Uridine Diphosphate-Glucuronic Acid-independent Conversion of Bilirubin Monoglucuronides to Diglucuronide in Presence of Plasma Membranes from Rat Liver is Nonenzymic

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ABSTRACT Two routes have been proposed for conversion of bilirubin monoglucuronide to the diglucuronide: glucuronyl transfer (*a*) from UDP-glucuronic acid to bilirubin monoglucuronide, catalyzed by a microsomal UDP-glucuronyltransferase, and (*b*) from one molecule of bilirubin monoglucuronide to another (transglucuronidation), catalyzed by an enzyme present in liver plasma membranes. The evidence regarding the role of the latter enzyme for in vivo formation of bilirubin diglucuronide is conflicting. We therefore decided to reexamine the transglucuronidation reaction in plasma membranes and to study the conversion of bilirubin monoglucuronide to diglucuronide in vivo. Purified bilirubin monoglucuronide was incubated with homogenates and plasma membrane-enriched fractions from liver of Wistar and Gunn rats. Stoichiometric formation of bilirubin and bilirubin diglucuronide out of 2 mol of bilirubin monoglucuronide was paralleled by an increase of the IIIα- and XIIIα-isomers of the bilirubin aglycone, thus showing that dipyrrole exchange, not transglucuronidation, is the underlying mechanism. Complete inhibition by ascorbic acid probably reflects intermediate formation of free radicals of dipyrrolic moieties. The reaction was nonenzymic because it proceeded independently of the protein concentration and heat denaturation of the plasma membranes did not result in decreased conversion rates. Collectively, these findings show spontaneous, nonenzymic dipyrrole exchange when bilirubin monoglucuronide is incubated in the presence of rat liver plasma membranes. Because bilirubin glucuronides present in biological fluids contain exclusively the bilirubin-IXα aglycone, formation of the diglucuronide from the monoglucuronide by dipyrrole exchange does not occur in vivo. Rapid excretion of unchanged bilirubin monoglucuronide in Gunn rat bile after injection of the pigment provides confirmatory evidence for the absence of a UDP-glucuronic acid-independent process.

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INTRODUCTION

In healthy humans and rats, bilirubin is converted mainly to glucuronides and excreted in bile as monoglucuronide (BMG,[1] a mixture of two positional isomers, Fig. 1) and, predominantly, as the diglucuronide (BDG) (1–5). The first metabolic conversion step involves transfer of a glucuronyl residue from UDP-glucuronic acid to bilirubin, catalyzed by a microsomal UDP-glucuronyltransferase (EC 2.4.1.17) (6–9).

Microsomal preparations from rat liver also catalyze UDP-glucuronic acid-dependent conjugation of BMG to form BDG (10, 11) and it is reasonable to assume that in vivo the same process is responsible, at least in part, for synthesis of BDG. As an alternative route, formation of BDG according to the disproportionation reaction 2 BMG $\rightleftharpoons$ B + BDG (Eq. 1) has been proposed recently (12–14). This transformation occurs in presence of plasma membrane-enriched preparations from rat liver (optimal pH 6.6) and has been claimed to be enzymic (12), but its mechanism is uncertain (14). Conceivably, transfer of glucuronyl residues between

[1] *Abbreviations used in this paper*: B, unconjugated bilirubin irrespective of the isomeric structure, BMG, bilirubin monoglucuronide, BDG, bilirubin diglucuronide, HPLC, high-performance liquid chromatography, N_2-fraction, plasma membrane-enriched subcellular fraction, TLC, thin-layer chromatography.

BMG molecules (transglucuronidation) (12) or dissociation into dipyrrole moieties and statistical recombination (dipyrrole exchange) could be involved (15, 16). The postulated enzyme has been named bilirubin glucuronoside glucuronyltransferase (EC 2.4.1.95) (13).

The hypothesis that UDP-glucuronic acid-independent transformation of BMG into BDG plays a role in synthesis of BDG in vivo (12–14) could not be confirmed by extensive analysis of biliary bile pigments formed in normal rat after injection of doubly-labeled BMG (17), but contradictory evidence has been communicated in a preliminary report (18). The homozygous Gunn rat offers a unique opportunity to test this hypothesis. Although this species congenitally lacks the ability to synthesize BMG from bilirubin (19, 20), conversion of BMG to BDG and bilirubin (eqn. 1) is reported to occur at comparable rates in presence of plasma membrane-enriched fractions from liver of either Gunn rats or Wistar rats (12, 13). In Gunn rats kept in darkness, unconjugated bilirubin is virtually not excreted into bile (21, 22) and is mainly metabolized to compounds that do not contain the bilirubin skeleton (23). Therefore, the biliary content of bile pigments with the IXα-skeleton is very low (21, 22) and secretion studies with BMG can be done with unlabeled material. Again, in vivo studies in this species supporting (13, 14) and disproving (17) UDP-glucuronic acid-independent conversion of BMG into BDG have been published.

In view of the present confusion in this area, we decided to reassess the UDP-glucuronic acid-independent conversion of BMG to BDG (eqn. 1), both in vitro and in vivo. As it seemed likely that some of the contradictory results have arisen from the pronounced instability of BMG (autoxidation and nonenzymic dipyrrole exchange) we first have reassessed some essential analytical procedures and defined conditions for proper handling of BMG preparations. Using plasma membrane-enriched material from liver of Wistar and Gunn rats, we addressed the following questions about the UDP-glucuronic acid-independent transformation of BMG into BDG in vitro: Does this conversion occur indeed, and if so, what is its mechanism (transglucuronidation or dipyrrole exchange)? Is the reaction catalyzed or spontaneous? Finally, the problem of in vivo conversion of BMG to BDG in Gunn rats was reexamined.

METHODS

Chemicals. Bilirubin was obtained from E. Merck, Darmstadt, West Germany. Glucaro-1,4-lactone H_2O was from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif., ethyl anthranilate from Fluka, Buchs, Switzerland, and Sephadex LH20 from Pharmacia, Div. Pharmacia Fine Chemicals, Uppsala, Sweden. Pentane-2-one (Union Chimique Belge, Drogenbos, Brussels, Belgium) was dried over $CaSO_4$ and redistilled before use. Glass plates precoated with silica gel (60F254, 0.25 mm, 20 × 20 cm from E. Merck.) were used for TLC. All other reagents were of analytical reagent grade.

Preparation of bilirubin glucuronides. BMG and BDG were isolated from bilirubin-enriched Wistar rat bile, and the mixture of glucuronides was purified by adsorption chromatography on Sephadex LH20 (10). BMG and BDG were isolated and repurified by TLC with chloroform/methanol/water (10:5:1, vol/vol/vol) as the solvent system. The BMG and BDG bands were scraped from the plate, eluted with methanol, and evaporated to dryness at 30°C under a stream of nitrogen. Analysis of ethyl anthranilate azo derivatives (24, 25) showed that the BMG preparations contained 0–4% BDG. The ratio, unconjugated azodipyrrole/azodipyrrole glucuronide was 0.99±0.03 (SD, n = 12), in close agreement with the expected value 1, for pure BMG. In one preparation the ratio was 0.85 corresponding to 92% of BMG and 8% of BDG. The BDG preparations yielded exclusively azodipyrrole glucuronide, indicating that the preparations were not contaminated with BMG.

In vivo experiments. Male Gunn rats (jj) (400–450 g body wt) were provided with a bile cannula and a jugular vein catheter under pentobarbital anesthesia (6 mg/100 g body wt, i.p.) and transferred to thermostated restraining cages where the body temperature was kept between 37.0 and 37.6°C. Continuous infusion (1.9 ml/h) of 0.16 M NaCl containing glucose (5%, wt/vol) was started and maintained during the experiment, except for the 15-min period during which BMG was administered. After recovery from anesthesia (usually 2–2.5 h after bile duct cannulation) three basal 10-min bile samples were collected ("control samples"). Only rats with a bile flow above 250 μl/100 g body wt/h were used. Freshly prepared dry BMG was redissolved in 0.1 M phosphate buffer, pH 7.4 (81.8 ml 0.5 M KH_2PO_4 and 59.2 ml 0.5 M NaOH brought to 1,000 ml with distilled water) containing 1 mM ascorbic acid. Without delay, 3 ml of BMG solution (207 and 287 nmol, respectively) was infused continuously over 15 min. Bile was collected in 20-min samples on ice in the dark for 2 h immediately following the beginning of BMG infusion. Each bile sample was weighed and analyzed immediately after collection. The total concentration of bilirubin conjugates (BMG + BDG) was determined by the ethyl anthranilate diazo-method of Van Roy and Heirwegh (24). Bile samples (diluted 21-fold with glycine-HCl buffer pH 2.7) were mixed with diazo-reagent and diazo-cleavage was allowed to proceed for 30 min at pH 2.7 at 20–25°C (24, 25). Under these conditions, reaction of BDG and BMG is complete and yields, respectively, azodipyrrole glucuronide (from BDG) and an equimolecular mixture of azodipyrrole and azodipyrrole glucuronide (from BMG). Reaction of unconjugated bilirubin is negligible if diazo-cleavage is performed at pH lower than or equal to 2.7 (24). The total azo color derived from a given sample then is a measure of the total amount of bilirubin conjugates. It is essential to buffer the sample first to pH 2.7 before adding the diazo-reagent, because otherwise the reaction mixture may temporarily be at pH above 3, causing diazo-cleavage of unconjugated bilirubin (24). Also, for bile, appropriate dilution (20-fold or more) is required because otherwise added or endogenous bilirubin may react to a considerable extent (26), probably owing to the reaction-promoting effect of bile salts. By subsequent TLC of the azopigment extracts (25), unconjugated azodipyrrole was separated from azodipyrrole glucuronide and the ratio: r = unconjugated azodipyrrole/azodipyrrole glucuronide (Eq.

2) was determined by densitometry (25). The conjugates injected contained no conjugating groups other than glucuronic acid. Because reaction of BMG and BDG was complete, the azodipyrrole glucuronide formed thus reflects the total amount of conjugating groups. On the other hand, because only BMG contributed significantly to the unconjugated azodipyrrole formed, the proportion R of glucuronides could be calculated from the formula: $R = BMG/(BMG + BDG) = 2r/(1 + r)$ (Eq. 3). In addition, the relative amounts of tetrapyrroles were determined by the alkaline methanolysis-TLC procedure of Blanckaert (5).

Immediately before and after infusion of BMG a 100-μl sample of the pigment solution was analyzed without delay by the same methods as the bile samples. The molecular ratio of ethyl anthranilate diazonium salt to ascorbic acid exceeded 30, resulting in complete diazo-cleavage. For calculation of the output of injected BMG, the samples obtained during the control and injection periods were diluted 21-fold. The azo color obtained for the control samples amounted to <2% of the values found after injection of BMG. This small correction was applied to calculate the output of BMG. TLC of the azo pigment extracts from control bile samples showed negligible amounts of unconjugated azodipyrrole.

Experiments in vitro. Male Gunn (jj) and Wistar rats (300–450 g body wt) were fasted for 16 h with water ad lib. The animals were killed by cervical dislocation, their livers were perfused, homogenized, and fractionated according to the procedure of Touster et al. (27). The plasma membrane-enriched fraction, N_2, present at the 7.5–37.2% (wt/wt) interphase in a discontinuous sucrose gradient, was identified by its marker enzyme activities and electron microscopy. The activities of 5′-nucleotidase and glucose-6-phosphatase were determined according to Weaver and Boyle (28), phospholipids by the method of Bartlett (29) after extraction according to Folch et al. (30), and protein according to Peterson (31). Electron microscopy was performed as described by Touster et al. (27). The fractions were stored at 4°C for no longer than 3 d.

Two incubation systems were used. Incubation system A (12): 1.25 ml of 25% liver homogenate (wt/vol) or N_2 fraction

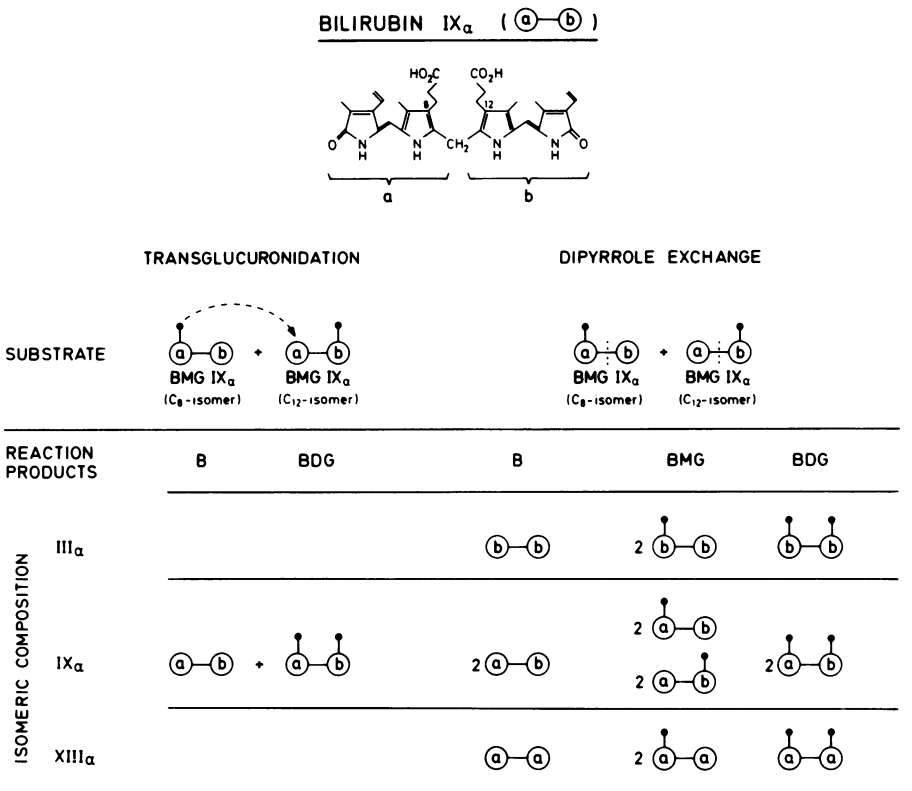

FIGURE 1 Structure of bilirubin-IXα and the two possible mechanisms of the disproportionation reaction: 2 BMG $\rightleftharpoons$ B + BDG. Bilirubin-IXα is an asymmetrical molecule with two different dipyrrolic moieties linked by a central methylene bridge. The two moieties are represented by ⓐ, containing anendo-vinyl group, and ⓑ, containing an exo-vinyl group. Bilirubin-IXα is consequently denoted as ⓐ-ⓑ, and the symmetrical molecules bilirubin-IIIα and -XIIIα as ⓑ-ⓑ and ⓐ-ⓐ, respectively. Transglucuronidation involves the transfer of a glucuronyl residue from one BMG molecule to another ("substrate"), forming BDG and unconjugated bilirubin (B) ("reaction products"). The isomeric composition of the reaction products does not change. The dipyrrole exchange mechanism consists in cleavage of the BMG molecules at either side of the central methylene bridge and statistical recombination of the dipyrroles formed. At equilibrium, the reaction products are a mixture of B, BMG and BDG in a 1:2:1 ratio. The isomeric composition of each reaction product is IIIα:IXα:XIIIα (1:2:1).

(~500 μg of protein) in 5 mM tris-HCl, pH 8.0, containing 0.25 M sucrose, 1.0 ml of 0.5 M KH_2PO_4-buffer, pH 6.4, and 2.75 ml of 9 mM glucaro-1,4-lactone were preincubated for 1 h at 25°C. Incubation system B (32): 0.5 ml N_2-fraction (0.613 μg of protein) and 1 ml of 0.1 M KH_2PO_4-buffer, pH 6.4 containing 10 mM glucaro-1,4-lactone were preincubated at 25°C for 1 h. To incubation mixture A, 1.18 ml of BMG solution (700 nmol, final concentration 119 μM) and to incubation mixture B, 0.5 ml of BMG solution (200–400 nmol, final concentration 100–200 μM) dissolved in 0.1 M Tris-HCl buffer, pH 7.8 was added to start the reaction. The final pH determined immediately after the addition of BMG or in parallel incubations was 6.48±0.04 (SD, n = 3) for incubation system A and 6.61±0.01 (SD, n = 9) for N_2-fractions in incubation system B. Addition of 1 mM ascorbic acid decreased the pH by only 0.05 pH units. The reaction proceeded at 37°C and 150-μl samples were removed before (control sample) and at several time points (0–16 min) after the addition of BMG. All determinations were done in duplicate. Total bilirubin was measured with diazotized *p*-iodoaniline (33). The bilirubin conjugates (BMG + BDG) were assayed by the ethyl anthranilate diazo-method (24, 25). After chromatographic determination of the ratio r (Eq. 2) the proportion of glucuronides was calculated from equation 3. The isomeric (IIIα, IXα, XIIIα) composition of bilirubin tetrapyrroles was determined by TLC (15) after alkaline hydrolysis (34). After spraying with 1% ascorbic acid in methanol the bilirubin IIIα-, IXα- and XIIIα-isomers were quantitated by densitometry at 434 nm, using a flying-spot densitometer (Vitatron, Dieren, The Netherlands). In selected samples the bilirubin isomers were also determined by HPLC on a silica column (250 by 4.6 mm, LiChrosorb Si 60, 5 μm, A. G. Merck, EM Laboratories, N. Y.) by development with chloroform/acetic acid (399:1, vol/vol) at a flow rate of 1.5 ml/min. On some of the incubation mixtures containing plasma membrane-enriched material from Wistar rat liver the bilirubins were determined by both the ethyl anthranilate diazo-method (24, 25) and the alkaline methanolysis-HPLC procedure (35).

Methodological approach

Demonstration of disproportionation of BMG according to Eq. 1 requires assessment of the proportion of the substrate, BMG, and of the reaction products, bilirubin and BDG. To determine the underlying mechanism (dipyrrole exchange or transglucuronidation) (Fig. 1) it is essential to measure the isomeric composition (IIIα, IXα, XIIIα) both before and after incubation, since dipyrrole exchange already occurs to a variable extent during isolation and purification of BMG. As shown in Fig. 2 the degree of disproportionation required to achieve a measurable change in the isomeric composition of bilirubin (present either in unconjugated bilirubin or in BMG and BDG) depends on the isomeric purity of the monoglucuronide. With BMG containing only the IXα-aglycone both the rates of disproportionation and isomerization are expected to change in parallel. The equilibrium state of bilirubin corresponds to a 1:2:1 proportion between IIIα, IXα, and XIIIα-isomers (50% of bilirubin-IXα). With BMG preparations that contain progressively larger fractions of the artificial IIIα- and XIIIα-bilirubin aglycones correspondingly larger extents of disproportionation and thus, in general, longer incubation periods will be required to achieve detectable enrichment of the IIIα- and XIIIα-isomers. In the extreme case where the aglycone of BMG would consist of an equilibrium mixture

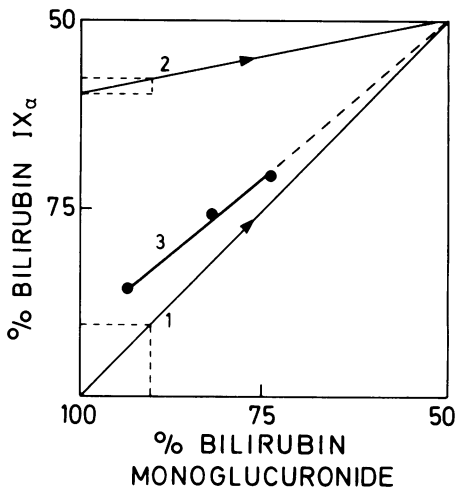

FIGURE 2 Correlation between the degree of advancement of the disproportionation reaction, 2 BMG ⇌ B + BDG and of the underlying dipyrrole exchange reaction. Starting from BMG containing exclusively the IXα-aglycone (lower left-hand corner), the change in composition follows line 1. For example, a 10% decrease of BMG corresponds to an identical fractional decrease of IXα-isomer. The equilibrium state corresponds to a 1:2:1 proportion of B:BMG:BDG (50% BMG) and a 1:2:1 proportion of IIIα-, IXα-, and XIIIα-isomers (50% IXα). Line 2 represents the changes in compositional coordinates when a pure BMG preparation (aglycone: IIIα/IXα/XIIIα, 1:3:1) is allowed to disproportionate. In this case for an identical degree of disproportionation, (10% decrease of BMG) as in the example given in case 1, the corresponding decrease of the IXα-isomer is only 2%, which is insufficient for detection by TLC. A pure BMG preparation that is fully isomerized with regard to its aglycone (50% IXα) (upper left-hand corner) would still undergo disproportionation but dipyrrole exchange would be undetectable by isomer analysis. Line 3 represents one of our experiments (Table III, experiment (*a*) with analysis of the isomers by the alkaline methanolysis-HPLC procedure. The three values were obtained at incubation times 0, 3, and 6 min.

(1:2:1) of IIIα-, IXα- and XIIIα-isomers it would be impossible to detect any exchange of dipyrroles by isomer analysis. Valid interpretation of kinetic studies of the disproportionation reaction of BMG further requires that autoxidation of bile pigments and hydrolysis of the conjugates are negligible. In the presence of N_2 fractions, at pH 6.6 at 37°C in absence of light, breakdown of rubins was absent over the first 8-min period of incubation as shown by a stable value of the *p*-iodoaniline azo color (Fig. 3a). Confirming the work of Jansen et al. (12) glucaro-1,4-lactone inhibited hydrolysis over the first 3–4 min (Fig. 4b), since in the presence of 1 mM ascorbic acid the bile pigment composition remained unchanged. The two basic conditions thus seem to be fulfilled over relatively short incubation periods (3–4 min).

A first analytical approach can be based conveniently on the ethyl anthranilate diazo-method because, under appropriate reaction conditions (see above), the azo color measures the sum of BMG and BDG and a decrease thus reflects a proportionate increase in unconjugated bilirubin. Chromatographic determination of the ratio r, unconjugated azodipyrrole/azodipyrrole glucuronide (Eq. 2) further per-

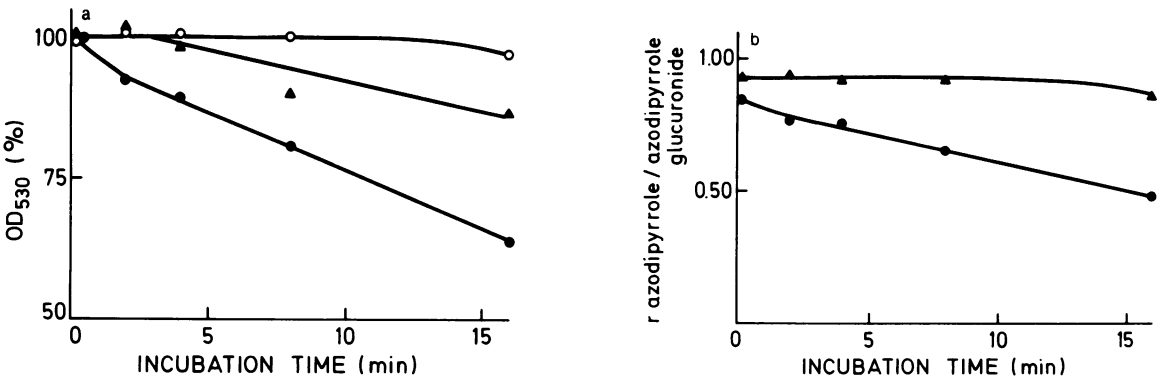

FIGURE 3 Change of the optical density (OD_{530}) (panel a) and of the ratio r, azodipyrrole/azodipyrrole glucuronide (panel b) as a function of time. BMG (200 μM final concentration) was incubated with N_2 fractions from Gunn rat liver at 37°C and pH 6.6 in incubation system A. Samples were removed and subjected to the ethyl anthranilate (●) or the *p*-iodoaniline diazo-procedures (○). BMG was also incubated in the presence of 1 mM ascorbic acid and subjected to the ethyl anthranilate diazo method (▲). Optical densities are given as the percentage of the values found at zero time. At zero time one BMG preparation (○) contained 8% of BDG. Stable values of *p*-iodoaniline azo color show absence of breakdown of the tetrapyrrole skeleton of bile pigments.

mits calculation of the proportion of mono- and diglucuronides (Eq. 3) and thus verification of the stoichiometry of the disproportionation. It is critical for these calculations that the unconjugated azodipyrrole is derived exclusively from BMG. As already mentioned, careful control of the pH of diazo-cleavage permits effective inhibition of reaction of unconjugated bilirubin. In addition, acid-catalyzed hydrolysis of the sugar-ester bonds did not occur at pH 2.7 because during diazo-treatment of pure BDG solely azodipyrrole glucuronides was formed (14 preparations tested). With purified BMG preparations the ratio r closely approximated the theoretical value 1.

Changes in isomeric composition can be monitored, e.g. by saponification (34) followed by TLC of bilirubin isomers (15). The alkaline methanolysis-HPLC procedure (35) offers a more direct approach because it permits determination of the concentrations of bilirubin and its mono- and diconjugates, and in the same run their isomeric compositions. In these procedures, the disproportionation and dipyrrole exchange reactions are stopped instantaneously by transferring the bile pigment containing samples (*a*) to a slightly acidic aqueous medium (12, 16) in the diazo-method (24, 25), (*b*) to methanol containing 60 mM ascorbic acid (15) in the alkaline methanolysis procedures (34, 35), and (*c*) to a strongly alkaline medium containing 60 mM ascorbic acid (15) in the alkaline hydrolysis procedure (34).

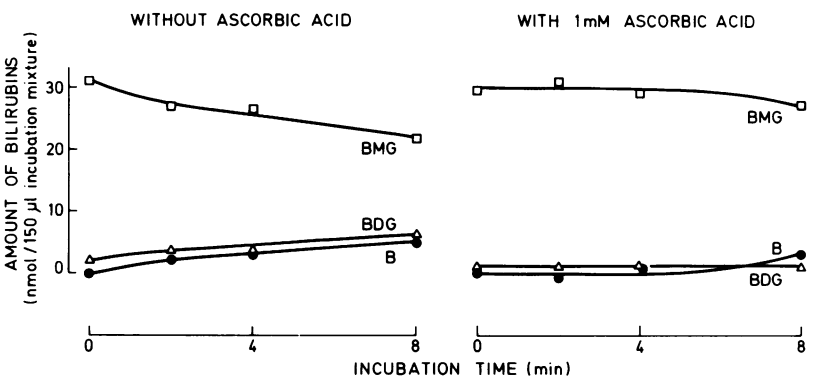

FIGURE 4 Concentration changes of bile pigments. BMG (200 μM final concentration) was incubated with N_2-fraction from Gunn rat liver at 37°C at pH 6.6, without (panel a) and with 1 mM ascorbic acid (panel b). Total bilirubin conjugates (BMG + BDG) were assayed by the ethyl anthranilate diazo-procedure. The amount of BMG and BDG were calculated from equation 2 and 3 after TLC of the ethyl anthranilate azo derivatives. Total bilirubins (B, BMG, BDG) determined by the *p*-iodoaniline diazo method, and the sum of BMG plus BDG were used to estimate unconjugated bilirubin. The changes in concentration (panel a) are compatible with stoichiometric formation of bilirubin and BDG from 2 mol of BMG. Hydrolysis of BMG to bilirubin only occurred after 4 min incubation (panel b).

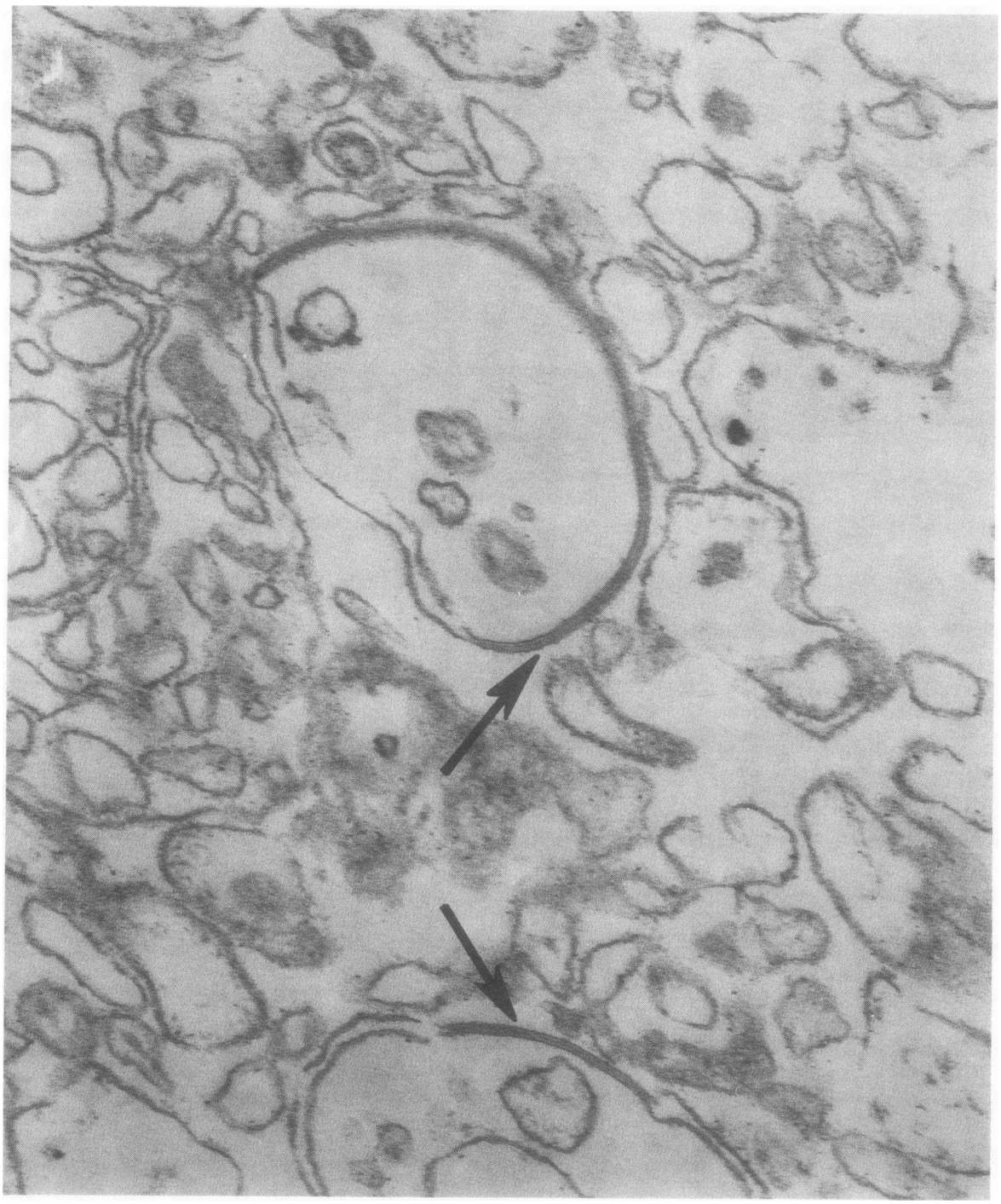

FIGURE 5 Electron microscopy of the plasma membrane-enriched fraction (N_2) from Wistar rat liver. ×92,000. Two gap junctions are indicated by arrows.

RESULTS

Experiments in vitro. Marker enzyme activities of subcellular fractions from liver of Gunn rats and Wistar rats agreed satisfactorily with published values (12, 13). In particular, for the N_2-fractions the activity of 5'-nucleotidase (μmol/mg protein per 20 min) was 53 and 60 (two Wistar rats) and 66±9 (SD, three Gunn rats), as compared to 74 (12) and 70 (13), respectively. Low activity of glucose-6-phosphatase (~0.3 μmol/

TABLE I
*Isomeric Composition of Bilirubin Aglycones during Incubation of BMG with N_2-fractions from Gunn Rat Liver**

	Experiment					
	1		2		3	
	No ascorbic acid				1 mM ascorbic acid	
Incubation time	IX	III + XIII	IX	III + XIII	IX	III + XIII
min	%		%		%	
0	78	22	74	26	71	29
2	66	34	64	36	73	27
4	64	36	65	35	71	29
8	63	37	60	40	74	26
16	59	41	58	42	73	24

* BMG (200 μM) was incubated at 37°C and pH 6.6 in incubation system A. At the indicated time intervals duplicate samples were removed and subjected to alkaline hydrolysis followed by TLC.
Abbreviations used in this table: IX, bilirubin IXα; III, bilirubin IIIα; XIII, bilirubin XIIIα.

mg protein/20 min) demonstrated negligible contamination by microsomal material. The ratio, phospholipid phosphorus/protein (μmol/mg) of 1.17 is comparable to the value, 0.92 given by Touster et al. (27). Electron microscopic examination of N_2-fractions from Gunn and Wistar rat liver revealed primarily smooth membranes without ribosomes. A few gap junctions (arrows in Fig. 5) could be clearly observed.

During incubation of plasma membrane-enriched N_2 fractions from Gunn rat liver (incubation system A) with BMG the total bilirubin content was stable over the first 8 min, with only 3% color being lost after 16 min (Fig. 3a). In contrast, total conjugates as measured by the ethyl anthranilate method (Fig. 3a) decreased steadily, the loss of total conjugates after 16 min amounting to 35%. The reaction was accompanied by an approximately parallel decrease of the ratio *r* (Fig. 3b). Comparison of the amounts of bilirubin and BDG produced showed stoichiometric disproportionation of BMG (Eq. 1) up to 3–4 min (Fig. 4a). The reaction was paralleled by an increase of the IIIα- and XIIIα-isomers of the bilirubin aglycone (Table I) proving exchange of dipyrrole moieties. Ascorbic acid (1 mM) completely inhibited formation of BDG from BMG (Fig. 4b) and exchange of dipyrrole moieties (Table I) over the first 8 min. Exactly similar observations were made for Wistar rat liver (Table II). Dipyrrole exchange was confirmed by the more direct alkaline methanolysis-HPLC procedure (Table III).

Incubation of liver homogenates from Wistar and Gunn rats with BMG at pH 6.5 resulted in formation of BDG with a conversion rate of 4.1 and 21 nmol/mg protein per min compared to 47±9 (SD, $n = 4$) for N_2-fractions (incubation system A). As for the N_2-fractions, dipyrrole exchange was confirmed by azopigment analysis and simultaneous analysis of the bilirubin α-isomers. Again, addition of ascorbic acid (final concentration 1 mM) to the incubation mixtures resulted in complete inhibition of the reaction.

Incubation of 100-206 μM BMG with N_2-fractions from Gunn rat liver (0–427 μg protein/ml) and from Wistar rat liver (0-613 μg protein/ml) did not show the linear increase of the conversion rate expected for

TABLE II
Incubation of BMG and N_2-fractions from Wistar Rat Liver at pH 6.6 and 37°C in Incubation System A*

Incubation time	$OD_{500}^{1\,cm}$‡	r	Bilirubin IXα
min			
0	0.363	0.952	92
4	0.356	0.881	82
8	0.345	0.778	79
16	0.322	0.743	73

* Final concentration of BMG: 155 μM.
‡ Obtained after ethylanthranilate diazo method.
Abbreviations used in this table: r, azodipyrrole/azodipyrrole glucuronide.

TABLE III
*Incubation of BMG with N_2-fractions from Wistar Rat Liver**

Experiment	Incubation time	B	BMG	BDG	Bilirubin α-isomers IX	Bilirubin α-isomers III + XIII
	min		%			%
a	0	4	94	2	86	14
	3	13	83	4	77	23
	6	19	73	8	71	29
b	0	4	92	4	84	16
	3	14	80	6	75	25
	6	20	70	10	70	30

* BMG (173 μM) was incubated with N_2-fractions from Wistar rat liver at 37°C and pH 6.6 in incubation system B. At the indicated time intervals samples were removed and subjected to the alkaline methanolysis-HPLC procedure. Experiment a was done with 'natural' N_2-fraction; in experiment b N_2-fraction was heated for 10 min at 100°C before preparing the incubation mixture.

a catalyzed reaction (Fig. 6). If anything, the data suggested slight inhibition of the spontaneous rate of conversion by the added membrane material. Also, heating the N_2-fractions for 10 min at 100°C did not affect the rat of BDG formation for material from Wistar rat liver and was stimulatory with preparations from Gunn rat liver (Table IV).

Stability of BMG. To define conditions suitable for injecting BMG, the stability of the pigment was investigated in aqueous solutions at pH 7.4 (Table V). Within 2 h the total concentration of conjugates decreased by 21% with a concomitant decrease of the *r* values. Close agreement was found with *r* values calculated on the assumption that the change involves disproportionation of BMG. In contrast, in presence of 1 mM sodium ascorbate at pH 7.4 and 25°C only 5% of BMG was lost after 2 h.

In vivo experiments. Two BMG preparations containing 0 and 2% of BDG, respectively, were dissolved in phosphate buffer containing ascorbic acid and injected in individual Gunn rats. Rapid excretion resulted, more than 95% of bilirubin conjugates being recovered in bile after 40 min. As shown by both azo pigment analysis and alkaline methanolysis, no BDG was formed from BMG either during the infusion or the passage of BMG through the liver (Table VI).

DISCUSSION

Jansen et al. (12) have reported the presence in liver plasma membranes of an enzyme catalyzing transformation of 2 mol of BMG into 1 mol of bilirubin and 1 mol of BDG. These studies were performed with the N_2-fraction obtained from liver homogenates of Wistar rats according to the procedure of Touster et al. (27). Similar activity has been detected in Gunn rat liver (13). In contrast, in the present work, spontaneous disproportionation of BMG (Eq. 1) at pH 6.6 was not enhanced by addition of N_2 fractions from liver of either Gunn rats or Wistar rats (Fig. 6), as would be expected for a catalytic reaction. Also, inactivation of the postulated enzyme by heating either had little effect on the reaction rates or produced some increase (Table IV). Slight inhibition of the reaction by added membrane material (Fig. 6) suggests that the rather low rates observed with liver homogenates could be

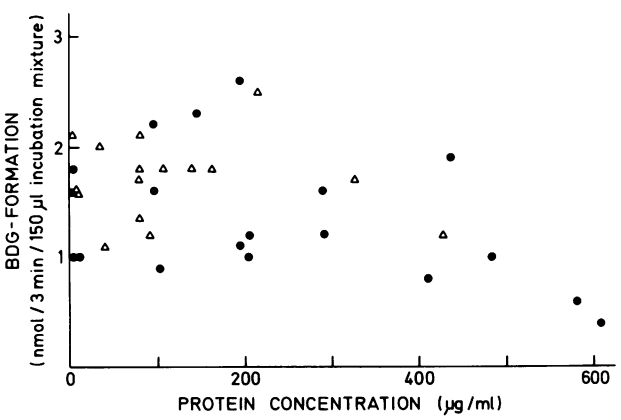

FIGURE 6 Rate of formation of bilirubin diglucuronide (BDG) as a function of protein concentration of N_2-fraction from Gunn rat (△) and Wistar rat liver (●). BMG (100–206 μM, final concentration) was incubated for 3 min with various amounts of N_2-fractions at 37°C and pH 6.6 (seven experiments with three N_2-preparations from Gunn rat liver, six experiments with three N_2-preparations from Wistar rat liver). BDG was quantified by the ethyl anthranilate diazomethod.

TABLE IV
*BDG-formation from BMG after Heat Denaturation of N_2-fraction**

	BDG formed		Protein concentration
	Pretreatment of N_2-fraction		
	10 min, 0°C	10 min, 100°C	μg/ml
	nmol/3 min/150 μl incubation mixture		
Wistar	1.2	1.2	204
Wistar	2.6	2.4	193
Gunn	1.8	3.4	140
Gunn	1.2	2.0	91

* To pretreated N_2-preparations buffered glucaro-1,4-lactone was added and the incubation was started (system B). The final concentration of BMG was 120–200 μM.

due to inhibition by cytosol protein. Gordon and Goresky (11) could not demonstrate any disproportionation of BMG in presence of N_2 fractions from liver of Sprague-Dawley rats. However, their incubation conditions may not have been optimal to examine this reaction. The protein concentration (1 mg/ml) of the incubation mixtures was rather high compared to 80 μg/ml used in the standard system (12) and according to our findings (Fig. 6) might cause inhibition of the disproportionation. Moreover, the BMG concentration was only 40 μM compared to 112–136 μM in the standard system (12). On the other hand, it is possible that in the incubation system used by Gordon and Goresky (11) the IXα-structure of bilirubin was stabilized so that no nonenzymic processes were observed.

The mechanism of the observed conversion has been described as sugar transfer (transglucuronidation) from one mole of BMG to another (12). From our results a transglucuronidation mechanism can clearly be ruled out. Stoichiometric formation of BDG and bilirubin out of 2 mol of BMG was paralleled by a decrease of IXα-isomers and an increase of the IIIα- and XIIIα-isomers. The evidence given in support of a transglucuronidation mechanism (12) appears to be unconvincing. Not the initial isomeric composition of the BMG preparation tested by Jansen et al. (12), but only differences in composition found before and after incubation were reported. Therefore, it is impossible to know how closely the isomeric composition of the bilirubin aglycone of the BMG preparation used approached the equilibrium state (1:2:1). Perhaps more importantly, the incubation periods used were rather short (3 min) and probably were insufficient for significant enrichment in IIIα- and XIIIα-isomers, leading the authors to postulate that transglucuronidation must underly the observed disproportionation reaction. In the present work, both the disproportionation reaction and the formation of bilirubin-IIIα and -XIIIα isomers were inhibited completely by the free-radical scavenger, ascorbic acid (15) during incubation of BMG with liver homogenate or plasma membrane-enriched N_2 fractions. Therefore, the underlying reaction mechanism is dipyrrole exchange proceeding probably via formation of free radicals.

It is questionable whether the UDP-glucuronic acid-independent formation of BDG plays any role in vivo. The data in support of the transglucuronidation hypothesis (12) on one hand are not convincing. On the other hand, BDG formation by nonenzymic dipyrrole exchange, as was shown by the present study in vitro, must be insignificant in vivo, because unconjugated and conjugated bilirubins present in biological fluids show nearly exclusively the IXα-isomeric structure (15). Our in vivo studies confirm the observation of Blanckaert et al. (17) that in Gunn rats formation of BDG from BMG does not occur and lend support to the conclusion that UDP-glucuronic acid-dependent synthesis of BDG, which has been validated experimentally (10), is the only plausible pathway of in vivo formation of BDG. The discrepancy of our in vivo results of those to other authors (13, 14) is as yet unexplained.

The conclusions from the present work are in sharp contradiction with those of other authors (12–14). Although it is conceivable that both our Wistar and homozygous Gunn rats are deficient in bilirubin glucu-

TABLE V
*Stability of BMG at 25°C and pH 7.4 in the Absence or Presence of Sodium Ascorbate**

	pH of incubation mixture				
	7.44			7.42	
	No ascorbate added			1 mM ascorbate	
Incubation time	OD_{546}	r	r′	OD_{546}	r
min	%			%	
0	100	1.02	1.00	100	1.00
30	91	0.83	0.82	99	1.01
60	87	0.68	0.74	98	0.98
90	82	0.65	0.64	96	0.97
120	79	0.57	0.58	95	1.01

* BMG (10 μM) was incubated at 25°C in 0.1 M phosphate buffer pH 7.4 in the dark. Optical density was determined by the ethylanthranilate method.
Abbreviations used in this table; r, azodipyrrole/azodipyrrole glucuronide; r′, theoretical ratio azodipyrrole/azodipyrrole glucuronide, calculated from the decrease of total conjugated bilirubin (OD_{546}), assuming dipyrrole exchange to be the reaction mechanism.

TABLE VI
*Composition of Injected BMG-solution and Biliary Excretion in Homozygous Gunn Rats**

	Rat 1					Rat 2				
	EA		AM			EA		AM		
	BMG	BDG	B	MM	DM	BMG	BDG	B	MM	DM
	%			%		%			%	
BMG-solution										
before infusion	98	2	0	100	0	100	0	0	100	0
after infusion	97	3	0	100	0	98	2	0	100	0
Rubins excreted in bile in 40 min	98	2	0	100	0	99	1	1	98	1

* 207 nmol BMG were infused into rat 1 and 287 nmol BMG into rat 2. Of the injected pigments 98 and 95%, respectively, were recovered in bile in 40 min.

Abbreviations used in this table: EA, ethylanthranilate diazo procedure; AM, alkaline methylanolysis procedure; MM, bilirubin monomethylester; DM, bilirubindimethylester.

ronoside glucuronyltransferase, such an explanation seems highly unlikely. It is suggested that some unsuspected technical failure in the approach(es) used by the proponents of the transglucuronidase hypothesis (12–14) is at the basis of the discrepant results. It should be emphasized that the pronounced lability of bilirubin conjugates, in particular of BMG, may easily give rise to artifacts and lead to erroneous conclusions. Some of these problems can be overcome by taking advantage of the stabilizing effect of ascorbic acid (Table V).

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