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Relationships Between Serum Bilirubins and Production and Conjugation of Bilirubin

Studies in Gilbert's Syndrome, Crigler–Najjar Disease, Hemolytic Disorders, and Rat Models

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The pattern of serum bilirubins was determined in serum of humans and rats with unconjugated hyperbilirubinemia due to increased pigment load or defective hepatic conjugation. Bilirubin ester conjugates were present in all serum samples tested and were identified as bilirubin 1-O-acyl glucuronides. In Gilbert's syndrome, the concentration of total conjugates was comparable to the values in healthy control subjects. Because the concentration of unconjugated pigment was increased, the fraction of conjugated relative to total bilirubins was markedly decreased. Sera from patients with Crigler–Najjar disease differed from those with Gilbert's syndrome by the higher unconjugated bilirubin levels and the undetectability of diconjugated bilirubins. A striking finding was that in hemolytic disease, the concentration of both monoconjugates and diconjugates was enhanced in parallel with the increase of unconjugated pigment. Therefore, the fraction of conjugated relative to total bilirubins remained within the normal range. As in Gilbert's syndrome, heterozygote R/APfd-j/+ rats with impaired hepatic bilirubin conjugation exhibit an increased unconjugated bilirubin level in serum, whereas the concentration of total conjugates was comparable to the values in normal rats. In serum of normal rats loaded intraperitoneally with unconjugated bilirubin, both unconjugated and mono- and diconjugated bilirubins were increased in parallel so that the ratio of unconjugated to esterified pigment remained unaffected. Decreased hepatic conjugation or increased bilirubin load was associated with a lower percentage of diconjugates relative to total conjugates both in human and rat serum. The present results are consistent with a compartmental model in which there is bidirectional transfer across the sinusoidal membrane for unconjugated bilirubin as well as for the bilirubin glucuronides. Because typical patterns of serum bilirubins are found in Gilbert's syndrome and patients with hemolytic hyperbilirubinemia, determination of esterified bilirubins in serum is of value to study the pathophysiology and the differential diagnosis of unconjugated hyperbilirubinemia.

The major bile pigment in serum of healthy adults and patients with defective bilirubin conjugation or hemolytic disease is unconjugated bilirubin. Previous studies have indicated that the plasma concentration of this pigment varies directly with the bilirubin production rate and inversely with the plasma bilirubin clearance rate (1). By contrast, little is known on the determinants of the level of conjugated bilirubins in serum of individuals with unconjugated hyperbilirubinemia, largely because sufficiently sensitive and specific methods for mea-

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surement of the various serum bilirubin fractions have not been available. For example, numerous reports have documented the inaccuracy and nonspecificity of the measurement of "direct-reacting" and "indirect-reacting" bilirubin using conventional diazo-methods (2,3), and even the highly specific alkaline methanolysis procedure combined with normal-phase "high-pressure" liquid chromatography (3,4), which permits measurement of individual esterified bilirubins in serum, was found to be too insensitive to detect, let alone to measure, conjugated bilirubins in normal serum (3,5).

We recently have devised an improved, highly sensitive liquid-chromatographic method for the specific measurement of the monoesterified and diesterified bilirubins and demonstrated that bilirubin monoglucuronides and diglucuronides amount to ~4% of the total serum bilirubins in healthy men and women (6).

In the present study, we have identified and measured the monoesterified and diesterified bilirubins in serum of humans and rats with unconjugated hyperbilirubinemia caused by either defective hepatic bilirubin conjugation [Gilbert's syndrome, Crigler–Najjar disease, heterozygote R/APfd-j/+ rat with deficient bilirubin uridine 5′-diphosphate (UDP)-glucuronyltransferase activity] or bilirubin overload (hemolytic disease, exogenous unconjugated bilirubin administration). Furthermore, the relationship between the concentrations of individual pigment fractions in serum and bilirubin production or hepatic conjugation rate was investigated. Esterified bilirubins were demonstrated in all serum specimens and identified as bilirubin 1-*O*-acyl monoglucuronides and diglucuronides. Our findings are consistent with the concept that even in the absence of impaired biliary secretion, a fraction of the esterified bilirubins formed in the liver normally refluxes from hepatocyte to plasma, as was previously demonstrated for the unconjugated pigment (7,8). Hence, changes in the level of conjugated bilirubin in the hepatocyte may be paralleled by corresponding changes in the concentration of conjugated pigment in the circulation. Such reflux would explain (a) the presently documented increase of serum concentration of bilirubin glucuronides in patients with hemolysis and in rats with bilirubin overload, in whom the hepatic production of conjugates is increased, and (b) the normal plasma levels of conjugated bilirubins found in individuals with Gilbert's syndrome and in the heterozygote R/APfd-j/+ rats, in whom a normal rate of in vivo conjugation of bilirubin is sustained at the expense of an increased unconjugated bilirubin concentration in liver and plasma.

Materials and Methods

Human Sera

Blood samples were obtained after an overnight fast from 22 individuals with Gilbert's syndrome (19 men, 3 women). The diagnosis was made on the basis of a chronic mild unconjugated hyperbilirubinemia in the presence of repeatedly normal liver function tests, normal fasting serum bile acids, and absence of hematologic abnormalities (normal reticulocyte count and haptoglobin level, normal hemoglobin electrophoresis, and normal erythrocyte enzyme activities and osmotic resistance). Sera were also obtained from 2 children and 1 adult with Crigler–Najjar syndrome type 1 (i.e., unresponsive to phenobarbital treatment). Blood specimens of 22 patients with uncomplicated hemolytic disease were collected after an overnight fast: 9 men with congenital spherocytosis, 6 (including 1 woman) with autoimmune hemolytic anemia, 2 men with thalassemia minor, 1 woman with paroxysmal nocturnal hemoglobinuria, 1 man with sickle cell disease, 1 woman with pyruvate kinase deficiency, 1 man with congenital dyserythropoietic anemia type II, and 1 woman with toxic hemolysis following dapsone intake. Of these 22 samples, only 14 hemolytic samples became available after development of the new high-pressure liquid chromatographic method (see below). Serum was stored at −70°C within 6 h after collection of the blood, and the samples were analyzed within 2 mo. No statistically significant changes of the concentration of the individual pigment fractions were detected within this period in serum specimens of which several aliquots were frozen at −70°C and analyzed at different time intervals. The study was approved by the University of Leuven Hospitals and Clinics Committee on Human Research.

Rat Sera

Male and female inbred Wistar-derived R/APfd rats (Proefdierencentrum, Catholic University of Leuven, Belgium) and heterozygote R/APfd-j/+ rats (F_1 generation of male R/APfd−j/j and female R/APfd rats), all 10–14 wk old, were used. R/APfd-j/j rats are totally deficient in bilirubin UDP-glucuronyltransferase activity (9). The factor jaundice (j) of the Gunn rat was introduced in the R/APfd rat by 16 generations backcross-intercross. The R/APfd-j/j rat is congenic with the R/APfd rat. Normal hepatocytes of the R/APfd rat can be transplanted without rejection in the R/APfd-j/j rat (9). The animals had free access to food and water, and were not starved before the experiment. Bilirubin overload was produced by a single intraperitoneal injection of unconjugated bilirubin (2 mg/100 g body wt) dissolved in 0.1 M NaOH and brought to pH 7.8 with 0.1 M HCl, given 5 h before death. To obtain blood and liver tissue, laparotomy was performed under light ether anesthesia and the animals were exsanguinated via the aorta abdominalis. Serum was stored at −70°C and analyzed within 1 wk after exsanguination. The liver was quickly excised, weighed, and stored at −20°C for a maximum of 1 mo before analysis. Determination of biliary excretion of bilirubins in rats with cannulated duct was performed as described elsewhere (10).

Analytic Methods

Specific measurement of unconjugated bilirubin and of bilirubin monoester and diester conjugates was performed with the alkaline methanolysis procedure combined with high-pressure liquid chromatography in the reverse-phase mode as previously described (6), except for the following modification. To prolong the lifetime of the column, the pigment residue was dissolved in 10 μl of chloroform and 100 μl of dimethyl sulfoxide instead of 100 μl of chloroform before injection in the chromatograph. Using 0.6 ml of sample, the detection limit for monoesterified and diesterified bilirubins was ~20 nmol/L for each one. Linearity of the assay was verified in the range of esterified bilirubin levels encountered in healthy individuals and in unconjugated hyperbilirubinemia, down to concentrations as low as 0.03 μM and 0.02 μM for, respectively, monoesterified and diesterified bilirubin. Artifactual formation of esterified bilirubins from unconjugated bilirubin of the sample does not occur in the alkaline methanolysis procedure. The coefficient of variation of determination of individual chromatographic peaks was between 5% and 8% for within-day analysis and between 6% and 13% for day-to-day analysis when the assay was applied to normal human serum (6). For each sample, the identity of the bilirubin ester peaks in the chromatogram was verified by taking a duplicate sample through the methanolysis procedure, except that methanol replaced the KOH/methanol reagent. In this blank, transesterification does not occur, and peaks corresponding with authentic methyl esters derived from sugar conjugates do not appear in the chromatogram. In another study on serum specimens in unconjugated hyperbilirubinemia, we could not detect bilirubin-protein conjugates and found no significant difference between the values for total bilirubin concentration assayed with a conventional diazo-method and the sum of the bilirubins detected by the present high-pressure liquid chromatography method (11). These data, and also those of others (12), indicate that bilirubin-protein conjugates are absent or negligible in samples of these types of patients.

Structure verification of the serum bilirubins was done by chromatographic and chemical tests on azoderivatives prepared by diazo-cleavage of the tetrapyrrolic bilirubins (6). The following diazo procedures, discussed in more detail elsewhere (13,14), were used. The p-iodoaniline (15) and ethyl anthranilate (16) diazo methods were employed to convert the bilirubins in 2 ml of serum to azoderivatives, which were separated by thin-layer chromatography (17). The δ-fraction in the chromatograms of p-iodoaniline azopigments comigrated with yellowish nonazopigment material. Rechromatography on silica gel-coated plates using chloroform/methanol/water (10:5:1, vol/vol/vol) was required to purify the azopigment before the δ-fraction could be determined. Nomenclature used for denotation of the various azopigment fractions is as previously described (17) and ethyl anthranilate azopigment reference materials are designated as proposed by Bergstrom and Blumenthal (18). Relative amounts of azoderivatives in chromatograms were determined by densitometry at 536 nm. Further structure verification of the ethyl anthranilate azoderivatives involved chromatographic analysis of the reaction products formed from isolated azopigment fractions consecutively methylated, acetylated, and subjected to alkaline ethanolysis by procedures described elsewhere (17).

The concentration of bilirubins was measured with the ethyl anthranilate diazo-assay (16). Assay of bilirubin UDP-glucuronyltransferase activity in digitonin-treated liver homogenate was performed according to Heirwegh et al. (19).

Statistical Analysis

Statistical significance of differences was analyzed with the nonparametric Mann–Whitney test using the 5% significance level as critical value of the test statistic (two-tailed hypotheses).

Results

Human Serum

Peaks with the retention times of bilirubin monomethyl ester and bilirubin dimethyl ester were present in all serum specimens of patients with Gilbert's syndrome or hemolytic disease (Figure 1*A*)

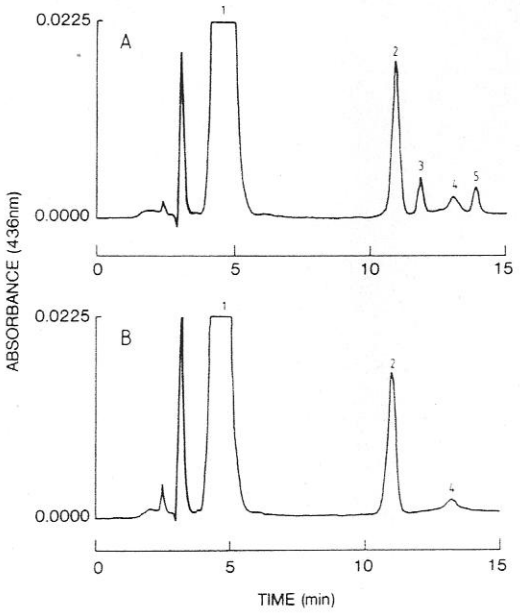

Figure 1. Chromatograms of pigments from serum of a patient with Gilbert's syndrome. *A*. Composition of the pigments extracted from serum after alkaline methanolysis. *B*. Same serum sample subjected to alkaline methanolysis procedure, except for the replacement of KOH/methanol reagent by plain methanol. 1, unconjugated bilirubin; 2, internal standard; 3, bilirubin monomethyl ester; 4, hemin; 5, bilirubin dimethyl ester. With the selected setting of the electronic integrator, adequate computation of the area under the complete peak was performed for each chromatographic fraction albeit the peak portion corresponding to $A^{1\,cm}_{436\,nm} > 0.0225$ could not be displayed in the traced chromatogram.

Table 1. Concentrations of Unconjugated Bilirubin, Monoesterified Bilirubins, and Diesterified Bilirubins in Serum of Healthy Men and Women (Taken From Reference 6) and of Patients With Unconjugated Hyperbilirubinemia

Group	n	Pigment concentration (μmol/L)			Esterified bilirubins (% of total bilirubins)	BDC (% of total esters)
		UCB	BME	BDE		
1. Healthy men	28	6.77 ± 2.67 (1.76–12.26)	0.10 ± 0.04 (0.03–0.22)	0.12 ± 0.06 (0.04–0.26)	3.5 ± 1.9 (1.2–9.3)	52 ± 13 (22–76)
2. Healthy women	14	5.39 ± 3.46 (2.42–14.86)	0.10 ± 0.07 (0.03–0.25)	0.09 ± 0.07 (0.02–0.26)	3.7 ± 2.3 (1.7–9.9)	50 ± 17 (14–78)
3. Gilbert's syndrome	22	31.50 ± 10.42[a] (18.07–58.29)	0.20 ± 0.09[a] (0.07–0.37)	0.09 ± 0.05 (0.04–0.22)	0.9 ± 0.4[a] (0.4–2.0)	33 ± 9[a] (12–49)
4. Crigler–Najjar disease	3	316; 263; 257	0.90; 0.48; 0.69	0; 0; 0	0.3; 0.2; 0.3	0; 0; 0
5. Hemolytic disorders	14	24.28 ± 7.42[a,b] (17.37–35.15)	0.61 ± 0.33[a,b] (0.24–1.19)	0.36 ± 0.22[a,b] (0.06–0.76)	4.0 ± 2.3[b] (1.7–9.7)	36 ± 10[a] (20–52)

BDE, diesterified bilirubin; BME, monoesterified bilirubin; UCB, unconjugated bilirubin. The given values correspond to mean ± 1 SD, and the range of the values is given in parentheses. Statistically significant differences ($p < 0.05$) are expressed as follows: [a] different from group 1; [b] group 5 different from group 3.

and were identified as being derived from bilirubin monoglucuronides and diglucuronides as follows. First, these two peaks were absent in the chromatogram obtained with pigment extracted from the corresponding serum sample treated with plain methanol instead of the KOH/methanol mixture (Figure 1*B*), which indicates that the bilirubin ester peaks were derived from corresponding bilirubin monoester and diester conjugates in serum. Second, the serum bilirubins were converted to p-iodoaniline and ethyl anthranilate azoderivatives, which were chromatographed or subjected to structural analysis, or both, to identify the conjugating group of the parent esterified bilirubins. A so-called δ-azopigment fraction with the chromatographic mobility of the reference p-iodoaniline azopyrromethene monoglucuronide accounted for ~2.3%–6.0% and 0.6%–1.2% of the total p-iodoaniline azopigment in patients with hemolytic disease (n = 9) and Gilbert's syndrome (n = 10), respectively. Verification of the glucuronide structure of the δ-azopigment fraction was carried out on the corresponding chromatographic fraction obtained by reaction of the serum bilirubins with diazotized ethyl anthranilate in six specimens of serum (three from patients with hemolytic disease and three from individuals with Gilbert's syndrome). Unlike the p-iodoaniline azoderivative, the ethyl anthranilate azopigment is sufficiently stable to permit the derivatization procedures needed for structural analysis. In each of the six samples, the identity of the δ-azopigment fraction with a mixture of the endovinyl and exovinyl isomers of azopyrromethene 1-*O*-acyl glucuronide was verified as follows. Methylation and subsequent acetylation of the δ-pigment resulted at each step in the formation of derivatives corresponding to those obtained from authentic reference azopyrromethene glucuronides after identical derivatization. Alkaline ethanolysis of each isomeric acetate derivative yielded the corresponding endovinyl and exovinyl ethyl ester isomers Azpm-8(Et) and Azpm-12(Et) (13,14,17,18).

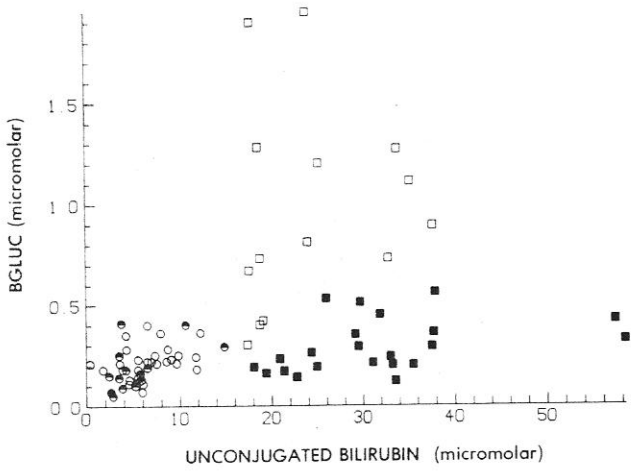

Figure 2. Relationship between the serum concentrations of unconjugated bilirubin and esterified bilirubins in healthy women (◓) and men (○), patients with Gilbert's syndrome (■), and patients with hemolytic disease (□). BGLUC, sum of monoesterified and diesterified bilirubins.

For the patients with Gilbert's syndrome, Crigler–Najjar disease, or hemolytic disease, concentrations of the various bilirubin fractions detected by high-pressure liquid chromatography in serum are summarized in Table 1 and Figure 2 and compared to the corresponding values previously found in healthy individuals. The concentrations of the monoesterified bilirubins and diesterified bilirubins ranged from 0.07 to 0.37 μmol/L and from 0.04 to 0.22 μmol/L, respectively, in the individuals with Gilbert's syndrome. Monoesters are slightly higher and diesters lower than the corresponding values

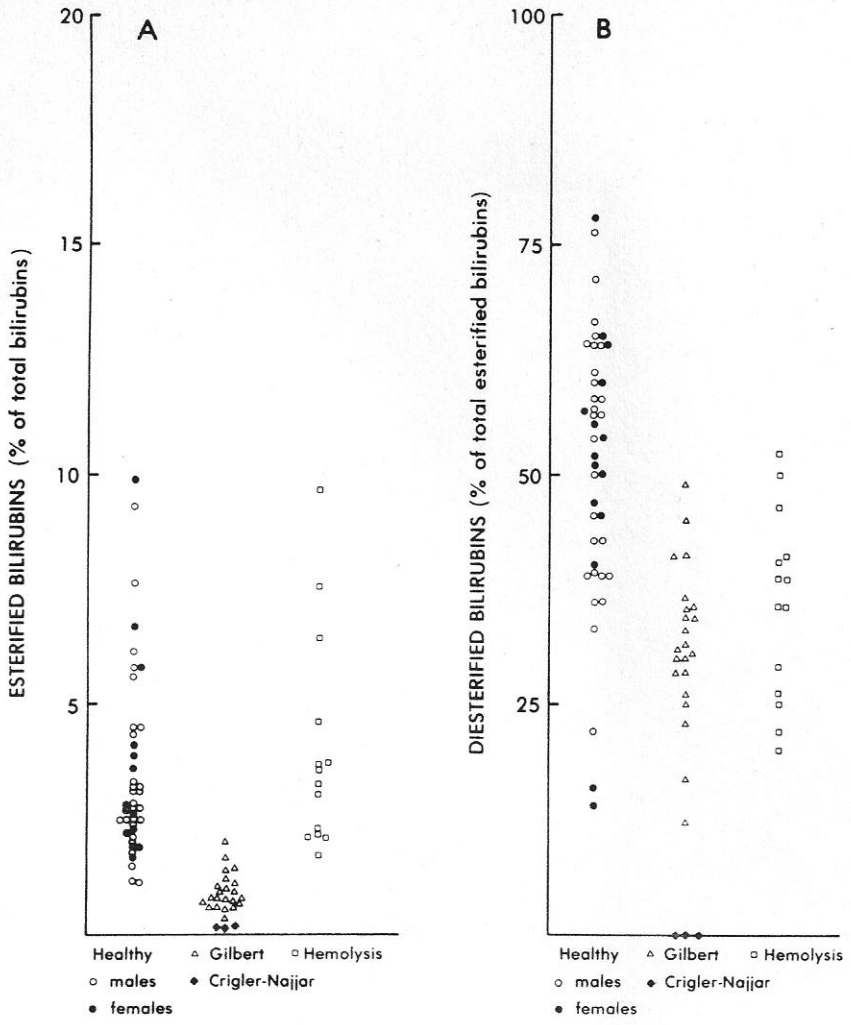

Figure 3. Composition of serum bilirubins in healthy women and men (taken from Reference 6), patients with Gilbert's syndrome or Crigler–Najjar disease, and patients with hemolytic disease. *A*, Total esterified bilirubins, as a percentage of total bilirubins. *B*, Diesterified bilirubins, as a percentage of total esterified bilirubins.

found in healthy adults (0.03–0.22 μmol/L for monoesters and 0.04–0.26 μmol/L for diesters). The increase of the unconjugated pigment caused the fraction of esterified bilirubin (between 0.4% and 2.0% of total serum bilirubins) to be distinctly smaller in Gilbert's syndrome than in the healthy controls (between 1.2% and 9.3%; Figure 3*A*) Whereas diesterified bilirubins were undetectable, a small peak with the retention time of reference bilirubin monomethyl ester was found for each of the three specimens from Crigler–Najjar patients. The monoesterified bilirubin peak was absent when the same serum samples were treated with plain methanol instead of the KOH/methanol reagent. This finding strongly supported our identification of this pigment fraction as authentic monoesterified bilirubin.

In contrast, the serum concentration of diesterified bilirubin (0.06–0.76 μmol/L) and particularly of monoesterified bilirubin (0.24–1.19 μmol/L) in patients with hemolytic disease tended to be elevated in parallel with the increase of unconjugated bilirubin. Therefore, and unlike in Gilbert's syndrome, the esterified fraction of serum bilirubins in the hemolysis patients was 1.7%–9.7% of total serum bilirubins, which is well within the range of values found in healthy adults (Table 1 and Figure 3*A*).

Rat Studies

Direct investigation of the quantitative relationships between the concentrations of the specific serum bilirubins, hepatic bilirubin conjugation, and bilirubin load was only possible in the rat model. First, the effect of hepatic bilirubin conjugation, assessed by measurement of bilirubin UDP-glucuronyltransferase activity, on the serum bilirubin concentrations was investigated by a comparative study of the pigment levels in healthy male and female R/APfd rats and in male heterozygote R/APfd-j/+ rats, which all had comparable biliary excretion rates of esterified bilirubins (0.66 ± 0.16 nmol/min · 100 g body wt, n = 22), used as a measure of endogenous bilirubin production. In agreement with

Table 2. Concentrations of Unconjugated Bilirubin, Monoesterified Bilirubins, and Diesterified Bilirubins in Serum of Male and Female R/APfd Rats, Heterozygote R/APfd j/+ Rats, and of Male R/APfd Rats Loaded With Unconjugated Bilirubin

Group	n	Pigment concentration (μmol/L)			Esterified bilirubins (% of total bilirubins)	BDC (% of total esters)
		UCB	BME	BDE		
1. Male rats	8	0.96 ± 0.05 (0.87–1.01)	0.06 ± 0.02 (0.04–0.08)	0.04 ± 0.02 (0.02–0.07)	9.3 ± 2.1 (6.4–12.4)	39 ± 8 (25–50)
2. Female rats	8	0.72 ± 0.05[a] (0.65–0.79)	0.05 ± 0.01 (0.03–0.07)	0.02 ± 0.01[a] (0.01–0.04)	8.6 ± 2.2 (6.2–12.2)	32 ± 7 (20–40)
3. Male (j/+) rats	6	1.23 ± 0.08[a] (1.08–1.29)	0.06 ± 0.02 (0.03–0.08)	0.02 ± 0.01[a] (0.01–0.03)	6.0 ± 1.3[a] (3.6–7.3)	25 ± 11[a] (11–38)
4. Male rats loaded with bilirubin	4	4.45 ± 1.03[a] (3.36–5.76)	0.51 ± 0.07[a] (0.44–0.60)	0.14 ± 0.03[a] (0.11–0.17)	12.6 ± 3.0 (10.5–17.2)	21 ± 2[a] (20–24)

BDE, diesterified bilirubin; BME, monoesterified bilirubin; UCB, unconjugated bilirubin. The given values correspond to mean ± 1 SD, and the range of the values is given in parentheses. [a] Statistically significant differences ($p < 0.05$) from group 1.

previous results (10), hepatic bilirubin UDP-glucuronyltransferase activity was significantly higher in female than in male rats (385 ± 39 nmol/min · 100 g body wt vs. 249 ± 17, n = 8). Male heterozygote R/APfd-j/+ rats exhibited only 64% (i.e., 159 ± 11 nmol/min · 100 g body wt, n = 6) of the enzyme activity found in the male rat. There was an inverse relationship between the serum concentration of unconjugated bilirubin and hepatic bilirubin UDP-glucuronyltransferase activity, whereas the mean level of total esterified bilirubins in serum was not significantly different for the three groups of animals (Table 2). Like in the patients with Gilbert's syndrome, the selective increase of the unconjugated bilirubin level in heterozygote R/APfd-/j+ rats resulted in a distinctly decreased fraction of esterified bilirubin relative to total bilirubin (Table 2).

The effect of increased production of esterified bilirubins on serum pigment levels was investigated in male R/APfd rats injected intraperitoneally with unconjugated pigment. After 5 h, both unconjugated and esterified bilirubins were markedly elevated (4.45 ± 1.03 and 0.65 ± 0.10 μmol/L, respectively), but the esterified bilirubin fraction (12.6% ± 3.0% of total serum bilirubins) was not significantly different from that in control rats (Table 2).

Composition of Esterified Bilirubins in Human and Rat Serum

Both monoesterified and diesterified bilirubins were detected in all human and rat serum specimens except for Crigler–Najjar disease (Table 1). Diesters usually slightly exceeded monoesters in the healthy adults, whereas monoesters predominated in serum of patients with Gilbert's syndrome or hemolysis (Figure 3*B*). Similarly, in rats, more monoesters were found in serum of male heterozygote and of bilirubin-loaded animals than in male controls (Table 2). Slightly less diconjugates were present in female than in male rat serum (Table 2).

Discussion

The present study has been made possible by the recent development of a highly specific and sensitive method that permits measurement of bilirubin and its monoesterified and diesterified derivatives in serum of healthy humans and rats and in patients and rats with unconjugated hyperbilirubinemia (6). Other methods for specific determination of individual bilirubin fractions seem to lack the sensitivity required for detection of the small amounts of esterified bilirubin present in normal human and rat sera (3,5,12,20,21). By the new method, both bilirubin monoconjugates and diconjugates have been identified previously in serum of healthy men and women (6). In the present study, the pattern of serum bilirubins was determined in patients with unconjugated hyperbilirubinemia associated with decreased hepatic conjugation or increased load of pigment, and was compared with our previous findings in healthy human adults. Serum bilirubins were also determined in four groups of rats used as models of some physiologic (sex) and pathological conditions (deficient hepatic conjugation and increased pigment load) characterizing the various human groups.

The concentration of unconjugated bilirubin in serum was found to be higher in male than in female rats. This sex difference is similar to that reported in humans for total serum bilirubins (22). Also, serum unconjugated bilirubin was higher in the heterozygote R/APfd-j/+ animals than in normal rats. This finding, together with the previously reported decreased hepatic bilirubin UDP-glucuronyltransferase activity (23) and reduced excretion of bilirubin diconjugates in bile (4,24), further supports the va-

lidity of the heterozygote R/APfd-j/+ rat as a model of Gilbert's syndrome. In these three groups of rats, the serum unconjugated bilirubin level was inversely proportional to hepatic bilirubin UDP-glucuronyltransferase activity. Interestingly, a similar relationship has been described between serum bilirubin levels and bilirubin clearance in normal human adults and in patients with Gilbert's syndrome (1). These differences could be visualized by using congenic strains of rats, characterized by low interindividual variability. Indeed, the heterozygote R/APfd-j/+ animals seem genetically identical to the R/A rats except for the presence of the mutant putative (j) genes, responsible for the deficiency of bilirubin UDP-glucuronyltransferase activity in the liver (9). Failure to detect a difference in serum unconjugated bilirubin levels among male and female Sprague–Dawley and heterozygote Gunn rats in a previous report (20) was probably due to the higher biological variability present in these animals of different strains.

Monoesterified as well as diesterified bilirubins were detected in all human and rat specimens and identified as 1-*O*-acyl glucuronides, except in serum of patients with Crigler–Najjar disease, where esterified bilirubin was present only in monoconjugated form. The source of the esterified pigment in plasma remains unknown, but is most likely to reside almost exclusively in the liver. Bilirubin UDP-glucuronyl transferase activity was not detectable in human kidney (25,26), and it is not likely, therefore, that extrahepatic conjugation of bilirubin is responsible for the observed esterified pigment concentration in human plasma, particularly if one considers the preliminary evidence for rapid hepatic removal of bilirubin glucuronides from plasma (27). It is not probable that the esterified bilirubins in plasma originate from the gut lumen as the esterified pigment, once excreted in bile, is not reabsorbed through the intestinal wall (28). Evidence for glucuronidation of bilirubin in extrahepatic tissues, i.e., in kidney and gut mucosa, has been obtained in rats and dogs but not in humans (25,26,29–31). It is conceivable that the occurrence of extrahepatic glucuronidation of bilirubin in rats but not in humans is at least partially responsible for the present finding that the esterified bilirubin fraction is considerably higher in rats than in humans (Tables 1 and 2).

The present observations are consistent with the new concept that a small fraction of the bilirubin esters formed in the liver normally refluxes into plasma (Figure 4). A similar assumption was made in a recently proposed compartmental model of bilirubin metabolism (32), and it readily explains the increased concentration of esterified bilirubins combined with a normal proportion of esterified pigment in serum when bilirubin overload causes overproduction of bilirubin glucuronides in the liver. This has been documented in the present study in the hemolysis patients and rats injected with unconjugated bilirubin, and has been suggested by some previous investigators on the basis of a slightly increased "direct-reacting" serum bilirubin level in hemolysis patients (33,34). Further support for this interpretation has recently been obtained using the isolated perfused normal rat liver. When unconjugated bilirubin was infused at a constant rate in the perfusate and a corresponding constant rate of biliary excretion of bilirubins was achieved, steady levels of monoesterified and diesterified bilirubins (~15% of total bilirubin) were detected in the perfusion fluid (Blanckaert N, Fevery J, Weisiger R, unpublished observations)

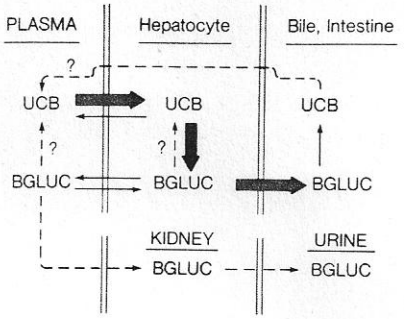

Figure 4. Schematic representation of bilirubin metabolism. This biological model illustrates the proposed concept that transport of unconjugated (UCB) as well as of esterified bilirubins (BGLUC) across the liver plasma membrane is a bidirectional process. Hence, the amount of esterified bilirubin present in serum would depend on the amount formed in the liver, which in turn depends on the amount of unconjugated bilirubin reaching the liver. If one assumes that biliary excretion is not rate-limiting, the fractional exchange of esterified bilirubin between blood and liver will be constant. The model also depicts the previously postulated (32) hydrolysis of esterified bilirubins in the hepatocyte or plasma, or both, and the possible reabsorption of small amounts of unconjugated bilirubin formed in the gut lumen.

It must be emphasized that the present explanation dispells the classical belief that the increase of the direct-reacting bilirubin level in hemolysis patients is caused by saturation of the transport mechanism for canalicular secretion of conjugated pigment. The latter hypothesis is not consistent with our finding that the ratio of esterified to total bilirubin was normal in our patients and rats with unconjugated bilirubin overload. Also, the capacity of the liver to excrete the conjugated pigment in bile far exceeds the unconjugated bilirubin production rates usually encountered in patients with hemolytic

disease and even the maximal rate of bilirubin production that can be chronically sustained (1).

The production rate of bilirubin glucuronides, as assessed by measurement of the biliary output of bilirubins, was normal in the heterozygote R/APfd-j/+ rat, and it is generally believed that patients with this mild conjugation defect excrete conjugated bilirubins in bile at a normal rate (1). Collectively, our findings in the heterozygote R/APfd-j/+ rat and in Gilbert's syndrome can best be explained by making the reasonable assumption that a steady state must be reached in the body, in which the rate of bilirubin conjugation by the liver equals the rate of bilirubin production. In the presence of a decreased activity of the conjugating enzyme, such an equilibrium is only reached at an increased substrate concentration. The high serum concentration of unconjugated bilirubin and the characteristically decreased ratio of esterified to total bilirubin in Gilbert's syndrome seem to reflect this mechanism (Table 1).

In the foregoing discussion, various esterified bilirubins were considered as one entity, but this approach certainly represents an oversimplification, as actually both bilirubin monoesterified and diesterified pigments are present and are assumed to undergo bidirectional transfer between liver and plasma, most probably exhibiting different kinetic characteristics. Diesters constitute about 80% of total conjugates in normal human bile, and about 60% in rat bile. The percent of diesters is lower both in normal human (54% ± 11%) and rat serum (37% ± 8%), probably as the result of preferential excretion of diesters in bile or preferential reflux of monoesters in plasma. In the present work, the concentration of diesters and their relative amount (percentage of total esterified bilirubin) in serum of individuals with Gilbert's syndrome and of heterozygote R/APfd-j/+ rats were lower than in the healthy controls. Diesters were undetectable in serum of the patients with Crigler–Najjar disease. This finding is reminiscent of results obtained in previous studies in which a decreased ratio of diesters to total esters was found in bile of humans and rats with bilirubin UDP-glucuronyltransferase deficiency (4,24,35,36). Both observations in bile and serum point to a predominant formation of monoglucuronide in transferase deficiency states, in agreement with recently studied characteristics of the bilirubin UDP-glucuronyltransferase (37–39). In humans and rats with unconjugated bilirubin overload, serum concentration of diesterified bilirubin is increased but less so than that of the monoesters, the ratio being lower than in healthy controls. Similarly, in bile of rats loaded with a large amount of unconjugated bilirubin, monoesters prevail over diesters (4,24,35,40). This also might be explained by the characteristic behavior of the microsomal bilirubin UDP-glucuronyltransferase system, of which the predominant reaction product is bilirubin monoglucuronide when the bilirubin substrate concentration is high (37,41). The lower concentration of diesters found in female rat serum could be due to a faster biliary excretion of these compounds in the female, as has been reported for other organic anions (42,43). Clearly, additional work is needed to investigate the differences in formation and excretion of bilirubin monoesters and diesters.

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