes**

Details of the isolation procedure and a complete characterization of the isolated canalicular liver plasma membranes are published elsewhere (19). Here we report only on the purity of the membrane preparations. Intracellular marker enzyme activities were depleted in the canalicular liver plasma membranes with respect to homogenate (the relative enrichments of marker enzyme activities over homogenate were 0.1 to 0.7, except for acid phosphatase (relative enrichment = 1.1)). The relative enrichment factor for the microsomal marker NADPH-cytochrome C reductase activity was 0.4 ± 0.2 (mean ± S.D., n = 8). Since lysosomes and endoplasmic reticulum represent 2 and 24% of total rat liver homogenate protein (33), respectively, canalicular liver plasma membranes were more contaminated with microsomal membranes (24 × 0.4 = 9.6%) than with lysosomes (2 × 1.1 = 2.2%), despite the more favorable relative enrichment factor. Correspondingly, the contaminations with mitochondriod (succinate-cytochrome C reductase) and Golgi (galactosyltransferase) membranes were 1.6 and 0.4%, respectively. Canalicular liver plasma membranes were free of the basolateral markers (Na⁺/K⁺)-ATPase and glucagon-stimulatable adenylate cyclase activities, but were highly enriched with respect to homogenate in the "canalicular marker" enzyme activities leucinaminopeptidase (48-fold), γ-glutamyl transpeptidase (60-fold), alkaline phosphatase (71-fold), Mg²⁺-ATPase (83-fold), and alkaline phosphodiesterase I (116-fold). These data demonstrate the highest degree of purification so far reported for canalicular liver plasma membranes, with a total contamination with intracellular membranes below 15%.

**Glucuronidation of Bilirubin**

**Microsomes A**—In vitro incubations of bilirubin (12.5 μM) with native microsomes prepared in 0.25 M sucrose and 1 mM EDTA in the presence of UDP-glucurionate but in the absence of digitonin resulted in the formation of bilirubin monoglucuronide (Fig. 2). In the substrate bilirubin, in this case, the relative proportions of the isomeric forms bilirubin XIIIa, IXa, and IIIa were 8.9, 73.0, and 18.1%, respectively. Identical isomeric patterns were found in the bilirubin monoglucuronide product. Over 80% of the bilirubin substrate was monoglucuronidated in 30 min, but only a small proportion was converted to bilirubin diglucuronide (less than 4% at 30 min). In contrast, when microsomes were first treated with digitonin at 0.35 mg/mg of protein for 60 min at 4 °C and then incubated with bilirubin (12.5 μM) and UDP-glucurionate, approximately equal proportions of bilirubin monoglucuronide and bilirubin diglucuronide were formed (Fig. 3). When a bilirubin monoglucuronide preparation derived from bile (9.4-17.1 μM) rather than bilirubin was incubated with digitonin-treated microsomes, bilirubin diglucuronide was again formed and its formation was absolutely dependant upon the presence of UDP-glucurionate (Fig. 4). In neither of these reactions did the relative proportions of the isomeric forms of bilirubin (i.e. XIIIa, IXa, IIIa) change during the glucuronidation reaction. Importantly there was no significant dipyrrole exchange under the conditions of the reactions (Table III and Figs. 2-4). In addition, the isomeric patterns detected in our preparations of bilirubin monoglucuronide were not altered in differential fashion by incubating the substrate for 30 min at 37 °C in buffers at pH 7.85, 6.78, or 6.45; furthermore, during incubation in the assay system with boiled enzyme, the isomeric distribution of a second preparation of the bilirubin monoglucuronide species did not change. These findings emphasize

![Fig. 2. Rat liver microsomes incubated at 37 °C in the presence of bilirubin (12.5 μM), with or without UDP-glucuronate (2.8 mM) as outlined under "Experimental Procedures." The chromatographic tracings indicate that bilirubin IXa (three isomeric peaks at 31-32 min) is not conjugated in the absence of UDP-glucuronate (upper); whereas in the presence of UDP-glucuronate, there is a substantial conversion to bilirubin monoglucuronide (four isomeric peaks at 14-17 min).](http://www.jbc.org/)
Bilirubin Diglucuronide Formation in Rat Liver

DIGITONIN TREATED MICROSOMES

UDP-glucuronate absent

0 10 20 30 40
TIME (min)

DIGITONIN TREATED MICROSOMES

UDP-glucuronate present

0 10 20 30 40
TIME (min)

FIG. 3. Rat liver microsomes pretreated with digitonin (0.35 mg/mg of microsomal protein) and incubated at 37 °C in the presence of bilirubin (12.5 μM) with or without UDP-glucuronate (2.8 mM) as outlined under “Experimental Procedures.” The chromatographic tracing indicates that bilirubin IXα (three isomeric forms at 31-32 min) is not conjugated in the absence of UDP-glucuronate (upper); whereas in the presence of UDP-glucuronate, there are almost equal amounts of bilirubin monoglucuronide (four isomeric peaks at 14-17 min) and bilirubin diglucuronide (three isomeric peaks at 9-11 min).

DIGITONIN TREATED MICROSOMES

UDP-glucuronate absent

0 10 20 30 40
TIME (min)

DIGITONIN TREATED MICROSOMES

UDP-glucuronate present

0 10 20 30 40
TIME (min)

FIG. 4. Rat liver microsomes pretreated with digitonin (0.35 mg/mg of microsomal protein) and incubated at 37 °C in the presence of bilirubin monoglucuronide (9.4 μM) with or without UDP-glucuronate (2.8 mM) as outlined under “Experimental Procedures.” Bilirubin diglucuronide was formed only in the presence of UDP-glucuronate.

FIG. 5. Rat liver microsomes isolated from a hypotonic medium (1 mM NaHCO₃), pretreated with digitonin (0.35 mg/mg of microsomal protein), and then suspended in either 1 mM NaHCO₃ or in 0.25 M sucrose. Incubations were carried out at 37 °C in the presence of bilirubin monoglucuronide (9.4 μM) and UDP-glucuronate (2.8 mM) as outlined under “Experimental Procedures.” Bilirubin diglucuronide was formed only in the 0.25 M sucrose media.

TABLE III

Effect of pH and enzymic assay conditions on the isomeric forms of bilirubin monoglucuronide

| No. of experiments | Bilirubin species | % |
|--------------------|------------------|---|
|                    | XIIIa            | IXα₀ | IXα₁² | XIIIa |
| pH                 |                  |      |       |       |
| 7.85               | 6.2             | 47.7 | 36.5  | 10.2  |
| 6.78               | 5.1             | 49.1 | 35.2  | 10.7  |
| 6.45               | 5.5             | 47.7 | 35.1  | 11.6  |
| Blank              | 4               | Trace| 56.6 ± 2.4 | 43.4 ± 2.45 | Trace |
| Test               | 5               | Trace| 56.7 ± 3.2 | 43.3 ± 3.2 | Trace |

As with microsomes A (see above), >80% of the bilirubin was glucuronidated very efficiently. The conditions of the assay, however, did modify the activity. It was observed that, when these microsomes were resuspended in hypotonic NaHCO₃ (1 mM), only bilirubin monoglucuronide was formed; whereas, if they were suspended in isotonic sucrose, both bilirubin monoglucuronide and bilirubin diglucuronide were synthesized in their usual proportions (Fig. 5). These findings suggest that the addition of the second glucuronic acid moiety to bilirubin is somehow dependent on the integrity of certain as yet undefined structural properties of the microsomal membrane and that these properties are modified by changing the osmolality and ionic strength of the medium.

Canalicular Liver Plasma Membranes—As illustrated in Fig. 6, no evidence was found with the bile-derived bilirubin IXα monoglucuronide preparation that canalicular liver plasma membranes had any capacity to glucuronidate bilirubin IXα monoglucuronide. The reactions were carried out...
with or without digitonin treatment of the canalicular liver plasma membranes in the presence and absence of UDP-glucuronate at varying concentrations of bilirubin monoglucuronide (9.4–17.1 \( \mu \)M) and at different pH levels (6.5, 6.8, and 7.8). None of these conditions resulted in the conversion of bilirubin monoglucuronide to bilirubin diglucuronide, either by enzymic reactions or by dipyrrole exchange (Fig. 6 and Table IV). In order to document whether there was any destruction of the bilirubin monoglucuronide substrate or any loss of substrate during the extraction procedure, the amount of bilirubin monoglucuronide added at zero time and that recovered at the end of the incubation were quantitated. No losses occurred; recoveries were 99.0 ± 1.2% (S.D.). Bilirubin was also utilized as the substrate and no glucuronidation was observed.

The lack of reaction of the highly enriched liver canalicular membrane preparation with the highly purified bilirubin IXa monoglucuronide was, of course, puzzling. We therefore also used the partly randomized Jansen preparation of bilirubin monoglucuronide (13), which had been used in the original dismutase experiments and which contains small amounts of bilirubin diglucuronide, to determine whether with this small amount of bilirubin diglucuronide, no dismutase activity was detected in either canalicular or basolateral membranes. At pH 6.6, further scrambling of the substrate occurred, associated with increases in both bilirubin and bilirubin diglucuronide. The reaction recorded proceeded at the same rate in the presence of either hepatic canalicular or basolateral membranes (the latter were also harvested at the time of the isolation of the canalicular membranes (19)) and in the presence of appropriate blanks. All of the changes observed could be ascribed to scrambling; no dismutase activity was observed. The addition of 1 \( \mu \)M ascorbic acid to the reaction mixture inhibited the randomization; in its presence, no dismutase activity was found. The results confirm those reported earlier by Sieg et al. (16). They suggest that what was originally interpreted to be a dismutase activity was really randomization. The cause for the instability of the bilirubin monoglucuronide, when prepared and extracted as originally described by Jansen et al. (13), is not clear.

**DISCUSSION**

These studies confirm our earlier findings and those of others that rat liver microsomes will convert bilirubin in vitro into bilirubin diglucuronide (9–12, Table IV). This microsomal glucuronidation of bilirubin appears to occur in two steps with bilirubin monoglucuronide as an intermediate and uridine diphosphate glucuronate as the acceptor donor. The proposed canalicular bilirubin-glucuronoside glucuronosyltransferase (EC 2.4.1.95), which has been reported to convert bilirubin monoglucuronide to bilirubin diglucuronide by a dismutation in the absence of UDP-glucuronate (13, 14), was not detected in our highly purified canalicular or liver plasma membrane preparation. No evidence was found for induction of randomization of the tetrapyrroles by microsomes under the conditions of the reactions, nor was bilirubin detected as a product when highly purified bilirubin IXa monoglucuronide was utilized as a substrate. These data strongly support the concept that the hepatic endoplasmic reticulum is the major subcellular site of formation of bilirubin diglucuronide (16) and demonstrate that the underlying enzymic reaction is dependent upon UDP-glucuronate.

It is difficult to compare the original work postulating the canalicular dismutase reaction with the present study since the isomeric patterns of the substrates used and the products formed were not evaluated in the earlier reports (13, 14). Thus, the possibility exists that the reported in vitro formation of bilirubin diglucuronide by bile canalicular enriched liver plasma membranes was actually due to dipyrrolic exchange (16), and our observations indicate that with the original bilirubin monoglucuronide preparation prepared by Jansen et al. (13) some element, but not canalicular membranes, promotes this kind of randomization. When highly enriched canalicular liver plasma membranes and almost pure

**TABLE IV**

The capacity of hepatic microsomes and plasma membranes to glucuronidate bilirubin

- **n** is the number of incubations carried out. cLPM, canalicular liver plasma membranes. Values are expressed as mean ± S.D. of the mean.

| Subcellular Fraction | Substrate | UDP-glucuronate | Bile pigments detected at 30 min | % total bile pigments |
|----------------------|-----------|----------------|---------------------------------|----------------------|
|                      |           | Bilirubin (12.5 \( \mu \)M) | Absent | None | None | 100.0 |
| Microsomes (n = 11)  | Bilirubin monoglucuronide (9.4–17.1 \( \mu \)M) | Present  | 40.9 ± 11.4 | 42.3 ± 11.8 | 16.9 ± 8.5 |
|                      | Bilirubin monoglucuronide (9.4–17.1 \( \mu \)M) | Absent | None | 100.0 | None |
|                      | Bilirubin monoglucuronide (9.4–17.1 \( \mu \)M) | Present | 33.0 ± 1.2 | 67.1 ± 1.3 | None |
| cLPM (n = 6)         | Bilirubin | Absent | None | 100 | None |
|                      | monoglucuronide | Present | None | 100 | None |
bile pigment IXα monoglucuronide were used in the present investigation, however, no dipeptidase was observed (Table III and Figs. 2-4). The canicular liver plasma membrane fraction as isolated in this study was highly purified with respect to intracellular organelles and basolateral plasma membranes (19). The conservation of the capacity to form bile pigment diglucuronide in microsomes isolated from the same homogenates and under similar conditions as the canicular liver plasma membranes (microsomes B; Fig. 5) indicates that the subcellular fractionation procedure did not inactivate that glucuronidation activity, that the purified bile pigment IXα monoglucuronide molecules were freely utilizable by the conjugating mechanism, and that the purification procedure had not in some way introduced some stabilizing factor. Thus, our data strongly argue against the presence of a bile pigment-glucuronoside glucuronosyltransferase (dismutase) in bile canicular membranes of normal rat liver. Instead, they reinforce the thesis that microsomal UDP-glucuronate-dependent glucuronyltransferase activity quantitatively accounts for the generation of the predominant product characteristically found in bile, bile pigment diglucuronide.

Several recent findings now explain the earlier inability to detect significant in vitro formation of bile pigment diglucuronide by hepatic microsomes (34). First, the relative proportions of bile pigment monoglucuronide and bile pigment diglucuronide formed by microsomal UDP-glucuronosyltransferase are governed by the level of bile pigment present in the reaction mixture (9, 10). Thus, whereas at low concentrations of bile pigment (12 μM or less), bile pigment diglucuronide is the major conjugate, at higher levels of bile pigment (166 μM and greater), only bile pigment monoglucuronide is formed. These findings indicate that the assay conditions are crucial for the in vitro formation of bile pigment diglucuronide and that bile pigment can inhibit glucuronidation formation. Second, various alterations of the structural integrity of the microsomal membrane reveal a particular sensitivity of the microsomal UDP-glucuronosylate-dependent, second step bile pigment glucuronyltransferase to changes in its microenvironment (12). This sensitivity is further emphasized by the present observation that the changes produced by incubation of digitonin-treated microsomes in hypotonic (0.1 mM) NaHCO₃ buffer destroy the ability of the digitonin-treated membranes to add a second glucuronide in the presence of UDP-glucuronate. The exact nature of and the conditions required for the efficient coupling between the first and second step glucuronidation of bile pigment by hepatic microsomes are under further investigation. The major question which now arises is whether the glucuronyltransferase activities adding the first and second glucuronides to bile pigment are distinct and separate, even though both have now been shown to be localized to the hepatic microsomes.

In summary, the present study indicates that the hepatic endoplasmic reticulum is the major subcellular site of the enzymic formation of bile pigment diglucuronide and that the underlying enzymic reaction is dependent upon UDP-glucuronate. No enzymic conversion of a highly purified bile pigment IXα monoglucuronide to bile pigment diglucuronide was found with a highly purified canalicular enriched liver plasma membrane preparation.

Acknowledgments—We thank Ursula Sommerer and Janet Jane for their technical assistance and Margaret Mulherin for typing this manuscript.

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