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Identification of Glutathione Conjugates of the Dimethyl Ester of Bilirubin in the Bile of Gunn Rats

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SUMMARY

The Gunn rat, which is deficient in the UDP-glucuronosyltransferase for bilirubin, promptly excreted polar conjugates of the dimethyl ester of bilirubin in bile after intravenous infusion of this ester. The conjugates proved to be monoglutathione thioether adducts of the vinyl groups of the parent tetrapyrrole. High performance liquid chromatographic analysis of the conjugates as their dipyrrolic azosulfanilates demonstrated that only one of the dipyrroles of each tetrapyrrole was conjugated. The nonconjugated dipyrrole eluted as either the methyl endo- or exovinyl azodipyrrole. The amino acid composition of the pigments was consistent with that of a monoglutathione conjugate. NMR spectroscopy of the two major pigments demonstrated the loss of the proton signals of the C-18 vinyl group, indicating it to be the site of conjugation. Cation fast atomic bombardment tandem mass spectrometry demonstrated a molecular ion, $[M + H]^+$, of *m/z* 937, which fragmented with a loss of 307 atomic mass units, consistent with glutathione. A molecular ion of *m/z* 807 was observed for the conjugate treated with γ-glutamyltranspeptidase, consistent with the loss of glutamate. The mass spectrometry data indicated that the conjugates also contained a functional group whose mass was equivalent to hydroxyl, suggesting initial formation of an epoxide, which then reacts with glutathione. Pretreatment of the rat with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to induce cytochrome P-450 resulted in a 6-fold increase of the biliary excretion of the glutathione conjugates. Such induction also resulted in the excretion of a glutathione conjugate of bilirubin itself.

The major excretory pathway of bilirubin IXα into bile involves its prior conjugation, by hepatic microsomes, to glycosidic esters at the C-8 and C-12 propionic acid side chains (Fig. 1). In humans and most mammals, this biotransformation is catalyzed by microsomal bilirubin UDP-glucuronosyltransferase (EC 2.4.1.17), with UDP-glucuronic acid serving as the glycoside donor (1). Neutral ester glycosides of bilirubin with glucose and xylose are found in the bile of some mammals (2–4).

The importance of glucuronide formation for bile pigment excretion is well demonstrated by the absence of hepatic UDP-glucuronosyltransferase activity for bilirubin in the genetic disorders type 1 Crigler-Najjar syndrome of humans (5–7) and its animal model, the homozygous Gunn rat (8, 9). In both instances, nonconjugated hyperbilirubinemia develops within hours after birth and is life-long. Both disorders are autosomal recessive. The obligate heterozygotes, although nonjaundiced, have delayed clearance of injected bilirubin (10) and, when assayed, exhibit significantly decreased UDP-glucuronosyltransferase activity for bilirubin and other aglycones (6, 9, 11–13). Recent molecular biologic studies demonstrate an absence of the enzyme protein in the Gunn rat (14, 15) and in the Crigler-Najjar syndrome, as well as a possible mutated inactive enzyme protein in the Crigler-Najjar syndrome (16).

Despite the absence of the glucuronidation of bilirubin as an excretory pathway, both the deficient human and rat do excrete their bile pigments by alternative routes, albeit in amounts insufficient to prevent life-threatening hyperbilirubinemia. Intravenous administration of [^{14}C]bilirubin has demonstrated that 85% or more of the radiolabel is recovered as pigments in bile and feces (17, 18). By solvent extraction, these pigments partitioned in more polar solvent fractions than did nonconjugated bilirubin. The nature of these more polar products has been only partially described. Blanckaert *et al.* (19), in extensive studies of the dipyrrolic azoanthranilate derivatives of the bile pigments of Gunn rat bile, found traces of a dihydroxyl derivative of bilirubin at the C-18 vinyl group. Ostrow and coworkers (20) identified a dihydroxyl derivative of bilirubin that they believed involved the oxidation of the methine bridge carbons adjacent to the B and C pyrrole rings (Fig. 1).

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ABBREVIATIONS: TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin; DMB, dimethyl bilirubin; Me_2SO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; FAB, fast atomic bombardment; CAD, collisionally activated decomposition; COSY, correlation spectroscopy; amu, atomic mass units.

The formation of hydroxyl derivatives of bilirubin suggests that an alternative pathway for bilirubin excretion may involve the cytochrome P-450 dependent monooxygenase system of microsomes. Indeed, induction of cytochrome P-450 with TCDD in Gunn rats produces a 60% reduction in the animal's serum bilirubin concentration and an associated 89% decrease in the miscible bilirubin pool (21).

This report presents evidence that the homozygous Gunn rat can form and excrete thioether glutathione conjugates of the dimethyl ester of bilirubin *in vivo*, at its C-3 and C-18 vinyl groups, after the infusion of DMB.

Materials and Methods

Animals

Homozygous jaundiced (jj) Gunn rats were derived from our Madison colony, which was established in 1976 from breeder pairs kindly provided by Dr. Lois Johnson of the University of Pennsylvania. Non-jaundiced Wistar rats were purchased (Harlan Sprague Dawley Co., Indianapolis, IN).

Adult male animals (280–350 g) were anesthetized with pentobarbital by intraperitoneal injection of pentobarbital (35 mg/kg) and were surgically equipped with PE-10 cannulas of the jugular vein and common bile duct. The DMB (5 mg dissolved in 0.35 ml of Me_2SO) was infused intravenously over 20 min, and biliary excretions were quantified as previously described (22). Pentobarbital was used for anaesthesia, because it had been previously shown to have minimal effects on bile flow and bile pigment excretion (23).

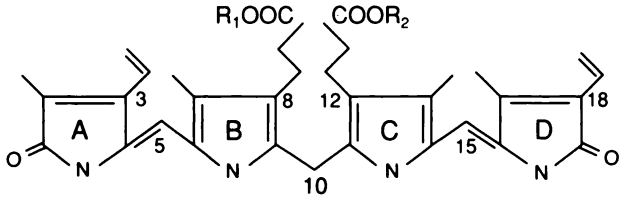
Induction of cytochrome P-450 in Gunn rats was done by a single intraperitoneal injection (10 μg/kg) of TCDD, dissolved in dioxane (25 μg/ml), 48–72 hr before use of the animals for study.

The DMB used for these experiments was synthesized as previously described (24).

Isolation and Chemical Identification of the Excreted Bile Pigments

The bile pigments from the infused animals were initially isolated from the bile by reverse phase HPLC, using a preparative Waters C18 μBondapack (7.8 mm × 30 cm) column, as recently described (25). Briefly, all samples were injected (400–800 μl) by using a Perkin-Elmer ISS-100 automatic sample injector equipped with a 1.0-ml sample loop. All elution solvents were prefiltered, deaerated with helium, and delivered by a Perkin-Elmer series 4 liquid chromatograph. The column was protected with a Waters Guard-Pak module containing a disposable PCSS C18 Guard-Pak precolumn insert. Column eluates were monitored with a Hewlett-Packard 1040A diode array detector controlled by a Hewlett-Packard 85B computer with an on-line Hewlett-Packard 3392 A integrator. The detector data were stored on a Bering series 3000 subsystem data-storage terminal and printed with a Hewlett-Packard 7470A graphic plotter. The pigments were monitored as their tetrapyrroles at 450 nm (band width, 4 nm) against a reference at 590 nm (band width, 10 nm). The bile was analyzed directly without any prior solvent extractions.

The mobile phase consisted of 0.1 M ammonium acetate (titrated to pH 4.85 with glacial acetic acid) and acetonitrile. An initial linear gradient from 30 to 40% (v/v) acetonitrile for 15 min was followed by a 10-min isocratic elution period. The acetonitrile was then linearly increased over 1 min to 80% followed by a 10-min isocratic elution. The initial composition of 30% acetonitrile was restored over 10 min, and a 15-min equilibration period was employed between injections. The flow rate was 4 ml/min.



Bilirubin IXα, (R_1, R_2 = H)

Fig. 1. Structure of bilirubin IXα.

The individual bile pigments were collected as their linear tetrapyrroles after passage through the detector into iced glass tubes (50 ml) under subdued light. Multiple (800-μl) injections of bile collected from one to three experimental animals were pooled when larger amounts of pigments were needed.

The subsequent HPLC analyses of the pigments either as their tetrapyrroles or as their derivatized dipyrrolic azosulfanilates employed a Waters reverse phase C18 μBondapack analytical (3.9 mm × 30 cm) column. The azoderivatives were monitored at 540 nm (band width, 4 nm) against a reference of 590 nm (band width, 10 nm). The mobile phase was the same as with the tetrapyrroles, but the profile consisted of an initial 10-min isocratic elution of 20% acetonitrile in 0.1 M ammonium acetate, pH 4.85, followed by a linear gradient of 20 min to 60% acetonitrile and a 5-min return to 20% acetonitrile. The flow rate was 1 ml/min.

For the purposes of this report, we studied the four unidentified pigments from Gunn rat bile eluting at 18, 19, 24, and 26 min and the bilirubin diglucuronide and C-8 bilirubin monoglucuronide from the Wistar rat bile eluting at 10 and 19 min, respectively (see Fig. 2). The samples were frozen at −70° overnight and subsequently lyophilized under vacuum (model 8; Labconco, Kansas City, MO) for 24 hr, while protected from light.

The amounts of the individual pigments, before lyophilization, were quantified by measurement of their total volume and absorbance at 450 nm in a Varian (DMA 80) dual-beam spectrophotometer, using 0.1 M ammonium acetate/acetonitrile (60:40, v/v) as a blank. An aliquot (0.4 ml) was converted to its dipyrrolic azosulfanilates by the addition of 0.1 ml of diazotized sulfanilic acid (21.0 mM in 0.7 M HCl). The reaction mixture was diluted to 1 ml with methanol, and after 30 min its absorbance was measured at 540 nm against 50% methanol. The pigment concentrations were calculated using the extinction coefficient ϵ_{540} = 60 mM^{-1} cm^{-1} for the azodipyrroles (26).

Analysis of the Lyophilized Pigments

Incubation with γ-glutamyltranspeptidase. The four major pigments of Gunn rat bile and the bilirubin monoglucuronide from the Wistar bile were dissolved in 0.05 M acetic acid/methanol (1:1, v/v). One to 3 nmol of pigment, as its bilirubin equivalent, were incubated with 1.15 units of γ-glutamyltranspeptidase, (EC2.3.2.2, Sigma Chemical Co. type II) (27) in a final volume of 0.2 ml containing 0.03 M Tris, pH 7.4, 0.04 M NaCl (27). The samples were incubated at 37° for 30 min, iced, and centrifuged at 8000 × *g* for 5 min in an Eppendorf tabletop centrifuge (Brinkman 5413). Each supernatant was divided so that 100 μl were analyzed directly by HPLC, as the tetrapyrrole, on the C18 μBondapack analytical column, as described above. The remainder of the supernatant was converted to the dipyrrolic azosulfanilates by addition of an equal volume (0.1 ml) of 21 mM diazotized sulfanilic acid in 0.7 M HCl. After 30 min, the latter sample was analyzed by HPLC as the dipyrrolic derivatives. Control samples included incubations without γ-glutamyltranspeptidase, as well as analyses of the freshly dissolved pigments without incubation.

The bile pigments of the Gunn and Wistar rats were also studied for potential hydrolysis by incubation of the bile, diluted in 0.06 M potassium phosphate buffer, pH 6.8, containing 1 mM ascorbic acid, with (a) β-D-glucuronidase (EC 3.2.1.31) (type IX, 1000 units) at 37° for 30 min, (b) α-D-glucosidase (EC 3.2.1.20) (type I, 16 units) at 37° for 120 min, and (c) β-D-glucosidase (EC 3.2.1.21) (type II, 16 units) at 37° for 120 min (28, 29). The three enzymes were from Sigma Chemical Co. (St. Louis, MO). After incubation, the samples were iced and centrifuged at 8000 × *g* for 5 min, and the supernatants were analyzed by HPLC as their linear tetrapyrroles, as described above.

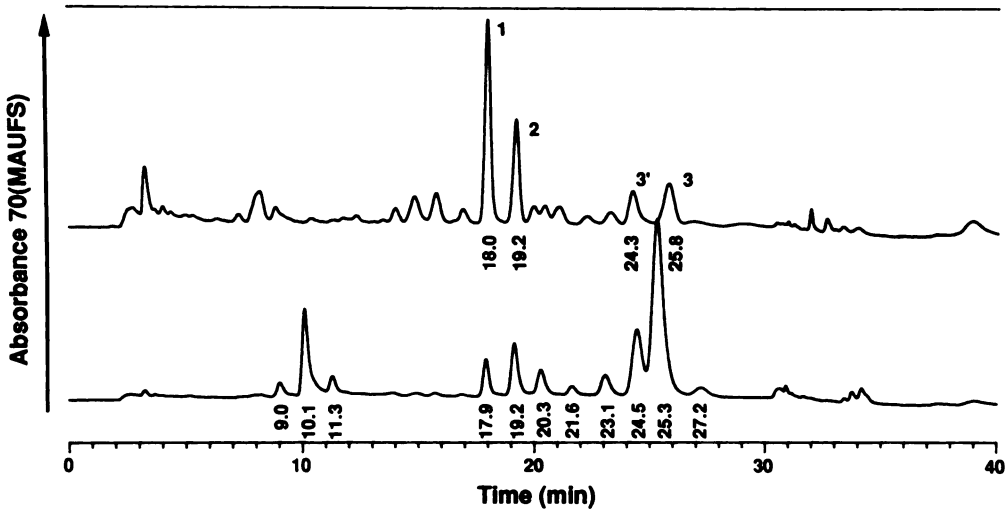


Fig. 2. Elution profiles of the bile pigments of a normal Wistar rat (*lower*) and homozygous jaundiced Gunn rat (*upper*) after the infusion of DMB. The bile samples were injected directly and monitored at 450 nm. The pigments were separated by reverse phase HPLC. The pigments eluted from the Wistar rat bile at 9.0, 10.1, and 11.3 min are the XIIIα, IXα, and IIIα bilirubin diglucuronides; those at 17.9, 19.2, 20.3, and 21.6 min are the XIIIα, C-8 and C-12 IXα, and IIIα monoglucuronides; and those at 23.1, 24.5, 25.3, and 27.2 min are XIIIα, C-8 and C-12 IXα, and IIIα monomethyl monoglucuronide diesters, respectively. The bile pigments eluted from the Gunn rat bile at 18.0, 19.2, 24.3, and 25.8 min (*1, 2, 3'*, and *3*) are the pigments identified as glutathione conjugates in this report. The elution times of DMB and bilirubin in this HPLC program occur at 34 and 38 min, respectively.

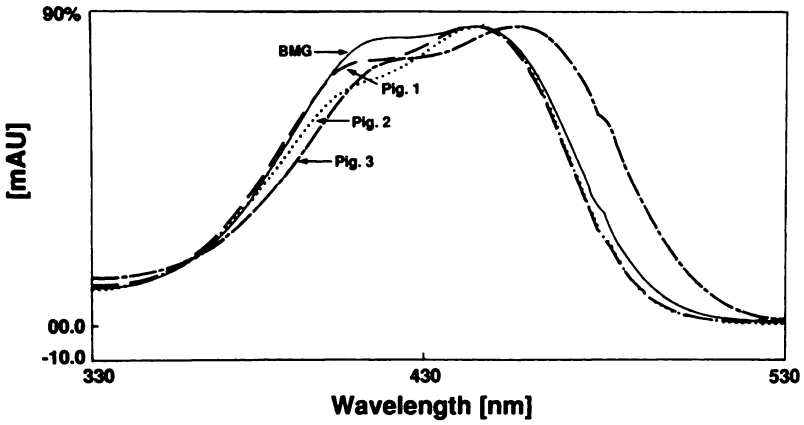


Fig. 3. Absorption spectra of pigments 1, 2, and 3 of Gunn rat bile, compared with that of bilirubin monoglucuronide (*BMG*). These spectra were derived from the diode array detector data of Fig. 2, in which the solvent compositions of acetonitrile and ammonium acetate, pH 4.85, were 60:40 (v/v). Each spectrum was plotted to give 90% full scale deflections at their respective attenuations. The extinction maxima are bilirubin monoglucuronide, 445 nm (68.5 mAU); pigment 1, 447 nm (522 mAU); pigment 2, 445 nm (383.0 mAU); pigment 3, 459 nm (127.7 mAU).

TABLE 1

Elution times of pigments 1, 2, and 3 (Fig. 2) before (A) and after (B) incubation with γ-glutamyltranspeptidase

	Elution time		
	Tetrapyrroles[a]	Azodipyrroles[b]	
	min		
Pigment 1			
A	16.7	14.5	33.2
B	19.8	19.9	33.2
Pigment 2			
A	18.0	13.6	33.3
B	20.0	19.8	33.3
Pigment 3			
A	23.7	22.4	33.9
B	28.6	24.3	33.9

[a] All of the pigments were analyzed by reverse phase HPLC on an analytical column, as described in Materials and Methods. The analysis of the tetrapyrroles before incubation with γ-glutamyltranspeptidase yields shorter elution times than shown in Fig. 2 because of the change in columns.

[b] The incubations of the pigments with γ-glutamyltranspeptidase were done using their tetrapyrroles, before conversion to their azodipyrrolic derivatives.

TABLE 2

Amino acid composition of the bile pigments from Gunn rat

Bile pigment			Recovery[a]		
Sample	Run	Amount analyzed[b]	Glutamic acid	Glycine	Cysteic acid
		nmol		*nmol*	
Pigment 1	A	1.0	0.84, 0.84	1.78, 1.88	0.96
	B	1.0	0.95, 0.84	1.28, 1.12	
	C	1.0	0.99, 1.09	1.08, 1.24	0.46, 0.46
Pigment 2	A	1.0	0.68, 0.62	1.48, 1.49	0.80
Pigment 3	A	1.0	0.84, 0.49	1.42, 1.22	0.53
	D	0.2	0.15	0.19	
Pigment 3'	A	1.0	0.07, 0.17	0.66, 0.86	0.22
	D	1.0	0.21, 0.38	1.60, 1.54	
DMB	A	1.0	0.05, 0.02	0.05	
Bilirubin	A	1.0	0.01	0.02	
Solvent	C		0.03, 0.08	0.11, 0.17	0.03

[a] The double columns under the individual amino acid recoveries represent duplicate samples of the pigments, rather than duplicate analyses.

[b] The amount of pigment sent for amino acid analysis was determined spectrophotometrically (ϵ_{450} = 60 mM^{-1} cm^{-1}).

Runs A-D represent the analyses of the pigments on 4 separate occasions.

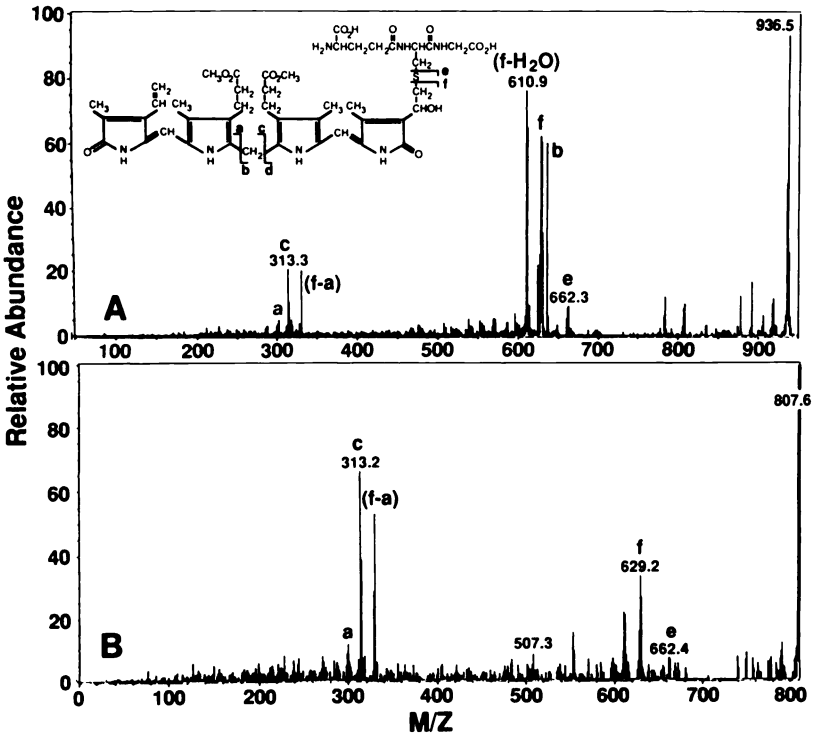


Fig. 4. CAD spectra of pigments 1 and 2 (A) and 3′ (B) after the mass selection of their precursor ion beams of *m/z* 936.5 and 807.6, respectively, by MS-1. Pigment 3′ was produced from pigment 1 by incubation with γ-glutamyltranspeptidase. The assigned *m/z* fragments are lettered in the *inset* of A. *a* = *m/z* 300, *b* = 636, *c* = 313, *d* = 622, *e* = 662, *f* = 629.

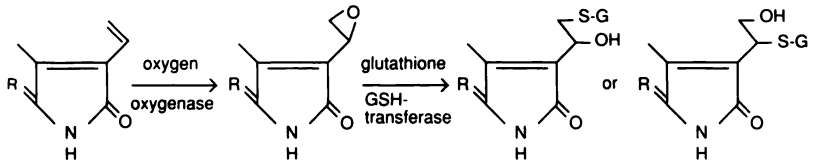


Fig. 5. Proposed formation of pigments 1 and 2.

Chemical analyses of the pigments for glucuronic acid were performed with carbazole (30) and naphthoresorcinol reagents (31).

Amino acid composition. Two tenths to 1 nmol of the four isolated pigments from the Gunn rat bile were transferred to nitric acid-cleansed tubes (3 mm × 5 cm), frozen at −70°, and lyophilized overnight. The amino acid analyses were performed on a Beckmann model 6300 amino acid analyzer at the Protein Structure Laboratory, University of California-Davis School of Medicine (Davis, CA). The analyses were done after hydrolysis with hydrochloric acid and/or performic acid oxidation before hydrolysis (30, 31). Blank samples consisted of the ammonium acetate/acetonitrile (60:40) eluate mixture used for separation of the pigment, as well as bilirubin and DMB dissolved in chloroform. All samples included internal standards of aminoguanidinopropionic acid in the 6 M HCl hydrolysates and β-thienylalanine in the performic acid oxidations.

Mass spectrometry. The plasma desorption mass spectrometry of the di- and monoglucuronides of bilirubin, as well as the pigments 1, 2, and 3 of the Gunn rat bile, as isolated in these studies, has been recently reported (34). In the present study, pigments 1, 2, and 3′, eluting at 18.0, 19.2, and 24.3 min, respectively (Fig. 2), and the tetrapyrrole products of pigments 1 and 2, isolated after incubation with γ-glutamyltranspeptidase, were lyophilized and analyzed by tandem mass spectrometry. For these analyses, cation FAB mass spectra were acquired on a JEOL HX110/HX110 tandem mass spectrometer (Tokyo, Japan) at the full accelerating voltage of 10 kV and under the control of a DA 5000 data system. The FAB gun was operated at 6 kV, with xenon as the FAB gas. The CAD spectra were obtained at a collision energy of 6 kV, by a *B/E* scan of MS-2, following mass selection of the precursor ion beam by MS-1. The intensity of the primary ion beam was attenuated by 70–80% by introduction of helium into the collision cell. For regular magnetic scans, MS-1 was operated at 1000 resolution and scanned upwards from *m/z* 300 to *m/z* 1100 in 45 sec. For tandem CAD scans, both MS-1 and MS-2 were operated at 1000 resolution (10% valley definition), and MS-2 was scanned upwards from *m/z* 0 to the precursor ion, which typically took 1 min. Samples were dissolved in 10 μl of distilled water, and 1 μl was applied to the stainless steel probe tip, followed by 1–2 μl of 3-nitrobenzyl alcohol.

Proton NMR spectroscopy. Pigments 1 and 2 (Fig. 2), chromatographically isolated from the bile of Gunn rats, were equilibrated with 99.98% D_2O and relyophilized. The pigments (0.1 mg), as well as crystalline bilirubin and DMB, were dissolved in 0.4 ml of d_6-Me_2SO (99.98% isotopic purity).

Proton NMR spectra were recorded at ambient temperatures on a GN-500 spectrometer (General Electric Co., Fremont, CA), operating at 500 MHz ^1H frequency in the pulsed Fourier mode with quadrature detection. Acquisition and data processing were under the control of the Nicolet 1280 data processor. The d_6-Me_2SO provided the internal lock signal and was also used as the internal reference (2.49 ppm) for chemical shifts. Spectra were acquired at a spectral width of 7000 Hz. In order to increase the receiver gain, the water signal at 3.2 ppm was suppressed by presaturation at that frequency (0.5 sec, 50 dB). The 16K transients of 200–300 scans were collected and multiplied by an exponential function (line broadening = 1), to increase the signal to noise ratio. The proton signals in the vinylic region were compared with those of previous studies (35, 36).

The proton-proton correlation (COSY) spectrum of pigment 1 from the Gunn rat bile was measured after purification of the pigment by HPLC. Ammonium formate was substituted for the ammonium acetate in the mobile phase of the chromatographic isolation. The isolated pigment was relyophilized after equilibration in D_2O, and 0.8 mg was

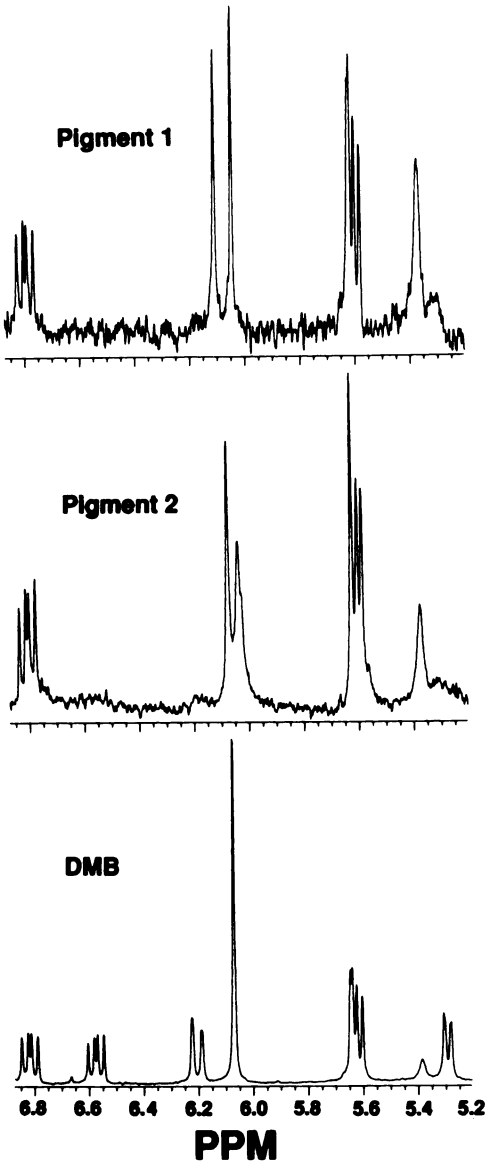


Fig. 6. Partial NMR spectra of DMB and pigments 1 and 2 isolated from bile after infusion of DMB into the Gunn rat. The characteristic proton signals of the endovinyl (C-3) and exovinyl (C-18) groups of DMB in deuterated Me_2SO are between δ 5.29 and 6.84 ppm, with the single proton signal of the 5 and 15 methine bridges at 6.07 ppm. The proton signals of the C-18 vinyl group are absent (δ 5.29, 6.20, and 6.57 ppm) in both pigments 1 and 2, whereas those of the C-3 vinyl group (δ 5.63 and 6.84) are clearly evident. The single proton signals of the 5 and 15 methine bridges at 6.07 ppm are split as a consequence of the asymmetry of the dipyrroles of pigments 1 and 2.

dissolved in 400 μl of d_6-Me_2SO. The spectrum was recorded using the standard COSY pulse sequence (37), with the addition of a presaturation pulse (200 msec) centered on the frequency (3.2 ppm) to suppress the water signal. The spectral width was 4500 Hz in both the F_1 and F_2 dimensions. The data were collected in a 1000 (F_1 dimension) × 512 matrix using 128 scans/trace, with a total acquisition time of 6 hr. The transformed data were displayed as a magnitude spectrum over 1000 × 512, after multiplication of the raw data with an unshifted sine bell squared function. The contour spectrum has been symmetrized.

Results

The HPLC elution profiles of the bile pigments after the infusion of 5 mg of DMB into Wistar and Gunn rats are illustrated in Fig. 2. All of the conjugates in the Wistar bile identified by the labeled compound elution times were hydrolyzed by the β-glucuronidase incubation, resulting in chromatograms that, after enzyme incubation, demonstrated only the presence of bilirubin and its monomethyl ester (data not shown). These results attest to their original β-D-glycoside structure. In contrast, the elution profile of the bile pigments from the Gunn rat remained unaltered, not only after β-glucuronidase incubation but also after incubation with α- and β-glucosidase. No evidence for the presence of glucuronic acid was found chemically with either the carbazole or naphthoresorcinol reagents when pigments 1, 2, 3′, and 3 were analyzed after their isolation by preparative HPLC. The simultaneous analysis of the isolated di- and monoglucuronides from the Wistar rat bile yielded the expected 2:1 and 1:1 molar ratios of glucuronic acid to bilirubin content (data not shown). Furthermore, none of the pigments from the bile of the Gunn rat exhibited transesterification in methanol (38) or amide formation when exposed to an NH_3 vapor overnight (39). Even though the HPLC profile of Fig. 2 indicates that the bile pigments from the Gunn rat are more polar than the infused parent compound, they proved not be ester glycosides.

The absorption spectra of pigments 1, 2, and 3 are compared with that of bilirubin monoglucuronide in Figure 3. The extinction maxima of pigments 1 and 2 at 447 and 445 nm closely coincide with that of the monoglucuronide at 445 nm, but the pigments have less absorbance at 420 nm. The extinction maximum of pigment 3 is at 459 nm and is readily distinguished from that of the other pigments.

The measurement of the bilirubin equivalents of the three pigments as their dipyrrolic azosulfanilates demonstrated a good correlation with the assumption that the original tetrapyrroles had extinction coefficients of ϵ_{450} = 60 mM^{-1} cm^{-1}, similar to bilirubin itself. Such results also indicated that both dipyrrolic halves of the linear tetrapyrrole were reactive with the diazotized sulfanilic acid and attest to the integrity of the central methene bridge at carbon 10 (see Fig. 1). Pigment 3 as its tetrapyrrole had an extinction coefficient 10% less at 450 nm, compared with that of its dipyrrolic azosulfanilate derivative at 540 nm. This discrepancy, as illustrated in Fig. 3, can be accounted for by the shift of its extinction maximum to 459 nm.

The effects of incubation of pigments 1, 2, and 3 of Fig. 2 with γ-glutamyltranspeptidase on their elution times by HPLC are summarized in Table 1. All three pigments, when analyzed as their tetrapyrroles, had later elution times. Pigment 3′ of Fig. 2 had the same elution time before and after incubation with the γ-glutamyltranspeptidase (data not shown), which coincided with that of pigments 1 and 2 after their incubation with γ-glutamyltranspeptidase, i.e., 19.9 min.

The elution times of the dipyrrolic azosulfanilate derivatives of the parent tetrapyrroles demonstrated that only one half of the tetrapyrrole was susceptible to the action of the γ-glutamyltranspeptidase; the nonconjugated half of the molecule eluted as either the methyl endo- or exovinyl azosulfanilate of DMB (33.2 or 33.9 min, respectively) before and after enzyme exposure. Pigments 1 and 2 showed later elution times, after enzyme exposure, of that dipyrrolic half of the molecule containing the C-18 exovinyl group, whereas its endovinyl-containing dipyrrole retained the same elution times characteristic of the methylated endovinyl azodipyrrole of DMB (25). When

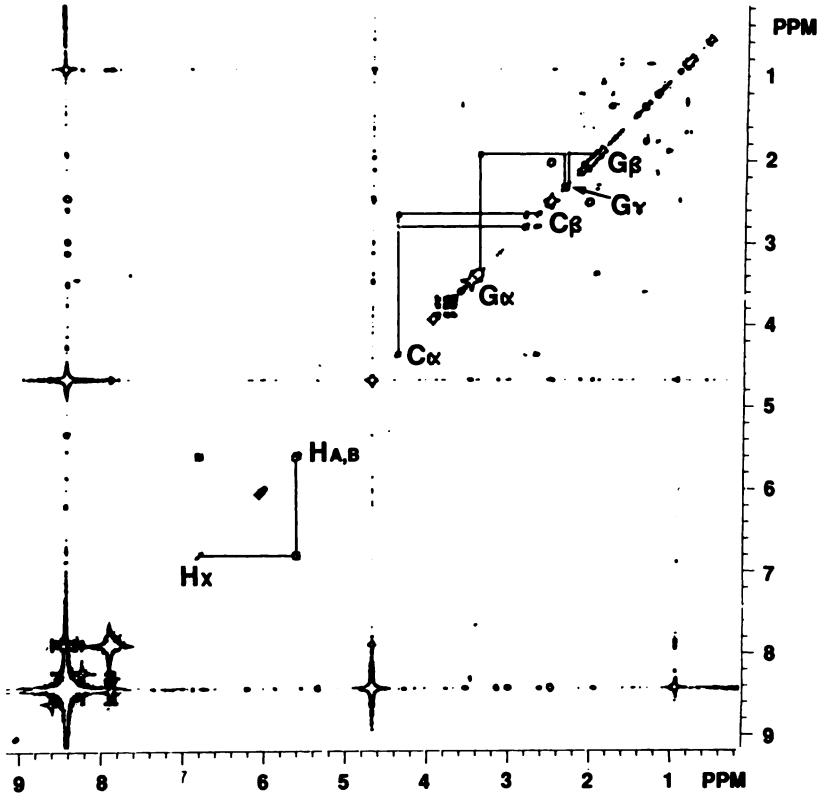


Fig. 7. COSY spectrum of pigment 1, using the standard COSY pulse sequence with the addition of a presaturation pulse (200 msec) centered on the frequency of water (3.2 ppm) to suppress its signal. The proton-coupling network of the glutathione moiety, with the exception of glycine, is indicated by the connecting lines. The α protons of glutamic acid (3.4 ppm) and cysteine (4.4 ppm) give the expected cross-peaks, with the respective β protons at 2 ppm and 2.6 and 2.8 ppm. Also drawn is the coupling pattern of the endovinyl system with a cross-peak at 6.8, 5.6 ppm, indicating the coupling of the AB protons with the HX proton.

TABLE 3

Influence of TCDD induction on the biliary excretion of conjugated bile pigment after infusion of DMB in Gunn rats

Each animal was infused with 5 mg of DMB in 0.35 ml of Me_2SO over 20 min. The data (mean ± standard deviation) are from the 60-min collections of bile following the completion of the infusion.

	Control ($n = 6$)	TCDD treated ($n = 6$)
Conjugated pigment (μg/100 g/min)	0.56 ± 0.20	3.14 ± 0.94[a]
Bile flow (μl/100 g/min)	4.85 ± 1.22	4.97 ± 1.09[b]

[a] The pigment excretion is significantly greater than in control animals ($p < 0.001$ by Student's t test for unpaired samples).
[b] The bile flows are not significantly different ($p > 0.1$).

pigments 1 and 2 were mixed as their azodipyrrolic sulfanilates after exposure to γ-glutamyltranspeptidase, they coeluted by HPLC. The elution times of 19.8 and 33.3 min of the dipyrroles corresponded to that of pigment 3′ (data not shown).

Pigment 3, in contrast, revealed that its nonconjugated half of the tetrapyrrole eluted as the exovinyl methyl azodipyrrolic sulfanilate of DMB (33.9 min) before and after enzyme exposure. Thus, the dipyrrolic half of the tetrapyrrole, which was susceptible to γ-glutamyltranspeptidase, contained the C-3 vinyl group.

The amino acid composition of the bile pigments isolated from the bile of the Gunn rats after infusion with DMB is summarized in Table 2. Qualitatively, all showed the presence of glutamic acid and glycine after HCl hydrolysis. Cysteine was demonstrated when analyzed by performic acid oxidation (runs A and C). No other amino acids were detected in nanomolar amounts. The results of duplicate samples are indicated by the double entries beneath the individual amino acid recoveries. The values for pigment 3′ include those for the compound isolated from bile (run A) and the product purified after the incubation of pigment 1 with γ-glutamyltranspeptidase (run D). Both samples reflect the marked reduction of the ratio of glutamic acid to pigment, compared with the values for pigments 1, 2, and 3. The recoveries of glycine for pigments 1, 2, and 3 of run A showed a significant molar excess, relative to the pigment, which cannot be satisfactorily explained, particularly because the molar ratios of glutamic acid/glycine/pigment were close to unity with runs B, C, and D.

The mass spectral analyses of the pigments are illustrated in Fig. 4. Both pigments 1 and 2 demonstrated identical $[M + H]^+$ ions of m/z 936.7, whereas that of pigment 3′ was m/z 807.6, whether purified from bile or after incubation of pigments 1 or 2 with γ-glutamyltranspeptidase. The difference of 129 amu between pigments 1 and 3′ is consistent with the loss of a glutamic acid residue from the glutathione moiety believed to be present in pigments 1, 2, and 3. The CAD spectra of pigments 1 and 2 were indistinguishable, as were those of pigment 3′, whether from bile or after *in vitro* exposure of pigments 1 and 2 to γ-glutamyltranspeptidase. The characteristic fragmentation patterns, shown in Fig. 4, provide the assigned decomposition products. Fragments m/z 300 (a), 313 (c), 329 ($f - a$), 611 ($f - H_2O$), 629 (f), and 662 (e) were common to both pigments 1 and 2, as well as pigment 3′. The fragment ion b at m/z 636 in Fig. 4A, which arises from the elimination of nonconjugated dipyrrole, appeared 129 amu lower, at m/z 507, in the spectrum of pigment 3′ (Fig. 4B). We do not, as yet, have satisfactory mass spectral data on pigment 3.

The mass spectrometry studies provided evidence not only that the pigments recovered from the bile were monoglutathione conjugates but also that both propionic acid side chains were still methylated, as in the parent DMB. Such data imply that the glutathione conjugation occurs at a site or sites on the

molecule other than the C-8 and C-12 propionic acid side chains.

Essential to the understanding of the mass spectrometry data was the inclusion of the additional mass of an hydroxyl ion (17 amu) that became incorporated in the DMB *in vivo*. Such incorporation of oxygen could involve the formation of an epoxide at the C-3 or C-18 vinyl side chains, mediated by a cytochrome P-450 monooxygenase, and its subsequent attack by reduced glutathione, as depicted in Fig. 5.

The unidimensional NMR spectra of pigments 1 and 2 revealed characteristic distinctions, compared with that of DMB, as illustrated in Fig. 6. The proton spectra of the DMB showed the expected two ABX systems characteristic of its exovinyl (C-18) and endovinyl (C-3) groups, between δ 5.29 and 6.84 ppm. However, the proton signals from the exovinyl (C-18) groups in both pigments 1 and 2 (δ A, 529; B, 6.20; X, 6.57) were absent, whereas those of the endovinyl (C-3) groups were readily detected. A consequence of the perturbation of the exovinyl system of pigments 1 and 2 was that the signals for the assigned two protons H-5 and H-15 (Fig. 1) became separated, whereas they have identical shifts, at δ 6.07 ppm, in DMB. In both pigments, one signal (presumably H-5) remained at 6.06 ppm, and the other (H-15) shifted downfield to 6.11 ppm in pigment 1 and upfield to 6.04 ppm in the instance of pigment 2. The NMR data suggested that the glutathione conjugation of pigments 1 and 2 occurred at the C-18 vinyl group. The HPLC data previously described suggested that the conjugation of glutathione in pigment 3 occurred at the C-3 endovinyl group. However, we do not as yet have confirmatory evidence for this assumption by NMR.

The COSY spectrum of pigment 1 is shown in Fig. 7. The proton couplings (with the exception of the protons attached to the glycine of the glutathione moiety) were readily assigned by examination of the coupling network. The proton resonance at 3.4 ppm assigned to the α proton of the glutamic acid residue was associated with a cross-peak at 2.0 ppm, indicating that this resonance is coupled to the two β protons of glutamic acid at 2.0 ppm, which in turn are coupled to the two γ protons at 2.4 ppm. The cysteine residue gave one cross-peak between the α proton at 4.4 ppm and the two β protons at 2.6 and 2.8 ppm. In addition to the proton-coupling pattern for the glutathione moiety, the coupling for the endovinyl system gave a cross-peak at 6.8, 5.6 ppm, indicating the coupling of the AB protons with the HX proton.

The results of *in vivo* induction of the cytochrome P-450 monooxygenase by TCDD in Gunn rats are summarized in Table 3. The data indicate an almost 6-fold increase in the rate of excretion of the bile pigment conjugates in the TCDD-induced animals at comparable flow rates of bile. By HPLC analysis, pigments 1, 2, and 3′ were the major excretory conjugates. In prolonged (3-hr) collections, 35% of the infused pigment was recovered in the bile, whereas only 4% is normally recovered in noninduced animals (22).

Discussion

The current results provide evidence that the homozygous jaundiced Gunn rat, which lacks the bilirubin UDP-glucuronosyltransferase, can excrete bile pigments as their glutathione conjugates.

One of us had previously reported (21) the excretion of bilirubin glucuronides when DMB was infused into jaundiced Gunn rats, using a strain of Gunn rat from a different colony. Although both strains showed a total absence of UDP-glucuronosyltransferase activity with bilirubin as substrate, the current colony of animals cannot make glucuronides of DMB either *in vivo* or *in vitro*, in contrast to the animals of the earlier study. Variations in the bile pigment metabolism in the strains of Gunn rats from different colonies have been reported by others. Blanckaert *et al.* (19) found traces of bilirubin glucuronides in the bile of their colony, and Watkins and Klaasen (40) reported some bilirubin glucuronide conjugation activity in their *in vitro* studies of Gunn rat liver from their colony. Similarly, discrepancies have also been reported in colonies of Gunn rats for the *in vitro* conjugation of *p*-nitrophenol (41) by liver microsomes. These discrepancies indicate the existence of multiple strains of Gunn rats, and such variation has contributed to the difficulties in interpreting the genetic defects of these jaundiced animals (42, 43).

The amino acid composition of the pigments (Table 2) and their mass spectrometric and COSY NMR analyses (Figs. 4 and 7) are consistent with the presence of a glutathione moiety. The loss of polarity of the pigments, by HPLC, after incubation with γ-glutamyltranspeptidase (Table 1) and the loss of glutamic acid by amino acid analysis (Table 2) also attest to the presence of glutathione. We believe the molar excess of glycine over pigment and glutamate for the analyses of pigments 1, 2, and 3 in run A of Table 2 was due to contamination. Such excess was not observed in the other runs, and by HPLC the pigments all had the same elution times. The possibility of an additional glycine conjugate that coeluted with the monoglutathione seems unlikely, particularly because part of the pool of pigment 1 used for the amino acid analysis was also used for the mass spectrometry study, and no molecular ion or CAD fragment consistent with a glycine conjugate was observed. The poor recovery of cysteine in four of the six analyses is probably related to interference by the pigment with the performic acid oxidation. On two occasions, we spiked samples of bilirubin with cysteine and had only a 50% recovery.

The HPLC analyses of the dipyrrole azosulfanilates confirmed that only one half of the molecule was conjugated with glutathione, because the nonconjugated azodipyrroles eluted as either the methylated endo- or exovinyl azodipyrrolic sulfanilate. The mass spectrometric analyses yielded molecular ions consistent with a monoglutathione conjugate (m/z 936), and the characteristic losses of 307 amu (residual m/z 629) are compatible with this interpretation. The presence of dipyrrolic fragment ions of m/z 313 and 300 indicated that both halves of the tetrapyrrole pigments were still methylated at the C-8 and C-12 propionic acid side chains, as seen with the parent DMB. Normally, bilirubin and its glucuronide conjugates exhibit dipyrrolic ions of m/z 287 and 300, characteristic of the fragmentation on either side of the C-10 carbon of the parent tetrapyrrole (34). The unidimensional NMR study demonstrated that for both pigments 1 and 2 the most likely site for the glutathione adduct was at the C-18 exovinyl group, because the characteristic proton signals were absent, whereas those of the C-3 vinyl group were present. The preferential reactivity of the C-18 vinyl group for sulfhydryl adduct formation has been previously demonstrated chemically by photon activation (44), as well as acid catalysis (45). We have not been able, for lack of sufficient pigment, to demonstrate by NMR that pigment 3 has its glutathione conjugation at the C-3 vinyl group. However, the

HPLC analyses of pigment 3 did demonstrate that the glutathione conjugation occurred on the dipyrrolic half of the tetrapyrrole containing the C-3 vinyl group.

In order to account for the molecular weights of the pigments, as measured by mass spectrometry, we assigned the additional mass of 17 amu as an hydroxyl group. Our postulate of an intermediate epoxide that was subsequently opened to a glutathione adduct was reinforced by the results obtained after induction of the oxygenase by TCDD. Such induction is known to promote the formation of epoxides, and the 6-fold increase in excretion of the conjugates in the rat was consistent with this hypothesis. Induction by TCDD involves the cytochrome P-450 family (1A1 or 1A2), which is known to be present in the Gunn rat (46). We have recently been able to synthesize pigments 1 and 2 *in vitro*, with isolated microsomes from TCDD-treated rats incubated with NADPH, DMB, and reduced glutathione (47), and also demonstrated the incorporation of oxygen (48).

Because the pigments reported were presumably derived from the infused DMB, one might question whether the DMB behaves *in vivo* as a xenobiotic rather than an analogue of bilirubin. However, we have found a candidate glutathione conjugate of bilirubin in the basal bile of TCDD-induced animals. This pigment co-migrated by HPLC with pigment 2 that had been previously demethylated by alkaline hydrolysis. The pigment also exhibited reduced polarity after incubation with γ-glutamyltranspeptidase *in vitro*.[1] More rigorous proof of structure is being pursued.

The current results demonstrate a plausible mechanism for an alternative pathway of bilirubin metabolism *in vivo*. The induction of cytochrome P-450 (1A1 or 1A2) of liver microsomes by TCDD promotes epoxide formation of the C-3 and/or C-18 vinyl side chains of bilirubin. Once formed, these epoxides can be attacked by glutathione, catalyzed by one of the glutathione transferases, and form the thioether conjugates, as currently reported. The catalysis of the epoxides by expoxide hydrolase to form dihydrols, as previously described by Blanckaert *et al.* (19), is also possible. Epoxide formation across the methine bridges at C-5 and C-15, as reported by Ostrow and co-workers (20) from their photooxidation studies, is another possibility. We did not find evidence of these latter pigments using DMB as an analogue of bilirubin. The fact that we found 90% of the 6-fold increase in total conjugated pigment excretion associated with TCDD induction accounted for by pigments 1, 2, 3, and 3′ is worthy of emphasis. The HPLC profiles were qualitatively similar to that of the Gunn rat bile in Fig. 2, which was from a noninduced animal, except that there was more of pigment 3′ than 3. We presume that pigment 3′ is derived from either pigment 1 or 2 but loses its glutamic acid *in vivo* from the action of biliary γ-glutamyltranspeptidase, which is comparable to the *in vitro* conversion of pigments 1 and 2 to 3′.

The current results may have application clinically to patients with lifethreatening hyperbilirubinemia, such as type 1 Crigler-Najjar syndrome. In a preliminary report (49), the administration of phenothiazine (chlorpromazine) to such a patient was reported to significantly reduce the serum bilirubin concentration. If confirmed, the induction of an alternative pathway for bilirubin excretion, as described above, may be feasible. The potential hepatotoxicity (50) and carcinogenicity (51) of such treatment will require evaluation before long term usage is considered.

[1] Odell, G. B., Shore, L. J., Murphy, C., and Fenselau, C. The identification of glutathione conjugates of bilirubin in the bile of Gunn rates. Manuscript in preparation.

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