JCI The Journal of Clinical Investigation

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

Johan Fevery, … , Norbert Blanckaert, Karel P. M. Heirwegh

J Clin Invest. 1977;60(5):970-979. https://doi.org/10.1172/JCI108877.

Research Article

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 […]

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

JOHAN FEVERY, NORBERT BLANCKAERT, and KAREL P. M. HEIRWEGH,
Laboratory for Liver Physiopathology, Department of Medical Research, University of Leuven, 3000 Leuven, Belgium
ANNE-MARIE PRÉAUX and PIERRE BERTHELOT, *Unité de Recherches U-99, Institut National de la Santé et de la Recherche Médicale, Hôpital Henri Mondor, 94010 Créteil, France*

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[1] activity (UDP-GTA)[2] 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 take 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]).

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).

Received for publication 16 July 1976 and in revised form 17 May 1977.

[1] UDP-glucuronosyltransferase, or UDP-glucuronate β-glucuronosyltransferase (acceptor-unspecific), EC 2.4.1.17.

[2] *Abbreviations used in this paper*: EA, ethyl anthranilate; PIA, *p*-iodoaniline; TLC, thin-layer chromatography; UDP-GTA, bilirubin UDP-glucuronosyltransferase activity.

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 phenobarbital 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 phenobarbital in the third child (S) could not be obtained. If one takes the response to phenobarbital and the development of

TABLE I

Characteristics of the Nine Children with Crigler-Najjar Disease

Child	Unconjugated bilirubin in serum	Kernicterus*	Response to phenobarbital*	Hepatic UDP-GTA‡	Biliary bilirubin		Bile analysis			
					PIA	EA	α_0-PIA§	α_0-EA‖ (pH 2.7)	(α_0-PIA–α_0-EA) (pH 2.7)	δ-EA¶ (pH 2.7)
	mg/100 ml			*μg/h/g*	*mg/100 ml*		*% of total azopigment*			
K	25.0	–	+	145	2.6	1.9	68	51	17	27
B	23.5	–	+	0; 0; 0; 252	2.9	1.2	73	52	21	24
S**		–		ND‡‡	93.0	62.0	68	56	12	23
M_1§§	24.0	+	–	0	0.9	0.4	72	60	12	17
M_2					2.9	1.2	73	52	21	15
D_1§§	19.6	+	–	ND	2.7	1.1	81	47	34	40
D_2§§					2.4	0.8	56	41	15	48
D_3					1.3	0.6	84	61	23	25
E	23.1	+	–	ND	3.2	1.3	99	77	22	trace
V_1					5.2	3.0	87	69	18	3
V_2	24.0	+	–	0	0.6	0.3	97	58	39	trace
V_3§§					1.1	0.7	90	80	10	trace
C	35.0	+	–	0	4.8	1.6	86	60	26	23
G_1	25.0	+	–	ND	7.1	4.0	87	70	17	9
G_2§§					8.5	3.2	86	66	20	18
G_3§§					5.3	3.4	83	68	15	30

* Absence (–) or presence (+) of kernicterus or positive response to treatment.

‡ Bilirubin UDP-glucuronosyltransferase activity.

§ Unconjugated azodipyrrole formed by coupling with PIA.

‖ Unconjugated azodipyrrole formed by coupling with diazotized EA, a system in which, at pH 2.7, only the conjugated bilirubin reacts. Therefore the difference (α_0-PIA minus α_0-EA) gives an estimate of the amount of unconjugated bilirubin present.

¶ Glucuronidated azodipyrrole formed by coupling with diazotized EA.

** Bile obtained by gallbladder puncture at surgery.

‡‡ Not determined.

§§ Samples taken under phenobarbital therapy. Subscripts after the letters refer to successive bile samples from the same patient.

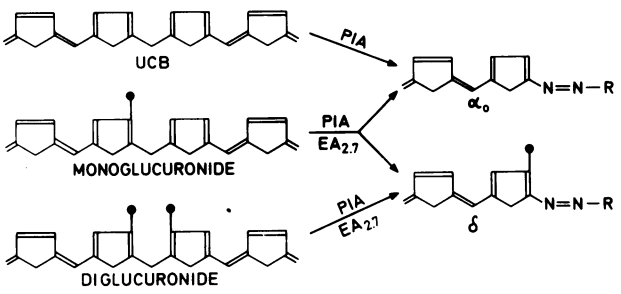

FIGURE 1 Schematic representation of diazo-cleavage of bilirubin, leading to dipyrrolic azopigments. Diazotized *p*-iodoaniline (PIA) reacts with both unconjugated (UCB) and conjugated bilirubin, whereas only the conjugates react with diazotized ethyl anthranilate (EA) at pH 2.7. In the PIA system, one molecule of UCB leads to the formation of two molecules of unconjugated azodipyrrole (α_0). In both PIA and EA systems: one molecule of bilirubin monoglucuronide produces one molecule of α_0 and one of conjugated δ-azopigment; diglucuronide yields only δ-azopigment.

kernicterus as discriminative features, then the former six children would belong to the so-called group I (22). In four of these patients, two consecutive bile samples were obtained, one before and one during treatment with phenobarbital, for periods varying from 1 mo to 1 yr.

Gallbladder bile was obtained after stimulation with intraduodenal magnesium sulfate (15 g) or intravenous (i.v.) injection of cholecystokinin (1 Ivy dog U/kg; CCK-PZ, GIH Unit, Karolinska Institute, Stockholm, Sweden). In all cases, a first sample (5–15 ml) taken before gallbladder stimulation was discarded because gastric juice present as a contaminant may induce artificial changes in the pattern of biliary bilirubin (23). The bile samples were analyzed either fresh or after storage at −20°C in the dark. Storage for at least 3 wk under these conditions did not alter the bilirubin composition, provided the samples were deep-frozen immediately after collection.

In the patients, but not in the controls, liver tissue was obtained by needle biopsy or at the time of splenectomy in the hemolytic patients. Specimens were assayed for bilirubin UDP-GTA immediately or after storage of the biopsy as such at −20°C. The activity remains unaltered for periods up to 6 wk at least under these conditions (24, 25).

Chemical methods. Bilirubin UDP-GTA was determined in digitonin-activated liver homogenates as described by Black et al. (25). In two children (C and B), transferase activity was measured by a slight modification of this method yielding essentially comparable results (26). β-glucuronidase activity was assayed with phenolphthalein as the substrate (27).

Bile pigments were assayed with various diazo-coupling techniques (Fig. 1). Before the assays, bile samples from the normal controls and adult patients were diluted 21 to 51-fold with distilled water. To obtain sufficient sensitivity in the assay, bile of patients with Crigler-Najjar disease was diluted only 3 to 11-fold, except in the child S (101-fold). Conjugated bilirubin was determined by treatment of diluted bile with diazotized ethyl anthranilate (EA) at pH 2.7 (28, 29). Total bile pigment was determined by coupling with diazotized *p*-iodoaniline (PIA) in the presence of a reaction accelerator (30). To detect any acid-labile conjugating bonds, bile was treated with diazotized EA at pH 6.0 (31); this also promotes reaction of unconjugated bilirubin-IXα if present.

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 (α_0; from bilirubin-IXα), of azodipyrrole β-D-monoxyloside (α_2), β-D-monoglucoside (α_3), 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 α_0-EA (pH 2.7) over total azopigment was calculated, thus permitting the determination of the relative amounts of mono- and diconjugated 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 α_0 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 mesoazodipyrrole (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 azodipyrrole 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 α_0-EA

Each mole of monoconjugated biirubin-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 α_0-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 α_0-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.

(*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 α_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-IXα. By TLC of the methyl ester of azopigment α_0 any contamination with mesoazodipyrrole can easily be detected. (35).

(*c*) Acid-labile conjugating linkages could be present in some bilirubin-IXα diconjugates. If split during diazo-coupling at pH 2.7, then some monoconjugated bilirubin-IXα could be formed. Inasmuch as such cleavage would be slower at less acidic pH, bile samples were treated in parallel with diazo-reagent at pH 2.7 (standard system) and at pH 6.0, and the α_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-IXα are found in human bile (23). In confirmation of previous data (28) and assays of urine (40) and serum (41), unconjugated bilirubin-IXα (10–20 mg/100 ml) added to diluted bile (5 to 51-fold) reacted only to a negligible extent. In particular the α_0-fractions were unchanged. It is likely, therefore, that reaction of endogenous unconjugated pigment can be disregarded. Even if it reacted completely its concentration 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 α_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 α_0-azopigment equal to, or higher than, 50% (Tables I and II), thus indicating that pigments other than monoconjugated bilirubin-IXα contributed to the formation of the α_0-azodipyrrole. In these bile samples, unconjugated bilirubin-IXα could be a major source of α_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-IXα 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 α_0-EA (pH 2.7) fraction.

Coupling with Diazotized PIA

In this procedure both unconjugated and conjugated bilirubin-IXα are transformed into azo-derivatives (Fig. 1). The α_0-PIA can thus derive from unconjugated and monoconjugated bilirubin-IXα. With bile from Crigler-Najjar patients, additional unconjugated azodipyrrole could originate from unconjugated, tetrapyrrolic breakdown products of bilirubin-IXα retaining an unmodified dipyrrole moiety (43).

TABLE II

Unconjugated Azopigment α_0 Obtained with Various Diazo-Procedures from Bile of Controls and Patients with Either Gilbert's Syndrome or Crigler-Najjar Disease*

	α_0-EA (pH 2.7)	α_0-EA (pH 6.0)	α_0-PIA
Controls			
V	13	13	14
Mo	7	8	9
L	12	10	11
Le	14	12	7
M	16	16	13
Adults with Gilbert's syndrome			
Bb	24	23	21
VdR	32	29	25
VR	26	25	26
WM	20	22	19
Sw	26	28	30
Children with Crigler-Najjar			
V_1	69	77	87
V_2	58	82	97
M_2	52	71	73
D_2	42	48	56

* The percentages of the α_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-IXα

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-IXα, but possibly also some conjugated pigment, and yellow breakdown products of bilirubin-IXα. Furthermore, if unconjugated bilirubin-IXα is relatively important, then both the bile pigment concentration and the α_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 μg of bilirubin-IXα 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±280 (1 SD, [2])

TABLE III

Unconjugated Bilirubin-IXα (UCB) in Bile of Normal Individuals and Patients with Either Gilbert's Syndrome or Hemolysis or Crigler-Najjar Disease

	Total bilirubin concentration in bile	Concentration of UCB in bile	UCB as % of total diazopositive material	Difference (α_0-PIA*−α_0-EA‡) (pH 2.7)
	mg/100 ml	*mg/100 ml*		
Normals				
(10 individuals)	3.3–60.9	0–0.35	0–0.8 (but in one: 2.05)	0–2.1
Gilbert's syndrome				
B	29.5	0.02	0.07	−2.5
Be	10.2	0.30	3.00	0.5
Hemolysis				
VdP	45.0	traces	0	−2.2
H	402.9	0.002	traces	−3.0
Crigler-Najjar disease				
V_1	5.2	2.45	47	18
V_2	0.6	0.34	57	39
V_3	1.1	0.60	54	10
G_3	5.3	1.59	30	15

* Unconjugated azodipyrrole formed by coupling with diazotized PIA.

‡ Unconjugated azodipyrrole formed by coupling with diazotized EA at pH 2.7.

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 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 azodipyrrole (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 α_0-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 α_0-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, α_0-PIA on the average exceeded α_0-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 α_0, both as the free acid and as its methyl ester, showed that the azopigment corresponded exclusively to unconjugated azodipyrrole. A critical analysis of comparative assays with three diazo-methods (Table II) excluded

the formation of azodipyrrole 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. α_0-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 α_0 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 α_0 corresponded to unconjugated azodipyrrole, 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 azodipyrrole is formed at pH 2.7 (Table I). However, unconjugated azodipyrrole must also have been formed from sources other than monoconjugates in those samples where α_0-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 quantitate 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 choloroform extraction followed by PIA azopigment analysis, amounted to 30–57% of total diazo-positive material. These values may slightly overestimate the unconjugated azodipyrrole 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 α_0-EA (pH 2.7), the increase seen in unconjugated azodipyrrole α_0 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.

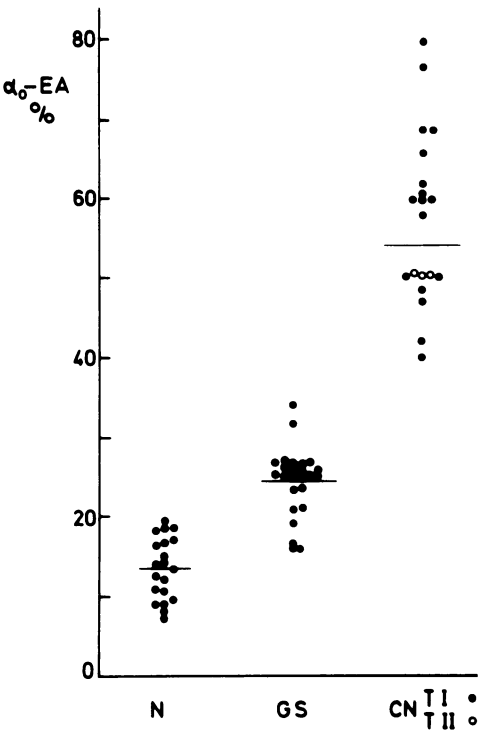

FIGURE 2 The percentage of α_0 unconjugated azodipyrrole formed with diazotized EA at pH 2.7 in bile from normal adults (N), from patients with Gilbert's syndrome (GS), and from patients with Crigler-Najjar (CN) disease of type I (●) or type II (○).

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 azocolor 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 concluded unequivocally that bilirubin-IXα glucuronide was present in the bile of patients D, V, and G. This observation was further supported by mass spectrometric 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 progress (45).

Azopigments α_2 and α_3 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 conclusion has been reached for human bile (Fevery, unpublished work). In the bile of patients with Gilbert's syndrome, azopigments α_2 and α_3 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 α_3, ranging from 3–17%, were found in bile from children with Crigler-Najjar disease.

DISCUSSION

Gilbert's syndrome or "constitutional hepatic dysfunction," is characterized by chronic, often mild, unconjugated 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 unconjugated 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 treatment 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 near-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 Gilbert'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 unconjugated hyperbilirubinemia.

The analysis of bile showed that conjugated bilirubin-IXα was present in all samples examined, including 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-azopigment); 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 usefulness of differentiation based on response to phenobarbital (group II) or lack of response and development of kernicterus (group I) was confirmed (22). However, 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 α_0 (unconjugated 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 patients with liver disease and cholestasis, but were

accompanied by other specific changes in biliary bilirubin composition (23). The α_0-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 α_0-fraction was not increased in patients with hemolysis in the absence of associated Gilbert's syndrome (Table IV), determination of the α_0-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

TABLE IV

Comparison of the Percentage of Unconjugated Azodipyrrole, UDP-GTA in Hepatic Biopsies and Serum Bilirubin Levels in Six Patients with Hemolysis and in Normal Individuals

	α_0-EA (pH 2.7)*	UDP-GTA	Total bilirubin in serum	Diagnosis
	% of total azopigment	*mg/h/g liver*	*mg/100 ml*	
Patients				
Ve	13.1	1.242	3.8	Spherocytosis
Vm	11.3	1.065	2.8	Spherocytosis
P	10.6	2.260	2.6	Spherocytosis
G	23.2	0.316	3.5	Autoimmune hemolysis
VdP	26.3	0.418	2.3	Spherocytosis
S	25.7	0.234	4.5	Spherocytosis
Normals (20 individuals)	13.6±3.9	1.100±0.280	<1.0	—

The liver biopsies were taken in the patients at time of splenectomy.
* Unconjugated azodipyrrole formed by treatment of bile with diazotized EA at pH 2.7.

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 disconjugated 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 monoglucuro-

nide. In the case of a two-enzyme system, an associated deficiency of the enzyme catalyzing conversion of mono- to diconjugated 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.

ACKNOWLEDGMENTS

We thank Doctors Brun, Dodion, Eggermont, Götze, Kline, Lévy, Nussbaumer, Rozenkranz, Sinaasappel, and Thaler who generously provided us with bile samples of children with Crigler-Najjar disease. Our thanks are also due to Doctors De Groote, Dhumeaux, Hanoune, Métreau, Vandenbroucke, and Verwilghen for support and discussions, and to Dr. Compernolle for mass spectrometric analysis. We are greatly indebted to the nurses and to P. Leroy, D. Serpeau, and M. Vandevijver for their skillful help in collecting and analyzing the samples. We thank Dr. John Chapman for careful assistance in reviewing the manuscript and Mrs. M. Tassier for the preparation of the manuscript.

This work was performed with grants from Institut National de la Santé et de la Recherche Médicale, France, and from Nationaal Fonds voor Wetenschappelyk Onderzoek, Belgium.

REFERENCES

1. Fevery, J., N. Blanckaert, A. M. Préaux, K. P. M. Heirwegh, and P. Berthelot. 1973. Unconjugated and conjugated bilirubin pigments in bile in Gilbert's and Crigler-Najjar's syndromes. *Digestion.* **8:** 445–446A. (Abstr.)
2. Black, M., and B. H. Billing. 1969. Hepatic bilirubin UDP-glucuronyl transferase activity in liver disease and Gilbert's syndrome. *N. Engl. J. Med.* **280:** 1266–1271.
3. Felsher, B. F., J. R. Craig, and N. Carpio. 1973. Hepatic bilirubin glucuronidation in Gilbert's syndrome. *J. Lab. Clin. Med.* **81:** 829–837.
4. Fevery, J., J. De Groote, and K. P. M. Heirwegh. 1973. Quantitation of hepatic bilirubin conjugation. *In* The Liver: Quantitative Aspects of Structure and Function. G. Paumgartner and R. Preisig, editors. S. Karger, Basel. 203–209.
5. Bellet, H., and A. Raynaud. 1974. An assay of bilirubin UDP-glucuronyl transferase on needle-biopsies applied to Gilbert's syndrome. *Clin. Chim. Acta.* **53:** 51–55.
6. Métreau, J. M., F. Roudot, A. M. Préaux, D. Dhumeaux, and P. Berthelot. 1974. Dyserythropoiesis, a necessary condition for disclosing Gilbert's disease. *Digestion.* **10:** 315A. (Abstr.)
7. Felsher, B. F., and N. M. Carpio. 1975. Caloric intake and unconjugated hyperbilirubinemia. *Gastroenterology.* **69:** 42–47.
8. Kirshenbaum, G., D. M. Shames, and R. Schmid. 1976. An expanded model of bilirubin kinetics: effect of feeding, fasting, and phenobarbital in Gilbert's syndrome. *J. Pharmacokinet. Biopharm.* **4:** 115–155.
9. Zakim, D., and D. A. Vessey. 1974. Membrane dependence of uridine diphosphate glucuronyltransferase: effect of the membrane on kinetic properties. *Biochem. Soc. Trans.* **2:** 1165–1167.
10. Abou-el Makarem, M. M., and K. W. Bock. 1976. UDP-glucuronyltransferase in perfused rat liver and in microsomes: glucuronidation of bilirubin. *Eur. J. Biochem.* **62:** 411–416.
11. Billing, B. H., R. Williams, and T. G. Richards. 1964. Defects in hepatic transport of bilirubin in congenital hyperbilirubinaemia: an analysis of plasma bilirubin disappearance curves. *Clin. Sci.* (Oxf.). **27:** 245–257.
12. Barrett, P. V. D., P. D. Berk, M. Menken, and N. I. Berlin. 1968. Bilirubin turnover studies in normal and pathologic states using bilirubin-^{14}C. *Ann. Intern. Med.* **68:** 355–377.
13. Berk, P. D., J. R. Bloomer, R. B. Howe, and N. I. Berlin. 1970. Constitutional hepatic dysfunction (Gilbert's syndrome): a new definition based on kinetic studies with unconjugated radiobilirubin. *Am. J. Med.* **49:** 296–305.
14. Bloomer, J. R., P. D. Berk, R. B. Howe, and N. I. Berlin. 1971. Bilirubin metabolism in congenital nonhemolytic jaundice. *Pediatr. Res.* **5:** 256–264.
15. Crigler, J. F., and N. I. Gold. 1969. Effect of sodium phenobarbital on bilirubin metabolism in an infant with congenital, nonhemolytic, unconjugated hyperbilirubinemia, and kernicterus. *J. Clin. Invest.* **48:** 42–55.
16. Berk, P. D., J. R. Bloomer, R. B. Howe, T. F. Blaschke, and N. I. Berlin. 1972. Bilirubin production as a measure of red cell life span. *J. Lab. Clin. Med.* **79:** 364–378.
17. Frezza, M., G. Perona, R. Corrocher, R. Cellerino, M. A. Bassetto, and G. De Sandre. 1973. Bilirubin-H^3 kinetic studies: pattern of normal Gilbert's syndrome and hemolytic states. *Acta Hepato-Gastroenterol.* **20:** 363–371.
18. Auclair, C., G. Feldmann, J. Hakim, P. Boivin, J. Boucherot, and H. Troube. 1976. Bilirubin and paranitrophenol glucuronyl transferase activities and ultrastructural aspect of the liver in patients with chronic hemolytic anemias. *Biomedicine (Paris).* **25:** 61–65.
19. Heirwegh, K. P. M., J. Fevery, J. A. T. P. Meuwissen, J. De Groote, F. Compernolle, V. Desmet, and F. P. Van Roy. 1974. Recent advances in the separation and analysis of diazo-positive bile pigments. *Methods Biochem. Anal.* **22:** 205–250.
20. Kline, W., and A. Rosenkranz. 1971. Klinische und biochemische Studien zum Crigler-Najjar-Syndrom. *Monatsschr. Kinderheilk.* **119:** 555–558.
21. Götze, H., D. Sidiropoulos, F. A. Hess, and P. Berthelot. 1972. Das Crigler-Najjar-Syndrom: klinische, biochemische, morphologische und therapeutische Aspekte. *Helv. Paediatr. Acta.* **27:** 335–351.
22. Arias, I. M., L. M. Gartner, M. Cohen, J. Ben Ezzer, and A. J. Levi. 1969. Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency: clinical, biochemical, pharmacologic and genetic evidence for heterogeneity. *Am. J. Med.* **47:** 395–409.
23. Fevery, J., B. Van Damme, R. Mechiel, J. De Groote, and K. P. M. Heirwegh. 1972. Bilirubin conjugates in bile of man and rat in the normal state and in liver disease. *J. Clin. Invest.* **51:** 2482–2492.
24. Black, M., B. H. Billing, and K. P. M. Heirwegh. 1970. Determination of bilirubin UDP-glucuronyl transferase activity in needle-biopsy specimens of human liver. *Clin. Chim. Acta.* **29:** 27–35.
25. Duvaldestin, P., J. L. Mahu, and P. Berthelot. 1975. Effect of fasting on substrate specificity of rat liver UDP-glucuronosyltransferase. *Biochim. Biophys. Acta.* **384:** 81–86.
26. Frei, J. 1970. Multiplicity and specificity of UDP-glucuronyltransferase. I. Effect of divalent cations and

EDTA on the activity of UDP-glucuronyl-transferase assayed with bilirubin, 4-methylumbelliferone and *p*-nitrophenol. *Enzymol. Biol. Clin.* **11:** 385–401.
27. Rodriguez Garay, E. A., M. Del Rosario Spetale, and L. S. Morisoli. 1972. Relationship between bilirubin and β-glucuronidase activity in rats with experimental obstructive jaundice. *Clin. Chim. Acta.* **37:** 171–177.
28. Van Roy, F. P., and K. P. M. Heirwegh. 1968. Determination of bilirubin glucuronide and assay of glucuronyltransferase with bilirubin as acceptor. *Biochem. J.* **107:** 507–518.
29. Heirwegh, K. P. M., G. P. Van Hees, P. Leroy, F. P. Van Roy, and F. H. Jansen. 1970. Heterogeneity of bile pigment conjugates as revealed by chromatography of their ethyl anthranilate azopigments. *Biochem. J.* **120:** 877–890.
30. Van Roy, F. P., J. A. T. P. Meuwissen, F. De Meuter, and K. P. M. Heirwegh. 1971. Determination of bilirubin in liver homogenates and serum with diazotized *p*-iodoaniline. *Clin. Chim. Acta.* **31:** 109–118.
31. Fevery, J., G. P. Van Hees, P. Leroy, F. Compernolle, and K. P. M. Heirwegh. 1971. Excretion in dog bile of glucose and xylose conjugates of bilirubin. *Biochem. J.* **125:** 803–810.
32. Compernolle, F., F. H. Jansen, and K. P. M. Heirwegh. 1970. Mass-spectrometric study of the azopigments obtained from bile pigments with diazotized ethyl anthranilate. *Biochem. J.* **120:** 891–894.
33. Compernolle, F., G. P. Van Hees, J. Fevery, and K. P. M. Heirwegh. 1971. Mass-spectrometric structure elucidation of dog bile azopigments as the acyl glycosides of glucopyranose and xylopyranose. *Biochem. J.* **125:** 811–819.
34. Heirwegh, K. P. M., G. P. Van Hees, N. Blanckaert, J. Fevery, and F. Compernolle. 1976. Comparative studies on the structures of conjugated bilirubin-IXα and changes in cholestasis. *In* The Hepatobiliary System. W. Taylor, editor. Plenum Press, New York. 339–358.
35. Blanckaert, N., K. P. M. Heirwegh, and F. Compernolle. 1976. Synthesis and separation by thin-layer chromatography of bilirubin-IX isomers. *Biochem. J.* **155:** 405–417.
36. Brodersen, R., and I. Vind. 1963. A specific method for quantitative determination of unconjugated bilirubin and mesobilirubin. *Scand. J. Clin. Lab. Invest.* **15:** 225–232.
37. McDonagh, A. F., and F. Assisi. 1971. Commercial bilirubin: a trinity of isomers. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **18:** 315–317.
38. Fevery, J., P. Leroy, M. Van de Vijver, and K. P. M. Heirwegh. 1972. Structures of bilirubin conjugates synthetized in vitro from bilirubin and uridine diphosphate glucuronic acid, uridine diphosphate glucose or uridine diphosphate xylose by preparations from rat liver. *Biochem. J.* **129:** 635–644.
39. Heirwegh, K. P. M., J. A. T. P. Meuwissen, and J. Fevery. 1973. Critique of the assay and significance of bilirubin conjugation. *Adv. Clin. Chem.* **16:** 239–289.
40. Fevery, J., F. H. Jansen, J. A. T. P. Meuwissen, and K. P. M. Heirwegh. 1968. Bilirubin-like material in urine of normal adults. *Clin. Chim. Acta.* **21:** 401–410.
41. Franco, D., A. M. Préaux, H. Bismuth, and P. Berthelot. 1972. Extra hepatic formation of bilirubin glucuronides in the rat. *Biochim. Biophys. Acta.* **286:** 55–61.
42. With, T. K. 1968. Bile Pigments. Academic Press, Inc., New York. 324–328.
43. Blanckaert, N., J. Fevery, K. P. M. Heirwegh, and F. Compernolle. 1977. Characterization of the major diazo-positive pigments in bile of homozygous Gunn rats as the four isomers of bilirubin-IX and as one dihydroxyl and two monohydroxyl derivatives of bilirubin-IXα. *Biochem. J.* **164:** 237–249.
44. Boonyapisit, S. T., B. W. Trotman, and J. D. Ostrow. 1974. Unconjugated bilirubin (UCB) and hydrolysis of conjugated bilirubin (CB) in gallbladder bile of patients with pigment or cholesterol gallstones. *Gastroenterology.* **67:** 781A. (Abstr).
45. Blanckaert, N., F. Compernolle, J. Dodion, J. Fevery, and K. P. M. Heirwegh. 1976. Characterization of the bilirubinoid pigments in bile of children with Crigler-Najjar disease (CN) and of homozygous Gunn rats: the effect of phototherapy. *Gastroenterology.* **71:** 897.
46. Foulk, W. T., H. R. Butt, C. A. Owen, F. F. Whitcomb, and H. L. Mason. 1959. Constitutional hepatic dysfunction (Gilbert's disease): its natural history and related syndromes. *Medicine (Baltimore).* **38:** 25–46.
47. Arias, I. M., and I. M. London. 1957. Bilirubin glucuronide formation in vitro: demonstration of a defect in Gilbert's disease. *Science (Wash. D.C.).* **126:** 563–564.
48. Arias, I. M. 1962. Chronic unconjugated hyperbilirubinemia without overt signs of hemolysis in adolescents and adults. *J. Clin. Invest.* **41:** 2233–2245.
49. Blaschke, T. F., P. D. Berk, B. F. Scharschmidt, J. R. Guyther, J. M. Vergalla, and J. G. Waggoner. 1974. Crigler-Najjar syndrome: an unusual course with development of neurologic damage at age eighteen. *Pediatr. Res.* **8:** 573–590.
50. Okolicsanyi, L., J. Frei, P. Magnenat, and R. Naccarato. 1971. Multiplicity of UDP-glucuronyl transferase. III. UDP-glucuronyl transferase and β-glucuronidase activities assayed with different substrates in inherited and acquired human liver disease. *Enzyme (Basel).* **12:** 658–673.
51. Black, M., R. D. Perrett, and A. E. Carter. 1973. Hepatic bilirubin UDP-glucuronyl transferase activity and cytochrome P 450 content in a surgical population, and the effects of preoperative drug therapy. *J. Lab. Clin. Med.* **81:** 704–712.
52. Felsher, B. F., N. M. Carpio, M. M. Wooley, and M. J. Asch. 1974. Hepatic bilirubin glucuronidation in neonates with unconjugated hyperbilirubinemia and congenital gastrointestinal obstruction. *J. Lab. Clin. Med.* **83:** 90–96.
53. Gollan J. L., S. N. Huang, B. Billing, and S. Sherlock, 1975. Prolonged survival in three brothers with severe type 2 Crigler-Najjar syndrome: ultrastructural and metabolic studies. *Gastroenterology.* **68:** 1543–1555.
54. Gordon, E. R., E. A. Shaffer, and A. Sass-Kortsak. 1976. Bilirubin secretion and conjugation in the Crigler-Najjar type II. *Gastroenterology.* **70:** 761–765.
55. Schmid, R., and L. Hammaker. 1963. Metabolism and disposition of C^{14}-bilirubin in congenital nonhemolytic jaundice. *J. Clin. Invest.* **42:** 1720–1734.
56. Thaler, M. M., N. H. Dawber, J. Krasner, S. J. Yaffe, and L. Mosovich. 1973. Effects of phototherapy on bilirubin(B) metabolism and sulfobromophthalein (BSP) excretion in unconjugated hyperbilirubinemia. *Pediatr. Res.* **7:** 334A. (Abstr.)
57. Nakata, D., D. Zakim, and D. A. Vessey. 1976. Defective function of a microsomal UDP-glucuronyltransferase in Gunn rats. *Proc. Natl. Acad. Sci. U.S.A.* **73:** 289–292.
58. Jansen, P. L. M., J. R. Chowdhury, E. Fischberg, and I. M. Arias. 1976. An enzyme in liver plasma membrane which converts bilirubin monoglucuronide (BMG) to bilirubin diglucuronide (BDG). *Clin. Res.* **24:** 433A. (Abstr.)