On the Binding of Bilirubin and Its Structural Analogues to Hepatic Microsomal Bilirubin UDPglucuronyltransferase†

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ABSTRACT: Hepatic glucuronidation of the asymmetrical natural bilirubin molecule results in formation of two different positional isomers, bilirubin C-8 monoglucuronide and bilirubin C-12 monoglucuronide. In view of the existence of multiple isoforms of UDPglucuronyltransferase, which is the microsomal enzyme system responsible for bilirubin esterification, we performed kinetic analysis of microsomal glucuronidation of bilirubin and a number of its structural congeners to determine whether synthesis of the two monoglucuronide isomers involved two distinct substrate-binding sites or reflected two different modes of binding to a single catalytic site. Both isomers were found in all tested species (man, rat, guinea pig, sheep), but there were marked species differences in the C-8/C-12 ratio of monoglucuronide found in bile or formed by liver microsomes. Correspondence between in vivo and in vitro results for such regioselectivity of glucuronidation was excellent in each species. On the basis of our results of kinetic analysis of bilirubin esterification at variable pigment substrate concentrations and inhibition studies with alternative substrates, we postulate that both natural monoglucuronide isomers are synthesized at a single binding site. Possible mechanisms responsible for the markedly regioselective esterification of bilirubin by rat and sheep liver were investigated by study of glucuronidation of selected structural analogues of the pigment. Our results do not support explanations of regioselectivity of bilirubin glucuronidation in terms of (i) preferential binding of either the C-8- or C-12-containing dipyrrolic half of the asymmetrical bilirubin molecule or (ii) enantioselective complexation of bilirubin UDPglucuronyltransferase to one of the two chirality enantiomers of intramolecularly hydrogen-bonded bilirubin. Collectively, our findings suggest that the molecular form(s) of bilirubin able to engage in catalytically effective binding to UDPglucuronyltransferase does (do) not correspond with intramolecularly hydrogen-bonded conformers and that the nature of the β-substituents of the outer pyrromethenone rings is a key determinant of glucuronidation rate.

In mammals, conjugation with glucuronic acid is a crucial step in the metabolism of a vast array of endogenous and exogenous compounds. These conjugation reactions occur primarily in the liver and are catalyzed by a microsomal UDPglucuronyltransferase system (UDPGT;[1] EC 2.4.1.17) that comprises a family of enzymes with overlapping substrate specificities (Dutton, 1980; Burchell, 1981). An important substrate of this transferase system is bilirubin, the breakdown product of heme. Detoxication and effective elimination of bilirubin require hepatic esterification of at least one of its two propionic acid side chains. Three UDP-sugars, UDP-glucuronic acid (UDPGlcUA), UDP-glucose, and UDP-xylose, have been identified as potential cosubstrates for esterification of bilirubin in humans and in a wide variety of animals including mammals, avians, and reptiles (Fevery et al., 1972, 1977; Cornelius et al., 1975). The proportion of glucuronides, glucosides, and xylosides as well as the ratio of monoesters to diesters in the conjugated pigment excreted in bile appears to be species dependent. In man and most other species, glucuronides account for over 90% of the natural esterified bilirubins. Bilirubin glucosides and/or xylosides account for a major fraction of the esterified pigment in bile of dog, horse, and chicken.

Two positional isomers (C-8 and C-12; see Figure 1 and Table I) of bilirubin monoglucuronide (BMG) are formed in vitro in microsomal incubation systems and in vivo (Jansen & Billing, 1971; Blanckaert et al., 1979; Blanckaert, 1980). Esterification of intermediate BMG to form bilirubin diglucuronide (BDG) occurs in relatively few species, including humans, rats, cats, and dogs (Fevery et al., 1977). Synthesis of BDG does not result in any obvious benefit compared to synthesis of monoesters alone because disposal of bilirubin appears to occur effectively in species that excrete bilirubin exclusively in monoesterified form. Therefore, formation of monoconjugates by bilirubin UDPGT is the essential and critical step in bilirubin disposal, even in those species that excrete predominantly diconjugates in bile.

Since the hepatic UDPGT system comprises many forms of transferase that are differentially regulated and appear to be selective for different sets of substrates (Dutton, 1980; Burchell, 1981), the question arises whether formation of the two natural BMG isomers depends on discrete enzyme forms and/or different bilirubin-binding sites. There is evidence that glucuronidation of bilirubin depends on a singular member of

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[1] Abbreviations: UDPGT, UDPglucuronyltransferase; UDPGlcUA, UDP-glucuronic acid; BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Chapso, 3-[(3-cholamidopropyl)dimethylammonium]-2-hydroxy-1-propanesulfonate; TLC, thin-layer chromatography; Azpm, azopyrromethene; Man, mannose; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Table I: β-Substituents of Bilirubins Used as Substrates or Inhibitors of Bilirubin UDPGT[a]

compd	β-substituents							
	2	3	7	8	12	13	17	18
bilirubin	Me	V	Me	P	P	Me	Me	V
C-8 BMG	Me	V	Me	PGlcUA	P	Me	Me	V
C-12 BMG	Me	V	Me	P	PGlcUA	Me	Me	V
bilirubin IIIα	V	Me	Me	P	P	Me	Me	V
bilirubin XIIIα	Me	V	Me	P	P	Me	V	Me
mesobilirubin IXα	Me	Et	Me	P	P	Me	Me	Et
deuterobilirubin IXα	Me	H	Me	P	P	Me	Me	H
AcCyst-bilirubin IXα	Me	V	Me	P	P	Me	Me	AcCyst
bilirubin IXα ditauryl conjugate	Me	V	Me	Ptaur	Ptaur	Me	Me	V
bilirubin IXβ	Me	P	P	Me	Me	V	Me	V

[a] The β-substituents attached at carbons 2, 3, 7, 8, 12, 13, 17, and 18 (Figure 1) are shown. Abbreviations: Me = $-CH_3$; V = $-CH{=}CH_2$; H = $-H$; Et = $-CH_2CH_3$; AcCyst = $CH(CH_3)SCH_2CH(NHCOCH_3)COOH$; P = $-(CH_2)_2COOH$; Ptaur = $-(CH_2)_2CONH(CH_2)_2SO_3Na$; PGlcUA = P group esterified with glucuronic acid.

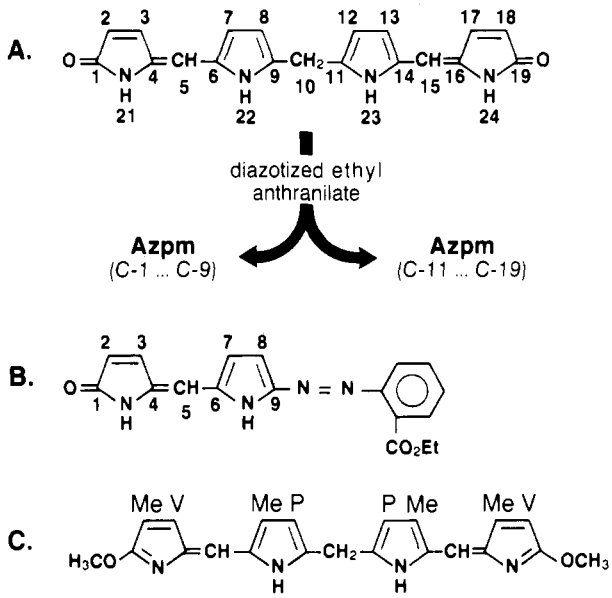

FIGURE 1: Structures of test pigments with bilinoid skeleton. The common bilinoid skeletal structure of the various test compounds and numbering of the carbon atoms (1–19) are depicted in panel A. The composition of the β-substituents in the various bilirubins used in this study is shown in Table I. In the diazo reaction, diazotized ethyl-anthranilate cleaves the tetrapyrrolic bilinoid molecule into two dipyrrolic azopyrromethene derivatives (Azpm), one of which is shown in panel B. Panel C depicts dimethoxybilirubin IXα.

the various UDPGT forms in liver. When the different transferases are separated, the capacity to glucuronidate bilirubin was found in only one form (Burchell, 1981; Chowdhury et al., 1986a,b). Further support for this notion comes from the observation that a mutant rat strain, the so-called homozygous Gunn rat, has no detectable hepatic UDPGT activity toward bilirubin whereas glucuronidation of all other major substrates of hepatic UDPGT appears to be either normal or only partially defective (Weatherhill et al., 1980; Mackenzie & Owens, 1981; Chowdhury et al., 1986a,b). Because the esterification activities at the C-8 and C-12 side chains of natural bilirubin are copurified in highly purified bilirubin UDPGT from rat liver (Chowdhury et al., 1986a,b; Burchell & Blanckaert, 1984) and selective impairment of either C-8 BMG or C-12 BMG synthesis is not observed in conditions characterized by genetic deficiency of bilirubin UDPGT such as in Gilbert syndrome, Crigler–Najjar disease, or the heterozygote Gunn rat (N. Blanckaert, unpublished results), it seems reasonable to hypothesize that both natural BMG isomers may be formed by a singular transferase.

This study represents a kinetic approach to investigate the question whether synthesis of C-8 BMG and C-12 BMG is a result of either binding and glucuronidation of bilirubin at two distinct substrate-binding sites in bilirubin UDPGT or reflects two different modes of interaction of bilirubin at a single catalytic site. Kinetic analysis of bilirubin conjugation was facilitated by development of an improved procedure for functional assay of bilirubin UDPGT in fully disrupted microsomes, in which the selective membrane permeability barrier for hydrophilic compounds is eliminated. Evidence is presented that both C-8 BMG and C-12 BMG are formed at a single substrate-binding site in microsomal bilirubin UDPGT, and consideration is given to possible mechanisms responsible for the observed regioselectivity of bilirubin glucuronidation.

Materials and Methods

Chemicals and Test Pigments. The following chemicals were used: human and bovine serum albumin factor V, D-mannose 6-phosphate (Man-6-P), bilirubin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), NAD^+, UDPGlcUA, UDPGlcNAc, and Lubrol 12A9 (Sigma, St. Louis, MO); the zwitterionic detergent Chapso (Bio-Rad, Richmond, CA); Emulgen-911 (Kao-Atlas, Tokyo, Japan); glucaro-1,4-lactone (A grade) and digitonin (Calbiochem, San Diego, CA); δ-amino[4-^{14}C]levulinic acid (2.15 Bq/pmol) (New England Nuclear, Boston, MA). All other chemicals were of reagent-grade quality. Aqueous solutions were made with deionized water. Glass plates precoated with silica gel 60 (5763/0025 or 5762/0025, from Merck Chemical Division) were used for TLC.

[^{14}C]Bilirubin (specific radioactivity between 0.67 and 0.85 Bq/pmol) was prepared biosynthetically (Ostrow et al., 1961). Deuterobilirubin IXα, mesobilirubin IXα, crude ditauryl conjugate of bilirubin IXα (Figure 1 and Table I), and biliverdin dimethyl ester were purchased from Porphyrin Products (Logan, UT). The following compounds were prepared as previously described: bilirubin IIIα and XIIIα (Blanckaert et al., 1980); dimethoxybilirubin IXα dimethyl ester (Blanckaert, 1980; Kuenzle, 1970); biliverdin (McDonagh & Palma, 1980); bilirubin IXβ (Blanckaert et al., 1976); the product ("AcCyst-bilirubin XIα", see Table I) of the addition reaction of *N*-acetyl-L-cysteine to the *exo*-vinyl group at carbon 18 of bilirubin (Mannito & Monti, 1973). Dimethoxybilirubin IXα [λ_{max} at 415 nm in both $CHCl_3$/CH_3OH (1:1 v/v) and 0.140 M Hepes buffer, pH 7.7] was obtained from its dimethyl ester by saponification (Blanckaert et al., 1976) and purified by TLC with chloroform/methanol (9:1 v/v). The unesterified pigment was freely soluble in 0.140 M Hepes buffer, pH 7.7, or in the homogenization buffer (0.25 M sucrose containing 1 mM EDTA and buffered to pH 7.4 with 5 mM Tris-HCl). [^{14}C]Bilirubin and commercially obtained mesobilirubin IXα and deuterobilirubin IXα were pu-

rified by TLC with chloroform containing 0.75% ethanol stabilizer as solvent. The commercial bilirubin IXα ditauryl conjugate preparation was purified by TLC with chloroform/methanol/water (10:5:1 by volume) to remove contaminating monotauryl conjugate.

Preparation of Microsomes and Collection of Bile. Liver microsomes were obtained after an overnight fast from adult male R/A Pfd rats (Leyten et al., 1986), homozygous Gunn rats, and guinea pigs. Sheep specimens were collected from adult females at a local slaughterhouse. Guinea pig and sheep bile were aspirated from the gallbladder. Human bile was collected from healthy volunteers, and human liver was obtained at surgery from patients who underwent cholecystectomy and had no hepatic or hematologic abnormality under protocols approved by the University of Leuven Hospitals Committee for Conduct of Human Research, as reported elsewhere (Fevery et al., 1983). Informed consent was given prior to surgery. Homogenization of liver tissue and preparation of washed microsomes were done as described (Vanstapel et al., 1986). Microsomes (40–50 mg of protein/mL) were kept under argon at −80 °C for up to 2 months. Under these storage conditions, activity and latency of both bilirubin UDPGT and Man-6-phosphatase did not change. Latency of Man-6-phosphatase in the thawed native microsomes was at least 95%, indicating that the microsomal membrane permeability barrier remained highly intact (Arion et al., 1976). Throughout this paper, such UDPGT preparations are denoted as "intact microsomes". Except when stated otherwise, UDPGT activities were measured in disrupted microsomes prepared by preincubation of a microsomal suspension (2 mg of protein/mL of homogenization buffer) for 60 min at 0 °C for 4 mM Chapso. Such pretreatment produces "fully disrupted microsomes" in which latency of Man-6-phosphatase is completely eliminated (Vanstapel et al., 1986). Disruption of microsomes with digitonin was performed as described elsewhere (Heirwegh et al., 1972).

Assays of Bilirubin UDPGT Activity. Two different methods were employed for determination of bilirubin glucuronides in incubation mixtures. The *o*-ethylanthranilate diazo method (Van Roy & Heirweigh, 1968) was used to determine V_{max} of glucuronide formation by bilirubin UDPGT, i.e., total bilirubin UDPGT activity. This assay accounts for total BMG synthesis (sum of C-8 and C-12 isomers) from bilirubin irrespective of whether or not BMG remains unchanged or is subsequently converted to BDG in the enzyme assay incubation mixture. This is so because diesterified bilirubin gives the same analytical response as bilirubin monoester. Because of its adequacy and simplicity for assay of total bilirubin UDPGT, the diazo method was selected for investigation of transferase activation by detergent and for selection of saturating concentrations of substrate and cosubstrate.

Two limitations of the diazo method prompted its replacement by a more elaborate radioassay using [^{14}C]bilirubin when kinetic analysis of bilirubin glucuronidation was performed. First, the sensitivity of the diazo method was insufficient for measurement of glucuronide formation at the low bilirubin substrate concentrations required in the kinetic studies. Second, kinetic analysis of C-8 BMG and C-12 BMG synthesis was only possible with a radioassay permitting specific determination of bilirubin, its individual C-8 and C-12 monoesters, and its diester.

Measurement of Total Bilirubin UDPGT. The enzyme assay incubation mixture (total volume of 2.0 mL) contained the following components: fully disrupted microsomes (500 μL of suspension/2.0 mL of final incubation mixture, to obtain a final concentration of 0.5–0.8 mg of microsomal protein/mL); 0.140 M Hepes buffer, pH 7.7; 6.25 mM $MgCl_2$; 10 mM NAD^+; 1 mM glucaro-1,4-lactone; 2 mM Chapso; 100 μM bilirubin; 50 μM albumin; UDPGlcUA, 0 or 5 mM for blank and test, respectively. Preparation of bilirubinate–albumin complex, mixing of the components, and incubation at 37 °C were done as described elsewhere (Vermeir et al., 1984). Except when stated otherwise, portions of 600 μL were withdrawn 3, 6, and 9 min after addition of UDPGlcUA and mixed immediately with 2 mL of ice-cold glycine/HCl buffer (0.4 M HCl adjusted to pH 2.7 with glycine). Esterified bilirubins were then measured by coupling reaction at 25 °C with diazotized *o*-ethylanthranilate (Heirwegh & Blanckaert, 1981). Enzyme activities with bilirubin IIIα, bilirubin XIIIα, mesobilirubin IXα, or deuterobilirubin IXα were assayed as described above except for replacement of bilirubin by the alternative substrate.

Radioassay of Bilirubin UDPGT Activity. The previously reported radioassay (Vermeir et al., 1984), with analysis of reaction products by TLC using chloroform/methanol/acetic acid (97:2:1 by volume), was employed with the following modifications. UDPGlcNAc and albumin were omitted, and fully disrupted microsomes (0.1–2.0 mg of protein/mL of total assay mixture) were used. The assay medium contained 2 mM Chapso, and the final concentration of [^{14}C]bilirubin was between 0.5 and 20 μM. Except when stated otherwise, radiolabeled pigment dissolved in 0.05 M NaOH containing 1 mM EDTA was added directly to the incubation mixture containing all components except UDPGlcUA.

Structure Analysis of Esterified Bile Pigment. Glucuronide formation in microsomal incubation mixtures containing bilirubin IIIα or XIIIα, mesobilirubin IXα, deuterobilirubin IXα, AcCyst-bilirubin IXα, or bilirubin IXβ was verified as follows. Test (containing UDPGlcUA) and blank (without UDPGlcUA) incubation mixtures were treated with diazotized *o*-ethylanthranilate (Van Roy & Heirwegh, 1968). The Azpm azo derivates (Figure 1) were subjected to TLC as described elsewhere (Heirwegh & Blanckaert, 1981; Blanckaert et al., 1977), with azopigments obtained by diazo reaction of the corresponding unesterified substrate pigment in the presence of propan-1-ol (Van Roy & Heirwegh, 1968) as chromatographic reference. The glucuronidated azo pigment fraction present as a new band in the chromatogram from the test incubation mixture but absent in the blank was subjected to alkaline methanolysis (Heirwegh & Blanckaert, 1981). This procedure resulted in conversion of glucuronidated azo pigment to the corresponding Azpm monomethyl ester. Thus, demonstration that methanolysis resulted in formation of a monomethyl ester derivative permitted verification of the glucuronide structure of the parent azo pigment. Moreover, this derivatization procedure allowed identification of the positional isomers in the glucuronidated azo pigment fraction because Azpm-8(Me) and Azpm-12(Me) can easily be separated by TLC (Blanckaert et al., 1976, 1977). Similarly, Azpm-7,3-(Me) and Azpm-3,7(Me) derived from bilirubin IXβ are readily distinguishable by TLC analysis (Blanckaert et al., 1977). For use as chromatographic references, Azpm monomethyl esters from the various test bilirubins were prepared by treatment of the corresponding unesterified Azpm derivatives with ethereal diazomethane (Blanckaert et al., 1976). *o*-Ethylanthranilate azo pigments are designated as proposed by Bergstrom and Blumenthal (Lightner, 1982).

Because biliverdin is diazo negative and dimethoxybilirubin IXα yielded markedly unstable azo derivatives, enzymatic incubation mixtures with these substrates were analyzed by

the alkaline methanolysis method and TLC of the reaction products with the corresponding monomethyl esters and dimethyl ester as chromatographic reference compounds. The reference pigments were obtained by partial saponification of the corresponding dimethyl esters as described (Blanckaert et al., 1976). Chloroform/methanol/water (40:9:1 by volume) and toluene/ethanol (25:2 v/v) were used as solvent systems for separation of free acid, monomethyl esters, and dimethyl ester of, respectively, biliverdin and dimethoxybilirubin IXα.

Isomeric composition of BMG isolated from bile (Fevery et al., 1983) was done by the alkaline methanolysis method (Blanckaert, 1980). The proportion of the two positional isomers in monoglucuronide formed from mesobilirubin IXα or deuterobilirubin IXα was assessed by the same method. Because for the deutero as well as for the meso pigments known reference tetrapyrrolic C-8 or C-12 monomethyl esters are unavailable, structural identification of the two monomethyl esters bands separated by TLC was done as follows. That the two bands in each case corresponded to an individual (C-8 or C-12) monomethyl ester was confirmed by analysis of their *o*-ethylanthranilate azo derivatives, which were prepared and analyzed by TLC as described (Blanckaert, 1980). Each band yielded equimolar amounts of a single isomer of unesterified azo pigment (Azpm-8 or Azpm-12) and a single isomer of methylated azo pigment [Azpm-8(Me) or Azpm-12(Me)]. Because reference azo pigments of known isomeric structure are available for the azo pigments derived from mesobilirubin IXα (Blanckaert et al., 1976), we were able to identify unambiguously the upper mesobilirubin IXα monomethyl ester band as the C-8 isomer and the lower one as the C-12 isomer. Due to unavailability of reference Azpm-8(Me) and Azpm-12(Me) deutero pigment positional isomers, assignment of isomeric structure to the two separated deuterobilirubin IXα monomethyl ester bands remained presumptive. In analogy with the chromatographic migration sequence for the monomethyl esters of bilirubin and mesobilirubin IXα, the fastest migrating band of the two deuterobilirubin XIα monomethyl ester isomers was tentatively identified as the C-8 isomer.

Other Determinations. Man-6-phosphatase activity expressed by native, untreated microsomes and by microsomes fully disrupted by pretreatment with Chapso was measured as reported elsewhere (Vanstapel et al., 1986). Total protein was assayed by a Coomassie Blue dye binding method with bovine serum albumin for calibration (Markwell et al., 1978). The concentration of Chapso in stock solutions was verified with the 7α-hydroxysteroid dehydrogenase method (Mashigue et al., 1976). Liquid scintillation counting was performed as described (Blanckaert, 1980). Kinetic analysis of microsomal bilirubin UDPglucuronyltransferase was carried out by determining initial rates of activity as a function of substrate concentrations (between 1 and 10 μM) at a fixed, saturating UDPGlcUA cosubstrate concentration in the presence and absence of a fixed inhibitor concentration. Initial velocity values were computed by linear least-squares regression analysis using the three time points in each assay. Then, double-reciprocal Lineweaver–Burk plots were constructed, and linear least-squares regression analysis was used to calculate apparent V_{max} values, Michaelis constants, and error estimates.

Results and Discussion

Activation of Bilirubin UDPGT in Detergent-Disrupted Rat Liver Microsomes. Two considerations led to use of fully disrupted microsomes in our kinetic studies. First, it has been postulated that the marked latency of transferase activity characterizing UDPGT of native microsomes may result from luminal orientation of the enzyme (Hallinan & de Brito, 1981; Mackenzie, 1986). Under such topological conditions, the lipid bilayer may act as a selective permeability barrier between the catalytic center and its substrates, especially the highly hydrophilic cosubstrate UDPGlcUA and possibly also polar pigment substrates such as bilirubin IXβ or dimethoxybilirubin IXα. To avoid interfering effects on enzyme kinetics by possible membrane translocation processes for substrates, we chose to use fully disrupted microsomes in which the membrane permeability barrier is eliminated. Second, untreated native microsomes are heterogeneous preparations composed of intact vesicles and disrupted microsomes, in which selective permeability is lacking and highly polar substrates have free access to luminally oriented microsomal enzymes (Arion et al., 1976, 1980). Because the catalytic activity of the nonlatent enzyme in disrupted microsomes is ~10-fold higher than that of the latent form of intact microsomal vesicles (see below), one can anticipate that the corresponding two kinetically different bilirubin UDPGT transferase forms must be considered in interpretation of kinetic data obtained with untreated microsomes, even in those preparations in which 95% of the microsomes are judged to be intact. We therefore preferred to perform kinetic analysis of bilirubin UDPGT in a homogeneous preparation of fully disrupted microsomes.

Diverse incubation systems using widely different bilirubin substrate concentrations (2–330 μM), with or without albumin, and various schemes of pretreatment of the microsomes have previously been employed for measurement of bilirubin UDPGT. For example, detergents like digitonin, Triton X-100, Lubrol, and Emulgen have been used for activation of the latent transferase, and markedly different effects have been reported, even when the same detergent was employed. For rat liver microsomes pretreated with digitonin, activation varied between less than 2-fold (Tavoloni et al., 1983; Whitmer et al., 1984) and up to 14-fold (Heirwegh et al., 1972).

Activation of total bilirubin UDPGT activity in microsomes pretreated with different detergents is shown in Figure 2. Maximal enzyme activity was limited to a narrow range of detergent concentrations for Lubrol and Emulgen. Both digitonin and Chapso gave a wider optimal concentration range, but maximal activation obtained with digitonin (about 7-fold) was lower than with the three other detergents (9–12-fold). These findings and the impurity of commercially available digitonin preparations, which may lead to batch-to-batch variability in its composition and activating properties, prompted us to select Chapso for further studies on the transferase system in detergent-disrupted microsomes.

Maximal bilirubin UDPGT activity obtained with microsomes disrupted with the optimal 4 mM Chapso concentration averaged 2.77 ± 0.191 nmol (mg of protein)$^{-1}$ min^{-1} (mean ± 1 SD; n = 6), which was between 11-fold and 17-fold (mean 15-fold) higher than the basal activity [0.18 ± 0.060 nmol (mg of protein)$^{-1}$ min^{-1}] found with the corresponding sealed native microsomes. This concentration of Chapso corresponded with the detergent concentration required to produce complete disruption of the microsomal membrane permeability barrier, as evidenced by total loss of latency of Man-6-phosphatase (Vanstapel et al., 1986).

As previously noticed for microsomal Man-6-phosphatase, dilution of Chapso by assaying bilirubin UDPGT of disrupted microsomes in an incubation medium that was not supplemented with detergent led to slight reversal of enzyme activation when Chapso fell below 1 mM (data not shown). Therefore, a final concentration of 2 mM Chapso was routinely

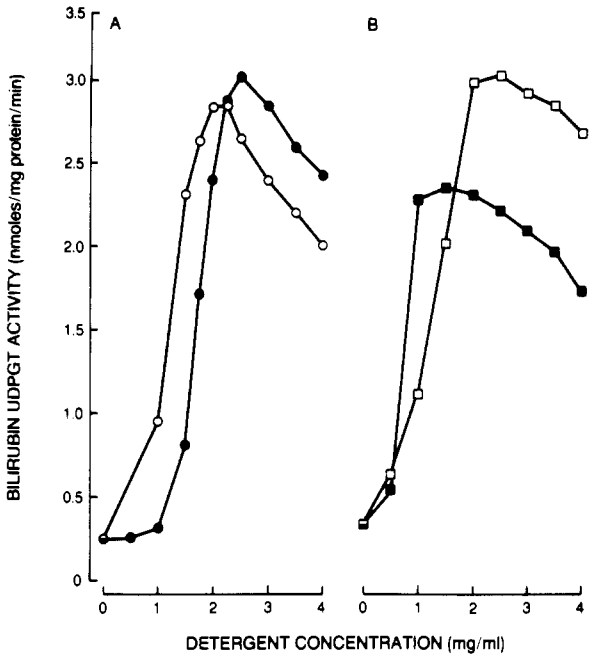

FIGURE 2: Activation profiles for bilirubin UDPGT in rat liver microsomes treated with detergent. Microsomes resuspended in buffer A (2 mg of protein/mL) were preincubated at 0 °C for 30 min with detergent in the concentrations shown in the abscissa. Except for the experiments with digitonin, detergent was added to the enzyme assay incubation mixture in order to obtain the same concentration as present during detergent pretreatment of the microsomes. Glucuronide formation was measured with the *o*-ethylanthranilate diazo method. Results obtained with Emulgen (○) and Lubrol (●) are shown in panel A; those with Chapso (□) and digitonin (■), in panel B.

employed for assay of total bilirubin UDPGT. For fully disrupted microsomes, product formation progress curves remained linear during at least 10 min at final Chapso concentration between 2 and 4 mM, indicating that no significant enzyme inactivation occurred in the enzyme assay incubation medium.

Total bilirubin UDPGT activity in fully disrupted microsomes was a linear function of the amount of microsomal protein for the tested concentration range between 0.1 and 2.0 mg of protein/mL of incubation mixture. In accordance with the conventional assay of bilirubin UDPGT (Heirwegh et al., 1972), bilirubin bound to serum albumin in a 2:1 molar ratio of pigment to protein was employed as substrate. Attempts to omit albumin from the incubation mixture resulted in erratic and imprecise emzyme activity values at bilirubin concentrations above 20 μM, possibly as a result of pigment aggregation and/or precipitation. That 100 μM bilirubin and 5 mM UDPGlcUA were saturating was demonstrated by the absence of any systematic or significant effect (variance less than 5%) on bilirubin conjugation rate when either the substrate concentration was changed between 50 and 150 μM or UDPGlcUA was varied between 3 and 10 mM. These findings are consistent with the results of monosubstrate kinetic analysis of BMG formation by fully disrupted microsomes (see below).

Assay Conditions for Kinetic Analysis of Bilirubin Glucuronidation by Rat Liver Microsomes. Albumin was omitted from the enzyme assay incubation mixture for monosubstrate kinetic analysis with bilirubin as variable substrate and in the inhibition studies because (i) possible interaction at the pigment-binding site(s) of albumin between bilirubin and inhibitors of bilirubin conjugation would have complicated interpretation of the kinetic findings and (ii) no difficulty was experienced in dissolving the pigment in the incubation mixture at the low concentrations (<20 μM) that are suitable for kinetic analysis. To test for possible aggregation and precipitation of bilirubin in the presence of fully disrupted microsomes prior to or during the enzyme radioassay, experiments were performed in which either the enzymatic reaction was started by addition of the [^{14}C]bilirubin substrate instead of UDPGlcUA or the incubation mixture containing all standard components except UDPGlcUA was preincubated for up to 1 h prior to starting the transferase reaction by adding UDPGlcUA cosubstrate. Indeed, if aggregation and/or precipitation of bilirubin occurred, one would expect to find a lower rate when the reaction was started with UDPGlcUA compared to when the pigment substrate was added last. When the enzymatic reaction was started with UDPGlcUA, one would also expect to observe curvilinearity in the product formation time curve due to a gradual decrease, by aggregation or precipitation, of the effective pigment substrate concentration. Instead, we found that time curves were consistently linear and that their slope was independent of the sequence in which bilirubin (tested with 2 μM) and UDPGlcUA were added. Bilirubin UDPGT activity remained stable even when a mixture containing all components except UDPGlcUA was preincubated under argon for up to 1 h at 0 °C prior to starting the enzymatic reaction (data not shown).

BDG formation was always observed and amounted to ~ 10–15% of the rate of total BMG formation. Because the amount of [^{14}C]bilirubin converted to BDG was small under our assay conditions, which were designed to optimize measurement of initial formation rates of the individual BMG isomers, kinetic data on BDG synthesis were insufficiently precise to permit computation of accurate kinetic parameters. In order to account for the small fraction of BMG that was further converted to BDG, BDG was added to the actual BMG concentration for computation of total BMG formation, i.e., total bilirubin UDPGT activity. Rates of formation of individual C-8 and C-12 isomers of BMG were not corrected for the unaccounted BMG fraction that had been converted to BDG because this neglect resulted in only a small negative bias. In fact, we were unable to determine the fraction of BDG that was derived from each individual isomer because kinetic data on the conversion of isolated C-8 BMG or C-12 BMG are unavailable.

Kinetics of C-8 BMG and C-12 BMG Formation by Rat Liver Microsomes at Various Bilirubin Concentrations. Initial reaction rates for C-8 BMG and C-12 BMG formation from bilirubin were determined at various substrate concentrations (1–10 μM). Double-reciprocal plots for C-8 BMG and C-12 BMG synthesis were linear ($r > 0.956$) over this range of substrate concentrations. Apparent V_{max} and $K_{bilirubin}$ values for total BMG formation were respectively 2.29 ± 0.55 nmol (mg of protein)$^{-1}$ min^{-1} and 0.92 ± 0.18 μM (mean ± 1 SD; $n = 8$). Rates of C-8 BMG formation markedly exceeded C-12 isomer synthesis. V_{max} for synthesis of C-8 BMG amounted to approximately two-thirds (mean ± 1 SD: 61.1% ± 2.1%; $n = 8$) of the corresponding value for total BMG (C-8 isomer + C-12 isomer) formation. The ratio of the two isomers was independent of the bilirubin substrate concentration. Thus, apparent $K_{bilirubin}$ values for individual formation of C-8 BMG (0.93 ± 0.19 μM; $n = 8$) and C-12 BMG (0.94 ± 0.22 μM; $n = 8$) were comparable. Predominant esterification by rat liver of one of the two propionic acid side chains of bilirubin and its structural isomers bilirubin IXβ and IXδ has been noted previously in vivo (Jansen & Billing, 1971; Blanckaert, 1980; Blanckaert et al., 1977) and under various in vitro assay conditions (Blanckaert et al., 1979; Whitmer et al., 1984). The ratio of the initial rates of glucuronidation at the C-8 and C-12

Table II: Isomeric Composition of BMG Formed in Vitro or in Vivo by Different Species[a]

species	in vitro: incubation conditions	in vitro: % C-8 BMG	in vivo: % C-8 BMG in bile
rat	50 μM bilirubin		65 ± 1.9 (n = 12)
	BSA, Chapso	62 ± 3.6 (n = 5)	
	BSA, digitonin	62; 66	
	HSA, digitonin	64	
	BSA, UDPGlcNAc	63 ± 3.2 (n = 5)	
	BSA, no activator	64 ± 2.6 (n = 4)	
sheep	50 μM bilirubin		31 ± 1.4 (n = 5)
	BSA, UDPGlcNAc	32.6 ± 1.10 (n = 4)	
	10 μM bilirubin		
	Chapso	30.2 ± 1.8 (n = 5)	
	digitonin	32.3; 35.6	
	UDPGlcNAc	31.0 ± 0.63 (n = 3)	
	no activator	31.7 ± 2.3 (n = 3)	
guinea pig	10 μM bilirubin		70 ± 2.9 (n = 3)
	UDPGlcNAc	73.0	
	no activator	67.3	
human	50 μM bilirubin		50 ± 3.5 (n = 4)
	BSA, digitonin	47.3; 50.2	

[a] Hepatic bilirubin UDPGT activity was assayed by the radioassay method. Liver homogenate was used in humans while microsomal preparations were employed for rat, sheep, and guinea pig. Incubation mixtures with rat microsomes contained 5 mM UDPGlcUA; those with enzyme preparations from the other species, 10 mM UDPGlcUA. When bovine (BSA) or human (HSA) albumin was included, the bilirubin/albumin molar ratio was 2:1. Presence of Chapso or digitonin in the incubation mixture indicates that an enzyme preparation disrupted by pretreatment with detergent (4 mM Chapso or 1 mg of digitonin/mg of protein) was used. When UDPGlcNAc was added, its concentration was 2 mM. The listed values for isomeric composition of BMG formed in vitro were obtained under conditions of zero-order kinetics for formation of each isomer.

side chains of bilirubin by rat liver microsomes was unaffected by detergent, UDPGlcNAc, or the presence of albumin in the enzyme incubation mixture and was comparable with the C-8/C-12 ratio of natural BMG in normal rat bile (Table II).

Inhibition of Microsomal Glucuronidation of Bilirubin by Alternative Substrates of Bilirubin UDPGT. Our finding of comparable apparent Michaelis constants ($K_{bilirubin}$) for the two individual reactions was consistent with involvement of a single catalytic center in the synthesis of C-8 BMG and C-12 BMG. Additional support for this hypothesis was obtained in inhibition studies with various structural analogues of bilirubin (Figure 1; Table III). All tested bilirubin congeners and even biliverdin inhibited formation of both BMG isomers from bilirubin without affecting the C-8/C-12 isomeric ratio. The Lineweaver–Burk plots obtained in the presence and absence of inhibitor were linear ($r > 0.955$) and intersected on the y axis, giving comparable apparent V_{max} values in the presence and absence of inhibitor (Table III). These findings are consistent with competitive inhibition. The apparent K_i values of the inhibitors (Table III) were calculated from the equation $K'_{bilirubin} = K_{bilirubin}(1 + i/K_i)$, where $K_{bilirubin}$ and $K'_{bilirubin}$ are the Michaelis constants found in, respectively, the absence and presence of inhibitor concentration i. Because asymmetry of the bilirubin molecule and structural difference between C-8 BMG and C-12 BMG reside exclusively in the positioning of the methyl and vinyl group substituents on the two outer pyrromethenone structures (Figure 1 and Table I), testing of compounds in which the asymmetry was eliminated (bilirubin IIIα and XIIIα) or a vinyl group was modified (mesobilirubin IXα, deuterobilirubin IXα, AcCyst-bilirubin IXα) as inhibitors was of particular interest. For example, if synthesis of C-8 BMG would have depended on binding of the *exo*-vinyl-bearing dipyrrolic half of bilirubin at one enzymatic site and C-12 BMG formation on interaction of the *endo*-vinyl-containing dipyrrolic half of the pigment at another independent catalytic site, then one would have expected to find selective competitive inhibition of synthesis of C-8 BMG and C-12 BMG by, respectively, bilirubin XIIIα and bilirubin IIIα. However, no such selective inhibition pattern was found with any one of the tested inhibitors as evidenced by the close agreement between the C-8/C-12 ratios observed in the presence or absence of inhibitor (Table III).

Interpretation of our kinetic data as indicating that the inhibitors bind at the same catalytic center of UDPGT as the natural bilirubin substrate is also supported by the following findings suggesting that all inhibiting pigments except bilirubin IXα ditauryl conjugate were alternative substrates and thereby depended exclusively on the bilirubin-conjugating form of UDPGT. Under incubation conditions that led to conversion of over 80% of the natural substrate bilirubin to glucuronidated pigment, all tested bilirubins with unconjugated propionic acid side chains and even biliverdin were glucuronidated by normal rat liver microsomes. Thus, azopyrromethene (Azpm) monoglucuronide was identified in the incubation mixtures containing UDPGlcUA and was undetectable in controls lacking

Table III: Effect of Various Inhibitors of Bilirubin Glucuronidation on Synthesis of C-8 BMG and C-12 BMG by Rat Liver Microsomes[a]

inhibitor	isomeric composition of [^{14}C]BMG (% C-8 isomer): −inhibitor	+inhibitor	apparent V_{max} ± SE (pmol mg^{-1} min^{-1}): −inhibitor	+inhibitor	K_i (μM)
bilirubin IIIα	64	64	1533 ± 85	1651 ± 197	1.03
bilirubin XIIIα	65	65	1791 ± 85	1369 ± 161	1.14
mesobilirubin IXα	62	69	1653 ± 159	1585 ± 206	1.53
deuterobilirubin IXα	62	62	2908 ± 145	2827 ± 156	6.24
bilirubin IXβ	66	62	2057 ± 75	2202 ± 136	1.86
dimethoxybilirubin IXα	60	60	1560 ± 37	1329 ± 285	0.87
bilirubin IXα ditauryl conjugate	59	60	2073 ± 41	2053 ± 61	7.40
AcCyst-bilirubin IXα	60	61	2194 ± 95	1947 ± 192	32.0
biliverdin	63	65	1884 ± 62	1718 ± 92	13.3

[a] With [^{14}C]bilirubin concentrations varying between 0.5 and 8 μM, kinetic analysis of synthesis of radiolabeled C-8 BMG and C-12 BMG was performed in the presence or absence of unlabeled inhibitor in the assay mixture. Inhibitor concentration was 10 μM except for biliverdin (50 μM) and AcCyst-bilirubin XIα (20 μM). The listed results pertain to individual experiments with each inhibitor. The isomeric composition of the BMG product is illustrated by expressing the apparent V_{max} for C-8 BMG synthesis as a percentage of the apparent V_{max} for total [^{14}C]BMG formation. Listed apparent V_{max} and K_i values refer to synthesis of total radiolabeled BMG.

UDPGlcUA when bilirubin IIIα or XIIIα, mesobilirubin IXα, bilirubin IXβ, AcCyst-bilirubin IXα, or deuterobilirubin IXα was used as substrate. Incubation of bilirubin IXβ yielded two glucuronidated Azpm fractions. The predominant one was identified as Azpm-3,7[GlcUA]. The second one corresponded with the diglucuronide Azpm-3[GlcUA],7[GlcUA]. Interestingly, the alternative monoglucuronide positional isomer, Azpm-7,3[GlcUA], was not formed by the rat liver microsomes. This regiospecificity of glucuronidation is in agreement with our previous findings of in vivo esterification of bilirubin IXβ and IXδ injected in rats with a bile fistula (Blanckaert et al., 1977). Enzymatic incubation mixtures containing either biliverdin or dimethoxybilirubin IXα as substrate were analyzed by the alkaline methanolysis method and TLC of the reaction products with the corresponding monomethyl esters and dimethyl ester as chromatographic reference compounds. Enzymatic formation of a monoglucuronidated derivative was demonstrated for both biliverdin and dimethoxybilirubin IXα while diglucuronide production remained undetectable.

That glucuronidation of the alternative substrates depended exclusively on bilirubin UDPGT was evidenced by our finding that esterification was undetectable when these substances were incubated with microsomes from homozygous Gunn rat liver, in which only the bilirubin-conjugating form of UDPGT is totally defective (Mackenzie & Owens, 1981; Chowdhury et al., 1986a,b; Jacobson et al., 1975).

Collectively, the results of the inhibition studies support the idea that both BMG isomers are formed by a single enzymatic site at which also the tested congeners of bilirubin bind and become glucuronidated if a free propionic acid side chain is available.

Dependence on Species of Regioselectivity of Bilirubin Conjugation in Vivo or in Vitro. To investigate the factors determining the relative glucuronidation rates at the C-8 and C-12 positions of natural bilirubin, regioselectivity was examined in different species. Marked differences in isomeric composition of BMG between species were observed in vivo as well as under various in vitro conditions (Table II). Our results in human liver are in agreement with previous preliminary work in which C-8 BMG and C-12 BMG formation by human microsomes was assayed without detergent pretreatment (Gollan et al., 1980). As stated above for rat microsomes, agreement was found also in sheep, guinea pig, and human liver between the C-8 BMG/C-12 BMG ratio in vivo, in bile, and in vitro. Moreover, the isomeric composition of BMG was unaffected by bilirubin substrate concentration, presence and type of detergent or albumin in the enzyme assay mixture, or addition of UDPGlcNAc, which is the presumed physiologic allosteric effector of microsomal UDPGT (Vessey & Zakim, 1978). As also noted above, similar agreement between in vivo and in vitro results was found when regioselectivity of conjugation of bilirubin IXβ was examined.

Collectively, these observations demonstrate that the regioselectivity exhibited by bilirubin UDPGT does not depend exclusively on structural properties of bilirubin per se or the pigment–albumin complex. The species dependence of regioselectivity indicates that there are marked differences between species in the mode(s) of interaction of bilirubin with the postulated unique catalytic center at which BMG is formed. Our results also suggest that the bilirubin-binding site of UDPGT is not significantly distorted under any of the tested enzyme assay conditions including those in which detergent-disrupted microsomes were used since the isomeric composition of the monoglucuronides of bilirubin or bilirubin IXβ formed in vivo was faithfully reproduced in vitro.

Dependence of Regioselectivity of Microsomal Glucuronidation on Structure of Pigment Substrate. Whether synthesis of C-8 BMG and C-12 BMG was a result of binding of bilirubin in two different manners to the postulated unique substrate-binding site of bilirubin UDPGT or corresponded with a single mode of substrate binding to the catalytic center with different rates of glucuronidation at C-8 and C-12 could not be determined from the kinetic analysis of BMG formation. In the former case, one would expect to find a difference in affinity for the two substrate–enzyme binding modes, but this would not result in two different apparent Michaelis constant values for C-8 BMG and C-12 BMG synthesis. Indeed, if as postulated a single substrate-binding site is responsible for formation of both isomers, each of the two substrate–enzyme binding modes would be nonproductive from the point of view of formation of the alternative product. For example, binding of bilirubin in the mode that results in conversion to C-8 BMG would exclude binding of substrate in a putative alternative C-12 BMG generating mode. Such a situation, in which glucuronidations at C-8 and C-12 are mutually exclusive events, would give rise to the same apparent Michaelis constant value for C-8 BMG and C-12 BMG formation. This K_m would be half the value of the harmonic mean of the K_m values for the two individual reactions (Ferscht, 1985). On the basis of these considerations, it can be concluded that our finding of indistinguishable K_m and K_i values for synthesis of C-8 BMG and C-12 BMG does not exclude the possibility of two different substrate–enzyme binding modes, with preferential binding in one of these two modes as the basis of the regioselectivity of bilirubin glucuronidation.

How could bilirubin possibly bind in two different modes to the catalytic center? Two potential explanations were considered. Does perhaps only one dipyrrolic half of the bilirubin molecule interact with the substrate-binding site and does C-8 BMG or C-12 BMG formation correspond to binding of, respectively, the *endo*-vinyl-bearing or *exo*-vinyl-containing part? In such case, one would expect to find marked differences in glucuronidation rate between bilirubin IIIα (containing only *exo*-vinyl-bearing halves, see Figure 1 and Table I) and bilirubin XIIIα (containing only *endo*-vinyl-bearing halves) in rat, in which formation of C-8 BMG and C-12 BMG occurs at markedly different rates. Such a simple mechanistic basis of regioselectivity is unlikely, however, because the esterification rates of these two symmetrical bilirubins were comparable to that of natural bilirubin: V_{max} values for bilirubin IIIα and XIIIα were respectively 2.18 ± 0.32 and 2.02 ± 0.45 nmol (mg of protein)$^{-1}$ min^{-1}, compared to a V_{max} value of 2.29 ± 0.55 nmol (mg of protein)$^{-1}$ min^{-1} for bilirubin (n = 3). Can perhaps predominant formation of either C-8 BMG or C-12 BMG be explained by preferential binding to the catalytic center of one of the two conformational enantiomers of natural intramolecularly hydrogen-bonded bilirubin? It has been postulated that such enantioselectivity in binding properties occurs in complexation by serum albumin to bilirubin (Bonnett et al., 1978; Lightner et al., 1986a,b). It presently is believed that three-dimensional shape and polarity of bilirubin and its structural analogues are determined by their ability and marked tendencies to form multiple intramolecular hydrogen bonds between the two propionic acid groups at C-8 and C-12 and the opposing pyrromethenone lactam C=O/NH and pyrrole NH groups. Six intramolecular hydrogen bonds occur in crystalline bilirubin and mesobilirubin IXα (Bonnett et al., 1978; Becker & Sheldrick, 1978; LeBas et al., 1980). Even when the propionic acid side chains are present in ionized form and two intramolecular hydrogen bonds

are thereby eliminated, four intramolecular hydrogen bonds that involve the carbonyl oxygens of the lactam groups may be formed, as was demonstrated in crystals and solutions of bilirubin salts (Mugnoli et al., 1983; Lightner et al., 1986a,b). Formation of these multiple intramolecular hydrogen bonds locks the pigment in a ridge-tile shape in which the molecule can assume either a right-handed or a left-handed screw sense-skewed chiral conformation. Both conformational enantiomers have been found in crystalline bilirubin and mesobilirubin IXα (Bonnett et al., 1978; Becker & Sheldrick, 1978; LeBas et al., 1980; Mugnoli et al., 1983). Both enantiomeric conformations are in dynamic equilibrium in solution, and recent studies indicate that there is preference for complexation of one of the two chirality enantiomers of bilirubin with cyclodextrin (Lightner et al., 1985) and albumin (Lightner et al., 1986a,b).

To determine whether preferential binding by UDPGT's catalytic center to one of the two chirality enantiomers of intramolecularly hydrogen-bonded bilirubin might provide an explanation for the marked regioselectivity of bilirubin glucuronidation in rat and sheep or the structural features responsible for the asymmetry of bilirubin, i.e., the different positioning of the β-substituents on the two outer pyrromethenone rings, were of importance for regioselective esterification, we examined microsomal formation rates of individual monoglucuronides from three different asymmetrical bilirubins: natural bilirubin, mesobilirubin IXα, and deuterobilirubin IXα (Table I). Current evidence indicates that the key structural elements that are required for formation of the multiple intramolecular hydrogen bonds and thereby determine the three-dimensional structure of bilirubin include (i) presence of two propionic acid side chains that must be located at C-8 and C-12, (ii) the *Z*-configuration C=C bonds at C-4 and C-15, and (iii) an sp^3 carbon at position 10 (Bonnett et al., 1978; LeBas et al., 1980). On the basis of this concept, it is reasonable to postulate that replacement of the vinyl substituents of natural bilirubin by ethyl groups or hydrogen atoms in, respectively, mesobilirubin IXα or deuterobilirubin IXα should not affect formation of intramolecular hydrogen bonds. Therefore, the same two chirality enantiomers should be present in the three test pigments. In keeping with this hypothesis, bilirubin, mesobilirubin IXα, deuterobilirubin IXα, and also bilirubin IIIα and XIIIα and AcCyst-bilirubin IXα all exhibit similar solubility properties and optical spectra while bilirubin IXβ and dimethoxybilirubin IXα, in which the intramolecular hydrogen bonds between the propionic acid side chains and lactam groups cannot be formed, are polar pigments and have different light absorbance spectra. If the possible enantioselective binding by the catalytic center to intramolecularly hydrogen-bonded conformers of the pigment substrate would be a key determinant of regioselectivity of bilirubin glucuronidation, one would not expect to find major differences in isomeric composition of monoglucuronides formed from bilirubin, mesobilirubin IXα, and deuterobilirubin IXα.

Explanation of regioselective conjugation in terms of enantioselective substrate–enzyme binding is inconsistent with our findings (Table IV), especially those for glucuronidation of deuterobilirubin IXα in sheep liver. In rat liver, predominance of glucuronidation at the C-8 side chain was even more pronounced for meso and deutero pigment than for natural bilirubin. In keeping with the slightly more marked conjugation regioselectivity for mesobilirubin IXα compared to natural bilirubin in rat liver, preference for C-12 monoglucuronide formation was slightly higher with mesobilirubin IXα than with bilirubin. Surprisingly, reversal of the C-8/C-12 ratio in monoglucuronide occurred when natural bilirubin was replaced by deuterobilirubin IXα. It should be noted here that even if our tentative assignment of the isomeric structure (C-8 or C-12) of the two deuterobilirubin IXα monoglucuronides formed by the rat and sheep liver preparations would have been incorrect, our results remain incompatible with enantioselectivity of enzyme–substrate binding being a key determinant of regioselectivity of conjugation because assignment of the alternative isomeric structure to the separated deuterobilirubin IXα monoesters would correspond to marked predominance of the C-12 isomer in deuterobilirubin IXα monoglucuronide formed by rat liver. The intriguing question as to how replacement of the vinyl β-substituents of bilirubin by hydrogen atoms exerts such remarkable effect on the regioselectivity and total conjugation rate remains unanswered.

Table IV: Regioselectivity of Glucuronidation of Bilirubin, Mesobilirubin IXα, and Deuterobilirubin IXα by Rat or Sheep Microsomes[a]

	V_{max} (nmol mg^{-1} min^{-1})	% C-8 BMG
rat		
bilirubin	2.22; 1.86; 2.67	62; 65; 63
mesobilirubin IXα	2.26; 1.92; 3.34	71; 81; 72
deuterobilirubin IXα	1.18; 1.00; 1.92	82; 89; 81
sheep		
bilirubin	1.21; 1.26	36; 31
mesobilirubin IXα	0.98; 1.39	23; 29
deuterobilirubin IXα	0.12; 0.21	77; 77

[a] Glucuronidation by fully disrupted microsomes was measured with the *o*-ethylanthranilate diazo method using saturating concentrations of pigment (100 μM) and UDPGlcUA (5 mM in rat, 10 mM in sheep). Isomeric composition of the formed monoglucuronides was determined by analysis of a portion of the enzymatic incubation mixture with the alkaline methanolysis procedure combined with TLC. Assignment of the C-8 or C-12 isomeric structure to the separated two pigment monomethyl esters was verified by TLC analysis of their *o*-ethylanthranilate azo derivatives, as described under Materials and Methods. Diglucuronide formation amounted to ~10–15% of the rate of total monoglucuronide formation in all incubation mixtures.

Our finding that marked differences in glucuronidation between bilirubins can occur despite the ability and pronounced tendency of these tetrapyrroles to form the same two conformational enantiomers by no means excludes the possibility that it is in these two conformational forms that bilirubin binds to the enzyme or even that preferential complexation of one of the two chirality enantiomers of intramolecularly hydrogen-bonded bilirubin occurs. However, our previous observations and the findings of this study indicate that bilirubins that cannot form the tightly knit system of multiple intramolecular hydrogen bonds characteristic of natural bilirubin and thereby adopt either of the aforementioned two enantiomeric conformations can in fact effectively bind to the postulated, unique substrate-binding site of bilirubin UDPGT. Specifically, bilirubin IXβ and dimethoxybilirubin IXα are efficient competitive inhibitors of bilirubin glucuronidation (Table III), and glucuronidation of both inhibitors is completely defective in Gunn rat liver, indicating that their conjugation depends exclusively on the bilirubin-conjugating action of UDPGT [see above and Blanckaert et al. (1977)]. Collectively, these findings suggest that the molecular form(s) of bilirubin engaging in binding to the catalytic site of UDPGT may not correspond to intramolecularly hydrogen-bonded conformers of the pigment and that, for unexplained reasons, the nature of the β-substituents attached to the outer pyrromethenone rings of bilirubins is a key determinant of glucuronidation rate.

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