Unconjugated Bilirubin and an Increased Proportion of Bilirubin Monoconjugates in the Bile of Patients with Gilbert’s Syndrome and Crigler-Najjar Disease

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ABSTRACT Bilirubin pigments were studied in the bile of 20 normal adults, 25 patients with Gilbert’s syndrome, 9 children with Crigler-Najjar disease, and 6 patients with hemolysis, to determine how a deficiency of hepatic bilirubin UDP-glucuronosyltransferase would affect the end products of bilirubin biotransformation.

In the bile from patients with Gilbert’s syndrome, a striking increase was found in the proportion of bilirubin monoconjugates (48.6±9.8% of total conjugates) relative to that in normal bile (27.2±7.8%). This increase was even more pronounced in children with Crigler-Najjar disease, in whom, even in the most severe cases, glucuronide could always be demonstrated in the bile. Furthermore, unconjugated bilirubin-IXα was unquestionably present in the bile of these children and amounted to 30–57% of their total bilirubin pigments (<1% in the controls). It was not possible to predict from the biliary bilirubin composition whether a child would respond to phenobarbital therapy or not. Bile composition was normal in patients with hemolysis, except when there was associated deficiency of hepatic glucuronosyltransferase. Therefore, the observed alterations were not a simple consequence of unconjugated hyperbilirubinemia.

The present findings suggest that Crigler-Najjar disease represents a more pronounced expression than Gilbert’s syndrome of a common biochemical defect. Hepatic bilirubin UDP-glucuronosyltransferase deficiency leads to decreased formation of diconjugates with an ensuing increase in the proportion of bilirubin monoconjugates in bile; in the most severe cases, an elevated content of biliary unconjugated bilirubin is also found.

INTRODUCTION

In both Gilbert’s syndrome and Crigler-Najjar disease, hepatic bilirubin UDP-glucuronosyltransferase activity (UDP-GTA) is significantly decreased (2–7), but does not correlate well with the serum bilirubin levels in the individual cases (2, 3, 6, 7). Indeed, other processes also influence the serum levels as illustrated, for example, by the fasting hyperbilirubinemia (7, 8), whereas the in vitro enzyme assay does not account of endogenous factors which may regulate the transferase activity in vivo (9, 10).

An interesting approach to both disorders is based on the delayed plasma disappearance rate of injected bilirubin (11–15). Such analysis also permitted the detection and quantification of the overproduction of bilirubin (13, 16, 17) which is often associated with Gilbert’s syndrome (6, 18). However, the precise meaning of the kinetic parameters is not yet known, as “the models are merely mathematical conceptualizations of the sites and rates of bilirubin elimination. They provide no information about the biochemical mechanisms involved” (cited from Bloomer et al. [14]).

1 UDP-glucuronosyltransferase, or UDP-glucuronate β-glucuronosyltransferase (acceptor-unspecific), EC 2.4.1.17.
2 Abbreviations used in this paper: EA, ethyl anthranilate; PLA, p-iodeanoline; TLC, thin-layer chromatography; UDP-GTA, bilirubin UDP-glucuronosyltransferase activity.

This work was presented in part at the 8th Annual Meeting of the European Association for the Study of the Liver, 7–8 September 1973, Vittel, France (1).

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Decreased hepatic bilirubin UDP-GTA and decreased hepatic clearance of labeled bilirubin can both be used for diagnosis as they yield indices of the conjugation rate of bilirubin-IXα (by far the predominant isomer in man). In the present work, another approach has been investigated. It is indeed reasonable to assume that the relative amounts of bilirubin-IXα and of its mono- and diconjugates in bile reflect the conjugating processes operating in the hepatocyte, provided that the secreted pigments are not significantly altered in the biliary system. For these reasons, we have analyzed the nature and amounts of conjugated and unconjugated bilirubins that are present in bile. Bile analysis was compared with the results of hepatic bilirubin UDP-GTA. Recent developments in methods for bile pigment analysis have made this approach possible (19).

METHODS

Bile collection and patients. Bile was obtained by duodenal intubation in 20 normal controls, 25 adults with Gilbert's syndrome, 8 children with Crigler-Najjar disease, and 6 patients with hemolytic jaundice who awaited splenectomy. The cause of the hemolysis was congenital spherocytosis in five, and autoimmune hemolysis in the sixth patient. In one additional child with Crigler-Najjar disease (child S), the bile sample was obtained by gallbladder puncture at time of surgery. The controls were informed patients examined for psychosomatic complaints or members of the laboratory. Gilbert's syndrome was defined as chronic unconjugated hyperbilirubinemia (>1.5 mg/100 ml) in the absence of overt hemolysis and of any other disease; these patients included 14 males and 11 females; their ages ranged from 15 to 64 yr.

The nine children with Crigler-Najjar disease were unrelated to each other; there were five males and four females, ranging from 1 mo to 3 yr of age; clinical data on some of them have been published (20, 21). The nine children with Crigler-Najjar disease (Table I) showed serum concentrations of unconjugated bilirubin ranging from 20 to 35 mg/100 ml. During the survey period, kernicterus developed in six children. In these six patients, administration of pheno-barbital failed to lower serum bilirubin, whereas it clearly decreased the hyperbilirubinemia in two of the remaining three children (20, 21). Exact data on the effect of pheno-barbital in the third child (S) could not be obtained. If one takes the response to pheno-barbital and the development of

<p>| TABLE I | Characteristics of the Nine Children with Crigler-Najjar Disease |
|-------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Child</th>
<th>Unconjugated bilirubin in serum</th>
<th>Kernicterus*</th>
<th>Response to pheno-barbital*</th>
<th>Hepatic UDP-GTA</th>
<th>Biliary bilirubin</th>
<th>Bile analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100 ml</td>
<td></td>
<td>mg/l g</td>
<td>% of total azapigment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>25.0</td>
<td></td>
<td>145</td>
<td>68</td>
<td>51</td>
<td>17</td>
</tr>
<tr>
<td>B</td>
<td>23.5</td>
<td></td>
<td>0; 0; 0; 252</td>
<td>73</td>
<td>52</td>
<td>21</td>
</tr>
<tr>
<td>S**</td>
<td>19.6</td>
<td></td>
<td>ND</td>
<td>81</td>
<td>47</td>
<td>34</td>
</tr>
<tr>
<td>M, §§</td>
<td>24.0</td>
<td></td>
<td>0</td>
<td>72</td>
<td>60</td>
<td>12</td>
</tr>
<tr>
<td>M</td>
<td>2.9</td>
<td></td>
<td>1.2</td>
<td>68</td>
<td>52</td>
<td>11</td>
</tr>
<tr>
<td>D, §§</td>
<td>2.4</td>
<td></td>
<td>0.8</td>
<td>73</td>
<td>52</td>
<td>11</td>
</tr>
<tr>
<td>D</td>
<td>1.6</td>
<td></td>
<td>0.6</td>
<td>84</td>
<td>61</td>
<td>23</td>
</tr>
<tr>
<td>E</td>
<td>23.1</td>
<td></td>
<td>1.3</td>
<td>84</td>
<td>61</td>
<td>23</td>
</tr>
<tr>
<td>V₁</td>
<td>24.0</td>
<td></td>
<td>0</td>
<td>99</td>
<td>77</td>
<td>22</td>
</tr>
<tr>
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<td>35.0</td>
<td></td>
<td>0</td>
<td>97</td>
<td>58</td>
<td>39</td>
</tr>
<tr>
<td>C</td>
<td>35.0</td>
<td></td>
<td>0</td>
<td>86</td>
<td>60</td>
<td>26</td>
</tr>
<tr>
<td>G₁</td>
<td>25.0</td>
<td></td>
<td>ND</td>
<td>87</td>
<td>70</td>
<td>17</td>
</tr>
<tr>
<td>G₂</td>
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<td></td>
<td>3.2</td>
<td>86</td>
<td>66</td>
<td>20</td>
</tr>
<tr>
<td>G₂</td>
<td>5.3</td>
<td></td>
<td>3.4</td>
<td>83</td>
<td>68</td>
<td>15</td>
</tr>
</tbody>
</table>

* Absence (−) or presence (+) of kernicterus or positive response to treatment.
† Bilirubin UDP-glucuronosyltransferase activity.
‡ Unconjugated azodipyrrrole formed by coupling with PIA.
§ Unconjugated azodipyrrrole formed by coupling with diazotized EA, a system in which, at pH 2.7, only the conjugated bilirubin reacts. Therefore the difference (αₚ-PIA minus αₚ-EA) gives an estimate of the amount of unconjugated bilirubin present.
¶ Glucuronated azodipyrrrole formed by coupling with diazotized EA.
** Bile obtained by gallbladder puncture at surgery.
11 Not determined.
§§ Samples taken under pheno-barbital therapy. Subscripts after the letters refer to successive bile samples from the same patient.
The azopigments formed were separated by thin-layer chromatography (TLC). The silica gel plates were first developed with benzene:ethyl acetate, 85:15 (vol/vol), to remove lipids and excess of diazoreagent. This washing procedure was followed by successive developments with chloroform:methanol:water, 65:25:3 (vol/vol/vol), for 10 cm and with chloroform:methanol, 85:15 (vol/vol), for 16 cm. The separated azopigments were quantitated either by densitometry or by photometric reading of methanol eluates (19).

Preparations of azodipyrrole (αeo from bilirubin-IXα), of azodiptyrrole β-D-monoxyloside (αd), β-D-monogalactoside (βo), and β-D-monoglucuronide (δ; from normal rat and dog bile), whose structures have all been previously established, were used as chromatographic references (19, 32–34). The ratio of the αo-EA (pH 2.7) over total azopigment was calculated, thus permitting the determination of the relative amounts of mono- and dicongjugated bilirubin-IXα (see below, Validity of methods).

The spectral purity of the separated azopigments was monitored by comparing the “characteristic spectra,” obtained in methanol, with those of pure reference compounds (35). Azopigment αo was identified as azodipyrrole by TLC separation into a mixture of the vinyl and isovinyl isomers of azodipyrrole, both as the free acids and as their methyl esters (19). Separation of the methyl esters allows easy differentiation from mesoaizodipyrrole (35). The δ-azopigment obtained from bile of three patients with Crigler-Najjar disease was further subjected to TLC, after formation of the methyl esters (35), and of the fully acetylated methyl esters (19). The corresponding derivatives of azodiptyrrole β-D-glucopyranuronoside obtained from normal rat bile (32, 34) were used as chromatographic references.

Unconjugated bilirubin-IXα was estimated by the use of chloroform extraction and spectrophotometry at 454 nm as described for serum by Brodersen and Vind (36). 17 bile samples were assayed immediately after collection; an additional sample had been stored at −20°C. To allow quantitation of any coextracted conjugated bilirubin, the chloroform extracts were reacted with diazotized PIA and the azopigments analyzed by TLC (23). In some instances, the chloroform extracts were applied directly to thin-layer plates and the bile pigments developed with chloroform:acetic acid, 99:1 (vol/vol) (37). Purified bilirubin-IXα served as a reference compound.

Validity of methods

COUPLING OF BILE PIGMENTS WITH DIAZOTIZED ETHYL ANTHRANILATE AT pH 2.7; SIGNIFICANCE OF THE AZOPIGMENT FRACTION αo-EA

Each mole of monoconjugated bilirubin-IXα gives rise to the formation of 1 mol of unconjugated and 1 mol of conjugated azodipyrrole (Fig. 1). Provided certain conditions are met, the azopigment fraction αo-EA (pH 2.7) multiplied by 2 equals the amount of monoconjugates expressed as a percentage of the total conjugated bilirubin-IXα (38, 39). This αo-EA (pH 2.7) fraction can assume the extreme values of 0 (no monoconjugate) and 50% (only monoconjugates), but theoretically should never exceed 50%. The analytical requirements are as follows:

(a) Conjugated bilirubin-IXα must react completely with the diazo-reagent. Previous work established that this condition is fulfilled for the reaction at pH 2.7 with diazotized EA (28) provided that bilirubin concentrations are <5 mg/100 ml as shown in the present work.

FIGURE 1 Schematic representation of diazo-cleavage of bilirubin, leading to dipyrrolic azopigments. Diazotized p-iodoaniline (PIA) reacts with both unconjugated (UCB) and conjugated bilirubin, whereas only the conjugates react with diazotized ethyl anthranilate (EA) at pH 2.7. In the PIA system, one molecule of UCB leads to the formation of two molecules of unconjugated azodipyrrole (αo). In both PIA and EA systems: one molecule of bilirubin monoglucuronide produces one molecule of αo and one of conjugated δ-azopigment; diglucuronide yields only δ-azopigment.
(b) Foreign color must not be present in the quantitated azopigment spots. Gross spectral impurity can easily be monitored by comparing "characteristic spectra" with those of pure reference compounds. Characteristic spectra are obtained by plotting the logarithm of the extinction (optical density) against the wavelength, thereby rendering the shape of the spectra independent of the concentration and optical path length (35). This test is especially indicated for azopigment $\alpha_0$ which moves near the solvent front. In general, TLC with respect to known reference compounds allows unequivocal localization of conjugated azodipyrroles derived from conjugated bilirubin-IXa. By TLC of the methyl ester of azopigment $\alpha_0$ any contamination with mesozopigyrrole can easily be detected.

(c) Acid-labile conjugating linkages could be present in some bilirubin-IXa disconjugates. If split during diazo-coupling at pH 2.7, then some monoconjugated bilirubin-IXa could be formed. Inasmuch as such cleavage would be slower at less acidic pH, bile samples were treated in parallel with diazo-reactant at pH 2.7 (standard system) and at pH 6.0, and the $\alpha_0$-fractions quantitated (Table II). Identical values were found when bile samples from normal adults and from Gilbert’s patients were analyzed.

(d) Formation of unconjugated azodipyrrole from other sources:

Normal controls and patients with Gilbert’s syndrome. In general, minute amounts of unconjugated bilirubin-IXa are found in human bile (23). In confirmation of previous data (28) and assays of urine (40) and serum (41), unconjugated bilirubin-IXa (10–20 mg/100 ml) added to diluted bile (5 to 51-fold) reacted only to a negligible extent. In particular the $\alpha_0$-fractions were unchanged. It is likely, therefore, that reaction of endogenous unconjugated pigment can be disregarded. Even if it reacted completely its contribution in fresh samples of both these types of bile is so low (Table III) (23) that the ratios would be barely affected. This is borne out by the similar amounts of $\alpha_0$-fractions obtained in the presence (PIA) or the absence (EA, pH 2.7) of accelerating substances (Table II).

Children with Crigler-Najjar disease. Treatment of bile with diazotized EA at pH 2.7 yielded percentages of $\alpha_0$-azopigment equal to, or higher than, 50% (Tables I and II), thus indicating that pigments other than monoconjugated bilirubin-IXa contributed to the formation of the $\alpha_0$-azodipyrrole. In these bile samples, unconjugated bilirubin-IXa could be a major source of $\alpha_0$-azopigment, because it was a relatively important component of diazo-positive material (Table III). Owing to the rather low dilutions of bile that could be applied, bile salts could have promoted its diazo-coupling at pH 2.7 (42). Another source of azodipyrrole is suggested by a recent study of bile from homozygous Gunn rats, in which at least 24% of total diazo-positive bile pigment was shown to be composed of diazo-positive unconjugated tetrapyrroles related to bilirubin-IXa but containing only one of both dipyrrole halves in unmodified form (43). In addition, a number of ill-defined pigments which also yielded some azodipyrrole were observed. It is suggested that similar pigments occur in bile from Crigler-Najjar patients, with an ensuing increase in the $\alpha_0$-EA (pH 2.7) fraction.

COUPLING WITH DIAZOTIZED PIA

In this procedure both unconjugated and conjugated bilirubin-IXa are transformed into azo-derivatives (Fig. 1). The $\alpha_0$-PIA can thus derive from unconjugated and monoconjugated bilirubin-IXa. With bile from Crigler-Najjar patients, additional unconjugated azodipyrrole could originate from unconjugated, tetrapyrrole breakdown products of bilirubin-IXa retaining an unmodified dipyrrole moiety (43).

**TABLE II**

<table>
<thead>
<tr>
<th>TABLE II Unconjugated Azopigment $\alpha_0$* Obtained with Various Diazo-Procedures from Bile of Controls and Patients with Either Gilbert’s Syndrome or Crigler-Najjar Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_0$-EA (pH 2.7)</td>
</tr>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>V</td>
</tr>
<tr>
<td>Mo</td>
</tr>
<tr>
<td>L</td>
</tr>
<tr>
<td>Le</td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td>Adults with Gilbert’s syndrome</td>
</tr>
<tr>
<td>Bb</td>
</tr>
<tr>
<td>VdR</td>
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<tr>
<td>VR</td>
</tr>
<tr>
<td>WM</td>
</tr>
<tr>
<td>Sw</td>
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<tr>
<td>Children with Crigler-Najjar</td>
</tr>
<tr>
<td>V₁</td>
</tr>
<tr>
<td>V₂</td>
</tr>
<tr>
<td>M₂</td>
</tr>
<tr>
<td>D₂</td>
</tr>
</tbody>
</table>

* The percentages of the $\alpha_0$ azopigment are expressed relative to the total azopigments resulting from treatment with diazotized EA at pH 2.7 or 6, and with diazotized PIA.

**DETERMINATION OF UNCONJUGATED BILIRUBIN-IXa**

The procedure is based on chloroform extraction. Correction for any coextraction of conjugated pigment was performed by treatment of the chloroform extracts with diazotized PIA and subsequent analysis of the azopigment distribution (23). As the uncorrected concentrations for normal bile and bile from Gilbert’s patients are already very low (in general <1% of total bile pigment), even a rough estimate of the correction term is adequate. Chloroform extracts from bile of Crigler-Najjar patients will not only contain unconjugated bilirubin-IXa, but possibly also some conjugated pigment, and yellow breakdown products of bilirubin-IXa. Furthermore, if unconjugated bilirubin-IXa is relatively important, then both the bile pigment concentration and the $\alpha_0$-fraction obtained with PIA will exceed the values obtained with EA at pH 2.7.

**RESULTS**

**Hepatic bilirubin UDP-GTA**

A liver biopsy was obtained from 18 of the 25 adults with Gilbert’s syndrome. UDP-GTA ranged between 42 and 494 $\mu$g of bilirubin-IXa conjugated per hour and per gram of wet weight of liver. This is far below the values found in normal individuals by the same method: $1,100 \pm 280$ (1 SD, [2])

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or 1,330±390 (6). In Crigler-Najjar disease, the activity of the enzyme was 0 in two children (M and V) and 145 in another (K; Table I). In two other patients, transferase activity measured by a modified version of the same method (26) was zero (C), and at successive assays, 0, 0, 0, and 252 (B).

Concentration of bile pigments in bile

Collection of duodenal bile fluid enriched with bile by means of intraduodenal magnesium sulfate or intravenous cholecystokinin inevitably leads to great individual variation in pigment concentration as a result of dilution by other secretions. Total bile pigment concentration in the samples was 4–194 mg/100 ml in the normals, 3–132 mg/100 ml in the adult Gilbert’s patients, and 0.6–8.5 mg/100 ml in eight of the Crigler-Najjar children. Bile obtained from child S by direct gallbladder puncture yielded 93 mg/100 ml. It is of interest that the concentrations calculated from the the method using diazotized PIA (total bilirubin) did not differ significantly from the values obtained with EA at pH 2.7 (conjugated bilirubin) in the normal individuals and in the adult patients with Gilbert’s syndrome. In contrast, in the bile samples of all the children suffering from Crigler-Najjar disease, total bilirubin concentrations greatly exceeded the values found with EA at pH 2.7, thus demonstrating the presence of unconjugated bilirubin in these samples (Table I).

Unconjugated azodipyrrrole (bilirubin-IXα monoconjugates) obtained from bile

Chromatographic analysis of EA azopigments formed at pH 2.7 in the bile of the control group, showed that αₐ-azopigment amounted to 13.6±3.9% (n = 20) of total azopigment. This proportion was significantly increased in patients with Gilbert’s syndrome, representing 24.3 ±4.9% (n = 25), P < 0.001. Markedly increased values (60.5±10.7) were obtained in the 16 bile samples from the nine children with Crigler-Najjar disease (Fig. 2). Determination of the αₐ-fraction with the two other diazo-methods (PIA and EA at pH 6.0) gave very similar values to those obtained with EA at pH 2.7 in the controls and in the adults with Gilbert’s syndrome; the differences never exceeded 4, except in two patients: one in the former group (Le) and one in the latter (VdR) in whom it was 7 (Table II). In contrast, in the 16 samples obtained from the nine children with Crigler-Najjar disease, αₐ-PIA on the average exceeded αₐ-EA (pH 2.7) by 20.13±7.78 (Table I). This again shows the presence of significant amounts of unconjugated bilirubin in the bile of children with Crigler-Najjar disease.

Bile of normal controls and of patients with Gilbert’s syndrome. Spectral and TLC analysis of azopigment αₐ both as the free acid and as its methyl ester, showed that the azopigment corresponded exclusively to unconjugated azodipyrrrole. A critical analysis of comparative assays with three diazo-methods (Table II) excluded

### TABLE III

<table>
<thead>
<tr>
<th></th>
<th>Total bilirubin concentration in bile</th>
<th>Concentration of UCB in bile</th>
<th>UCB as % of total diazopositive material</th>
<th>Difference (αₐ-PIA*–αₐ-EA) (pH 2.7)</th>
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</thead>
<tbody>
<tr>
<td><strong>Normals</strong></td>
<td>mg/100 ml</td>
<td>mg/100 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 individuals)</td>
<td>3.3–60.9</td>
<td>0–0.35</td>
<td>0–0.8</td>
<td>0–2.1 (but in one: 2.05)</td>
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<td><strong>Gilbert’s syndrome</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>0.02</td>
<td>0.07</td>
<td>–2.5</td>
</tr>
<tr>
<td>Be</td>
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<td>0.30</td>
<td>3.00</td>
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<td>VdP</td>
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<td>–2.2</td>
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<td></td>
<td>0.002</td>
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<td><strong>Crigler-Najjar disease</strong></td>
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<tr>
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</tr>
<tr>
<td>G₃</td>
<td>5.3</td>
<td>1.59</td>
<td>30</td>
<td>15</td>
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</table>

* Unconjugated azodipyrrrole formed by coupling with diazotized PIA.
† Unconjugated azodipyrrrole formed by coupling with diazotized EA at pH 2.7.
the formation of azodipyrrrole from diconjugates containing acid-labile conjugating bonds or from sources other than monoconjugates (see Validity of methods). Therefore, the amount of monoconjugates expressed as a percentage of total conjugated bilirubin-IXα (i.e. α0-EA [pH 2.7] multiplied by 2) was 27.2±7.8% (n = 20) and 48.6±9.8% (n = 25) in bile of normal adults and in that of patients with Gilbert’s syndrome, respectively (P < 0.001). It is likely that these values are valid for freshly secreted bile. Thus, the presence of specific deconjugating factors in the bile of the patients could be excluded on the following basis: equal volumes of normal bile and patient’s bile (four samples tested) were mixed and incubated at 37°C for 30 min. After diazo-treatment at pH 2.7 and TLC analysis of the products, the concentrations of azopigment α0 did not exceed the mean values calculated from assays performed on the individual bile samples. Furthermore, β-glucuronidase activity could not be detected in the bile from two normal controls nor in that from two patients with Gilbert’s syndrome. This observation is in agreement with the studies of Felsher et al. (3) in liver tissue and of Boonyapisit et al. (44) in bile from adult patients. We therefore conclude that the proportion of monoconjugates is significantly increased in the bile of patients with Gilbert’s syndrome or, conversely, that the excretion of diconjugated bilirubin-IXα is depressed.

Bile of patients with Crigler-Najjar disease. As with bile from normal adults and from patients with Gilbert’s syndrome, azopigment α0 corresponded to unconjugated azodipyrrrole, but its chemical significance is unclear (see Validity of methods). Obviously, all conjugated bilirubin-IXα could consist of monoconjugates as more than the required equivalent of unconjugated azodipyrrrole is formed at pH 2.7 (Table I). However, unconjugated azodipyrrrole must also have been formed from sources other than monoconjugates in those samples where α0-EA (pH 2.7) significantly exceeded the 50% value. Owing to lack of sufficient bile it has not been possible to analyze bile pigments directly by TLC in order to quantify the diconjugates present. However, a reasonable assumption is that the levels of diconjugates in the bile of these children are very low, or even absent.

Unconjugated bilirubin-IXα

The proportion of unconjugated bilirubin-IXα in bile, determined by PIA azopigment analysis of chloroform extracts, never exceeded 3%, and was usually below 0.8% in 10 normal subjects, two patients with Gilbert’s syndrome and two with hemolysis (Table III). In contrast, in four bile samples from two children with Crigler-Najjar disease, unconjugated bilirubin-IXα, estimated by chloroform extraction followed by PIA azopigment analysis, amounted to 30–57% of total diazo-positive material. These values may slightly overestimate the unconjugated azodipyrrrole fraction (see Validity of methods). However, in one case (G3), sufficient material was available for direct analysis of the chloroform extract by TLC, thereby permitting unequivocal determination of a component which migrated as unconjugated bilirubin-IXα. In comparison to α0-EA (pH 2.7), the increase seen in unconjugated azodipyrrrole α0 when bile samples from all the children were treated with diazo-reagent, under conditions promoting reaction of all bile pigments (EA pH 6.0 and PIA; Tables I and II), supports the contention that unconjugated bilirubin-IXα is of importance in the bile of Crigler-Najjar patients.

Conjugated azo-derivatives

All bile samples from the patients with Gilbert’s syndrome yielded abundant δ-azopigment on treatment with diazotized EA at pH 2.7. This
pigment amounted to 54.0±4.9% of the total azo-
color in bile samples from 12 patients, a value 
which is lower than that previously found for normal 
adults, 75.4±5.7% (23). δ-Azopigment was also formed 
upon diazo-treatment of bile from almost all patients 
with Crigler-Najjar disease (Table I). In three cases 
with severe type I disease (patients D, V, and G), the 
isolated δ-azopigments were further analyzed. On 
TLC, the methyl esters and the fully acetylated 
methyl esters of the δ-azopigment moved in each 
case as the corresponding derivatives of authentic 
azodipyrrole β-D-glucopyranuronoside. The identity of 
the unknowns and reference material was further 
supported by the parallel separation into their vinyl 
and isovinyl isomers. From the presently applied 
double-derivative formation technique, it may be con-
cluded unequivocally that bilirubin-IXα glucuronic 
was present in the bile of patients D, V, and G. This 
observation was further supported by mass spectrom-
etric analysis of the δ-azopigment found in the bile 
samples of children D and G. In view of the important 
implications of these results to our concepts of Crigler-
Najjar disease, an extensive organochemical and mass 
spectrometric study of bilirubin derivatives is in pro-
gress (45).

Azopigments α₂ and α₃ were found in all bile 
samples. In dog bile, they have been identified as the 
β-D-xylopyranoside and the β-D-glucopyranoside of 
azodipyrrole, respectively (31, 33). The same con-
clusion has been reached for human bile (Fevery, 
unpublished work). In the bile of patients with Gil-
bert’s syndrome, azopigments α₂ and α₀ amounted 
to 1.6±1.0 and 3.7±1.2% respectively, values which did 
not differ significantly from those found previously (23) 
for normal human bile, i.e. 1.2±0.4 and 3.5±0.8%. 
Somewhat higher amounts of azopigment α₀, ranging 
from 3–17%, were found in bile from children with 
Crigler-Najjar disease.

DISCUSSION

Gilbert’s syndrome or “constitutional hepatic dysfunc-
tion,” is characterized by chronic, often mild, uncon-
jugated hyperbilirubinemia in the absence of overt 
hemolysis (for a review, see reference 46). It has long 
remained a diagnosis made by exclusion of other 
diseases, and therefore depended on the extent of the 
investigations that had been performed. The recent 
demonstration of markedly decreased bilirubin UDP-
GTA, both in unactivated (5) and in digitonin-activated 
liver homogenates (2-4, 6, 7), complemented the 
previous observations of decreased transferase activity 
in some patients with more pronounced unconjugated 
hyperbilirubinemia (47, 48). In 1969, Arias et al. (22) 
described 16 cases of severe chronic nonhemolytic un-
conjugated hyperbilirubinemia which they proposed to 
subdivide into two groups. Group I was composed of 
the most severely affected children who developed 
kernicterus and whose condition did not improve upon 
treatment with phenobarbital. Their bile was “virtually 
colorless and contained only a trace of unconjugated 
bilirubin” (cited from Arias et al. [22]). In contrast, 
conjugates were detected in the bile of patients in 
group II, and their bilirubinemia decreased on treat-
ment with phenobarbital. The activity of hepatic 
bilirubin UDP-GTA was near to zero in both groups. 
Further aspects of the disease have been discussed 
recently by Blaschke et al. (49).

In the present study, several parameters of bilirubin 
metabolism have been investigated in both disorders. 
In our patients with Gilbert’s syndrome, transferase 
activity was 4–45% of the control values. Zero or 
non-zero activities were found in the children with 
Crigler-Najjar disease, in agreement with other work 
(22, 49, 50). This, however, does not offer an 
absolute diagnostic criterion, as values approaching 
zero were occasionally found in patients with Gil-
bert’s syndrome (50, 51) and in neonates without 
liver disease (52). However, for the present, transferase 
assays allow differentiation of Gilbert’s syndrome and 
Crigler-Najjar disease from other types of uncon-
jugated hyperbilirubinemia.

The analysis of bile showed that conjugated biliru-
bin-IXα was present in all samples examined, in-
cluding those obtained from the children with Crigler-
Najjar disease. The conjugating groups detected were 
glucuronic acid, glucose, and xylose. In seven of nine 
children with Crigler-Najjar disease, 9–48% of the EA-
azopigments contained glucuronic acid (δ-azopig-
ment); in two children (E and V), only trace amounts 
were found (Table I). Glucose residues (3–17%) were 
usually present in slightly higher relative proportions 
than in the bile from the controls. The present results 
indicate that the detectability or nondetectability of 
conjugated bilirubin-IXα (22) is not a reliable criterion 
for the differentiation of patients with Crigler-Najjar 
disease, although it should be noted that children E 
and V, whose bile contained only traces of glucuronide, 
belonged to the more severely affected group. The use-
fulness of differentiation based on response to pheno-
obarbital (group II) or lack of response and develop-
ment of kernicterus (group I) was confirmed (22). How-
ever, colorless bile was never observed in any of our 
children with Crigler-Najjar disease, even in the most 
severe cases.

The most striking feature of the present study was 
the highly significant increase in azopigment α₀ (un-
conjugated azodipyrrole) in the EA (pH 2.7)-treated 
bile from all patients with congenital nonhemolytic 
unconjugated hyperbilirubinemia. Minor increases in 
this pigment have occasionally been detected in pa-
tients with liver disease and cholestasis, but were
accompanied by other specific changes in biliary bilirubin composition (23). The $\alpha_r$-fraction was significantly higher in Crigler-Najjar disease than in Gilbert’s syndrome. A similar increase has been recently confirmed in four adults with Crigler-Najjar disease type II (53, 54). As the $\alpha_r$-fraction was not increased in patients with hemolysis in the absence of associated Gilbert’s syndrome (Table IV), determination of the $\alpha_r$-fraction may allow an easy, safe, and rapid diagnosis of bilirubin-IXα UDP-GTA deficiency (Fig. 2). In patients with Gilbert’s syndrome, the deficiency is clearly expressed in decreased formation and biliary secretion of diconjugated bilirubin-IXα.

In patients with Crigler-Najjar disease, our main finding was that conjugates were always present to some extent in bile, whatever the severity of the disease.

Evidence was found that these conjugates were mainly, and perhaps exclusively, bilirubin-IXα monoglucuronide, an observation which is in accordance with a study by Gordon et al. (54), who showed in addition that there was a definite decrease in the total biliary bilirubin output in these patients. This clearly indicates that the main abnormality in Crigler-Najjar disease is the decreased excretion of diconjugates, reflecting the transferase deficiency which is more pronounced in this condition than in Gilbert’s syndrome. In addition, the percentage of unconjugated bilirubin-IXα that we found in bile was typically increased to 30–57% of the total bile pigment. Such an increase has also been found by isotopical methods (55, 56). However, the concentrations of unconjugated bilirubin-IXα in the bile of patients with Crigler-Najjar disease were only one- to sevenfold the values found in normal adults or patients with Gilbert’s syndrome. This pigment therefore constitutes only a minor fraction of total heme breakdown. A comparable situation is found in Gunn rat bile where 31–40% of the biliary bile pigments is unconjugated bilirubin-IXα, although the daily excretion of this unconjugated pigment is barely higher than that of control Wistar rats and approaches only 3–4% of total heme turnover (43).

The observations reported in the present work raise several problems regarding the biochemical definition of the enzyme deficiencies involved in congenital, nonhemolytic, unconjugated hyperbilirubinemia. Firstly, a disproportion is apparent between transferase activities and the rates of formation of conjugated bilirubin-IXα in vivo. The near-zero GTA levels found occasionally in the liver of Gilbert’s patients in previous studies (50, 51), in the present work, and in neonatal liver (52), suggest that the standard enzyme assay which we have employed and which is optimized for normal liver tissue, may not always be adequate. The simplest explanation would appear to be that in Gilbert’s syndrome an abnormal transferase is present in the liver.

A situation of this sort seems to exist in Gunn rats whose liver has a normal amount of UDP-GTA acting on $p$-nitrophenol with an abnormally low affinity for UDP-glucuronic acid (57). The near-zero transferase activities typically found in the liver of Crigler-Najjar patients (Table 1) (22, 49, 50) are consistent with the low biliary output of bilirubin conjugates in this condition (54, 55). The difference in response towards phenobarbital found in type I and type II patients may also point to a fundamental difference in the type of transferase responsible for conjugating bilirubin-IXα.

How can the decreased excretion of diconjugated bilirubin-IXα be related to the documented bilirubin-IXα UDP-GTA deficiency? Too little is known about the biochemical mechanisms underlying the conjugation and excretion of bilirubin to warrant any thorough discussion. It may suffice to consider briefly a few of many possibilities which could explain our basic observations. If bilirubin-IXα were converted to its monoglucuronide and subsequently to its diglucuronide by a single enzymic site, then transferase deficiency would of course result in an increased bilirubin concentration in the cytosol. If one now assumes that unconjugated bilirubin-IXα binds more strongly to the enzyme than its monoconjugate, then a smaller fraction of the enzymic sites would be free to bind the monoglucuronide under conditions when a decreased amount of enzyme is available. As a consequence, a less rapid conversion to the diconjugate would ensue, together perhaps with a more efficient direct biliary elimination of the monoglucuronide.

### Table IV

<table>
<thead>
<tr>
<th>Patients</th>
<th>$\alpha_r$-EA (pH 2.7)*</th>
<th>UDP-GTA</th>
<th>Total bilirubin in serum</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ve</td>
<td>13.1</td>
<td>1.242</td>
<td>3.8</td>
<td>Spherocytosis</td>
</tr>
<tr>
<td>Vm</td>
<td>11.3</td>
<td>1.065</td>
<td>2.8</td>
<td>Spherocytosis</td>
</tr>
<tr>
<td>F</td>
<td>10.6</td>
<td>2.290</td>
<td>2.6</td>
<td>Spherocytosis</td>
</tr>
<tr>
<td>G</td>
<td>23.2</td>
<td>0.316</td>
<td>3.5</td>
<td>Autoimmune hemolysis</td>
</tr>
<tr>
<td>Vdp</td>
<td>26.3</td>
<td>0.418</td>
<td>2.3</td>
<td>Spherocytosis</td>
</tr>
<tr>
<td>S</td>
<td>25.7</td>
<td>0.234</td>
<td>4.5</td>
<td>Spherocytosis</td>
</tr>
<tr>
<td>Normals</td>
<td>13.6±3.9</td>
<td>1.100±0.280</td>
<td>&lt;1.0</td>
<td>—</td>
</tr>
</tbody>
</table>

The liver biopsies were taken in the patients at time of splenectomy.
* Unconjugated azodipyrolyle formed by treatment of bile with diazotized EA at pH 2.7.
nide. In the case of a two-enzyme system, an associated deficiency of the enzyme catalyzing conversion of mono- to conjugated bilirubin-IXα could explain the present data. However, even if the activity of the second enzyme is normal in vitro, as proposed by Jansen et al. (58) in studies of two patients with Crigler-Najjar type II, other mechanisms, such as accumulation of bilirubin-IXα within the hepatocyte, may inhibit the second step in vivo. Clearly, further investigation is necessary to elucidate the underlying mechanisms of glucuronide formation.

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