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Characterization of the Major Diazo-Positive Pigments in Bile of Homozygous Gunn Rats

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Bilinoid pigments in bile of homozygous Gunn rats (jj) were analysed either after formation of dipyrrolic ethyl anthranilate azo derivatives or as the unmodified parent tetrapyrroles. 1. T.l.c. of the azo derivatives revealed seven major unconjugated components which were structurally characterized by chemical tests, spectrophotometry and mass spectrometry. In addition, two minor components were identified as azodipyrrole (A+B)-glucoside and azodipyrrole (A+B)-β-D-glucuronide. 2. Extraction and t.l.c. of the tetrapyrrolic pigments showed 13 major yellow diazo-positive bands. Four of them, accounting for 59% of total diazo-positive material, were identified as unconjugated bilirubin-IXα, -IXβ, -IXγ and -IXδ. A fifth band (16%) was characterized as a mixture of two isomeric monohydroxyl derivatives and another band (8%) as a dihydroxyl derivative of bilirubin-IXα. 3. Although unconjugated bilirubin-IXα constitutes one-third of total diazo-positive material in bile of our strain of Gunn rats, the daily amount excreted represented only about 3–4% of daily bilirubin production. 4. Phototherapy caused a 2.2-fold increase in the biliary output of diazo-positive bilinoids, but did not affect markedly their composition. However, an additional diazo-negative pigment, accounting for one-third of total yellow colour, was observed but was not identified. Mass-spectral data on two dipyrrolic azopigments have been deposited as Supplementary Publication SUP 50076 (3 pages) with the British Library Lending Division, Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms given in *Biochem. J.* (1977) **161**, 1.

Crigler & Najjar (1952) described a human disease characterized by congenital non-haemolytic severe unconjugated hyperbilirubinaemia. Subsequent reports revealed considerable heterogeneity with regard to the clinical course, the biochemical findings, the response to treatment with phenobarbital and the mode of genetic transmission. On the basis of these criteria, a markedly (Type I) and a moderately (Type II) affected group have been distinguished (Arias *et al.*, 1969). A third group of patients with mild congential unconjugated hyperbilirubinaemia (Gilbert's syndrome) has characteristics related to those of the Crigler–Najjar patients (Foulk *et al.*, 1959). A similar, genetically determined non-haemolytic unconjugated hyperbilirubinaemia is present in an autosomal recessive mutant of the Wistar rat (Gunn, 1938). A common finding in the three groups of patients and in the Gunn rats is a decreased clearance of bilirubin-IXα from plasma (Schmid & Hammaker, 1963; Berk *et al.*, 1970). The severity of this deficiency determines the extent to which unconjugated bilirubin-IXα accumulates in the plasma and tissues and therefore the risk of developing bilirubin encephalopathy. Although the nature and number of the hereditary defects involved are as yet poorly defined, a deficiency in the mechanisms for conjugation of bilirubin-IXα is apparently the determining defect (Arias & London, 1957; Carbone & Grodsky, 1957; Arias *et al.*, 1969; Black & Billing, 1969; Fevery *et al.*, 1972).

A prerequisite for understanding both the normal and disturbed metabolism of bilirubin is a knowledge of the chemical structures of the bilirubin metabolites. In the present work the chemical characterization of bilinoids in bile of Gunn rats that were either illuminated or kept under dim light has been undertaken. In particular we have tried to answer the following questions. (*a*) Are acyl glycosides present in Gunn-rat bile? (*b*) What is the isomeric status and quantitative importance of the different bilirubin-IX isomers? (*c*) Can other diazo-positive pigments be isolated and identified?

A number of dipyrrolic azopigments have been derived from Gunn-rat bile. As, in general, they are unconjugated they have been indicated by symbols, G_1–G_7, different from the Greek-letter notation

adopted previously to denote azopigments derived from bile of man and various animals (Heirwegh *et al.*, 1974). In Blanckaert *et al.* (1976*b*), structurally known unconjugated azopigments derived from the bilirubin-IX isomers were denoted by a Roman numeral referring to the parent tetrapyrrole followed by a letter, A–G, indicating the nature of the azodipyrrole. In the present work the numeral has been omitted for convenience and the list of letter symbols enlarged (H, J, K) to include hydroxylated azopigments. For example, from bilirubin-IXα and -IXδ, and from tetrapyrrole V (Scheme 1) the azopigment pairs (A+B), (C+G) and (A+H) are obtained respectively. In the same way the mixture of isomeric azopigments obtained from bilirubin-IXα diglucuronide is represented by (A+B)-β-D-glucuronide. The structures of the dipyrrolic azo derivatives studied are shown in Scheme 2.

Materials and Methods

Chemicals

Saccharo-(1→4)-lactone (A grade) was purchased from Calbiochem (San Diego, CA, U.S.A.). β-Glucuronidase preparations from bovine liver (type B-3; 5.24 Fishman units/μg) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trisyl Z, *NO*-bis(trimethylsilyl)acetamide and *NO*-bis-(trimethylsilyl)trifluoroacetamide were from Pierce Chemical Co. (Europoort, Rotterdam, The Netherlands). 3-Ethyl-1-*p*-tolyltriazene and butane boronic acid were purchased from Aldrich–Europe (Janssen Pharmaceutica, Beerse, Belgium). The other chemicals were as specified in Blanckaert *et al.* (1976*b*).

Animals

Homozygous Gunn rats (jj) were obtained in 1962 from the late Dr. Castle (University of California, Berkeley, CA, U.S.A.). After an initial cross-breeding with Wistar R rats (JJ) the heterozygous offspring (jJ) was used to raise a pure line of Gunn rats. For the present work we have been using the 26th generation of pure inbred Gunn rats. Although hepatic bilirubin-IXα UDP-glucuronyltransferase activity is undetectable in our Gunn colony (Fevery *et al.*, 1972), they have only minimal neurological damage. In adult animals the concentration of unconjugated bilirubin-IXα in serum is about 100mg/ml. Their fertility is near-normal, averaging eight young per litter. Only males, weighing 300–350g, were used for the present work.

Methods

As far as possible all manipulations were done under dim light or in the dark. All centrifugations were carried out at room temperature (20–22°C) at $1000g_{av.}$. Routine buffers were prepared at room temperature as follows: glycine/HCl buffer, pH2.0, was 2M-HCl adjusted with solid glycine to pH2.0; glycine/HCl buffers, pH1.8 and 2.7, were 0.4M-HCl adjusted with solid glycine to the required pH; acetate/acetic acid buffer, pH5.2, was 0.2M-sodium acetate adjusted with 0.2M-acetic acid to pH5.2; citrate/phosphate buffer, pH6.0, was prepared with 36.0ml of 0.5M-citric acid and 63.9ml of 1M-Na_2HPO_4.

Preparation of experimental animals and collection of bile and serum. Under ether anaesthesia the rats were provided with an external biliary fistula; if light-treatment was to be applied, their torsos were fully shaven. The animals were placed in restraining cages and bile was collected in tared tubes placed on ice. In some experiments the rats were anaesthetized with ether at the end of the collection period and blood was collected from the aorta abdominalis for the preparation of serum. Bile and serum samples were stored immediately at −15°C in the dark until analysis (within 1 week).

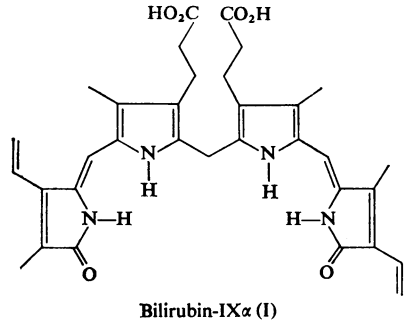
Illumination of experimental animals. The animals were illuminated with eight 20W Vita-Lite fluorescent lamps (Type Duro-Test CRI91, Philips, Eindhoven, The Netherlands), mounted in a reflecting canopy, and placed at a distance of 25cm from the animals. The average radiant power was 9.5μW/cm^2 per nm in the 425–475nm range.

Extraction and separation of bile pigments. Bile (1ml) was acidified with 8ml of glycine/HCl buffer, pH1.8. After further addition of 2ml of ascorbic acid solution (100mg/ml of a saturated solution of NaCl) and 2g of solid NaCl, extraction proceeded at 0°C with 8ml of ethanol/chloroform (1:1, v/v) (J. Fevery, unpublished work). With the system used near-quantitative extraction of yellow pigments was achieved. This is not the case for the extraction procedures published previously (Heirwegh *et al.*, 1975).

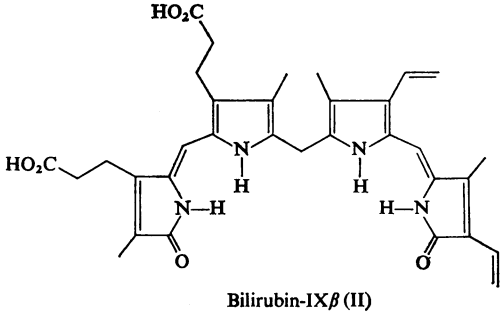
The organic extracts were applied to thin-layer plates (DC-Kieselgel F254, 5715/0025; Merck A.G., Darmstadt, Germany) and the pigments were separated by development with chloroform/methanol water (10:5:1, by vol.). Preparations of bilirubin-IXα, -IXβ, -IXγ and -IXδ (Blanckaert *et al.*, 1976*b*) served as reference substances.

Formation of ethyl anthranilate azo derivatives. Azo derivatives of bile pigments were prepared both from whole bile and from the individual pigments previously separated by t.l.c.

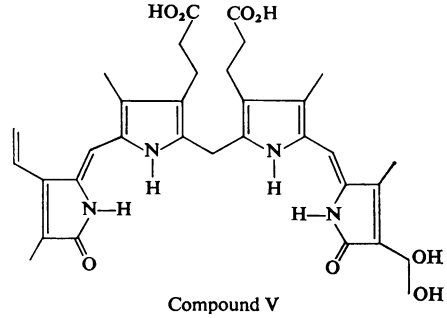
(1) The diazo-positive bilinoids in whole bile from Gunn rats did not react completely with the standard ethyl anthranilate reagent at pH2.7 (Van Roy & Heirwegh, 1968). Therefore we devised the following procedure, which produced complete azopigment formation: a 1% (v/v) suspension of ethyl anthranilate in 0.15M-HCl was prepared, and 0.6ml was



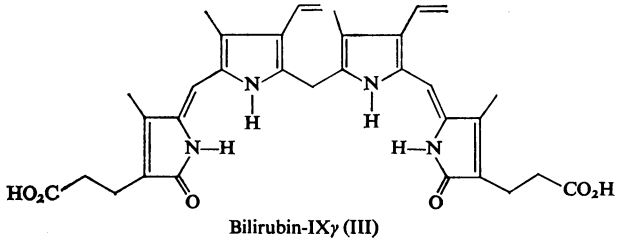
Bilirubin-IXα (I)
(A+B)



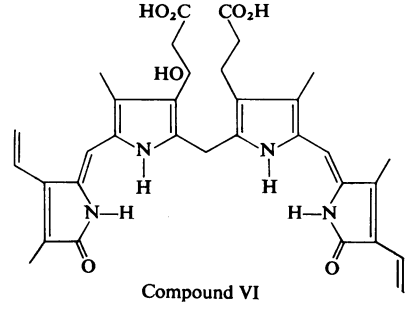
Bilirubin-IXβ (II)
(C+D)



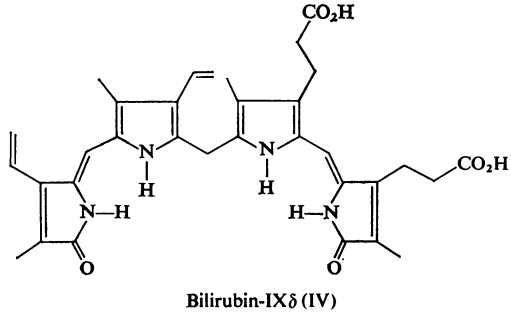
Compound V
(A–H)



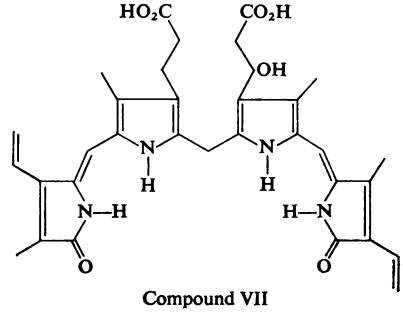
Bilirubin-IXγ (III)
(E–F)



Compound VI
(J–B)



Bilirubin-IXδ (IV)
(G–C)



Compound VII
(A–K)

Scheme 1. *Structures of the major diazo-positive bilinoids in bile of homozygous Gunn rats*
The pairs of capital letters below the formulae denote the two dipyrrolic azopigments obtained by treatment of each bilinoid with diazo reagent (see Scheme 2).

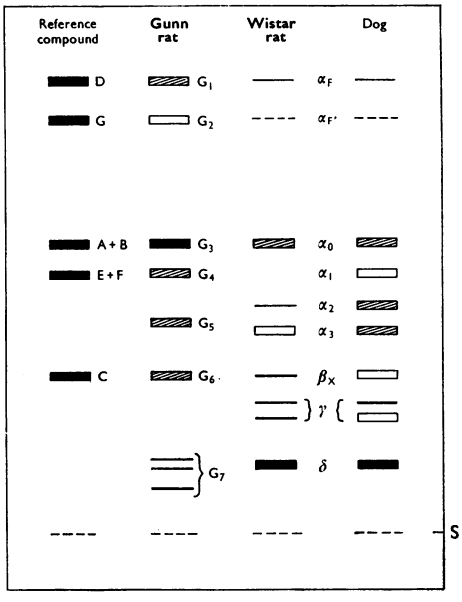


Fig. 1. *T.l.c. of dipyrrolic azopigments derived from unconjugated bilirubin-IX isomers and from bile of Gunn rats, Wistar rats and dogs*

T.l.c. was done as described previously (Blanckaert *et al.*, 1976*b*). Azopigment fractions obtained from Gunn-rat bile are indicated by G_1–G_7. The structures of the unconjugated reference compounds, A–G, are shown in Scheme 2. For comparison, chromatograms of azopigments from bile of Wistar rats and dogs and the corresponding Greek-letter notation of the chromatographic components (Heirwegh *et al.*, 1974) are also shown. The application line is indicated by S.

diluted with 9.4ml of water. To this was added 0.6ml of $NaNO_2$ solution (5mg/ml) and subsequently 5min later 0.2ml of ammonium sulphamate solution (10mg/ml). Diazo reaction was carried out by mixing 1vol. of bile with 1vol. of citrate/phosphate buffer, pH6.0, 0.5vol. of formamide/ethanol (1:1, v/v) and 1vol. of the diazo reagent. After 45min at 0°C, 1 vol. of ascorbic acid solution (10mg/ml) in glycine/HCl buffer, pH2.0, was added to destroy the excess of diazo reagent and to lower the pH for extraction.

The azopigments were extracted without delay by vigorous shaking with 2vol. of pentan-2-one, centrifuged and the A_{530} values of the organic phases were determined against pure solvent. Apparent concentrations of diazo-positive pigments were calculated by assuming ε_{530} for the unknown pigments to be the same as that of unconjugated bilirubin-IXα, 44.4×10^3 litre·mol^{-1}·cm^{-1} (Van Roy & Heirwegh, 1968). A correction factor was applied to take into account a decrease in the organic phase (vol. organic phase/vol. of extract = 0.9).

(2) Bile pigments separated by t.l.c. (see under 'Extraction and separation of bile pigments') were converted into ethyl anthranilate azopigments by the procedure of Blanckaert *et al.* (1976*b*).

T.l.c. of azo derivatives, chemical and enzymic tests. Except when stated otherwise, azopigments were separated and purified as described by Blanckaert *et al.* (1976*b*). Azo derivatives from bilirubin-IXα, -IXβ, -IXγ and -IXδ (Blanckaert *et al.*, 1976*b*) and from bile of normal rats and dogs (Heirwegh *et al.*, 1974) were used as references (Fig. 1, Scheme 2).

The purified azopigments were treated with ethereal diazomethane, NH_3 vapour, alkaline methanol or acetic anhydride/pyridine, and the reaction products were analysed by t.l.c. as described previously (Heirwegh *et al.*, 1974; Blanckaert *et al.*, 1976*b*). Hydrogenation was as described by Blanckaert *et al.* (1976*b*), except that the reaction time was 1min instead of 3min.

Preparation and analysis of ethyl esters by alkaline ethanolysis of conjugated azopigments were done as follows: the dried azopigments were dissolved in 0.5ml of ethanol and mixed with an equal volume of alkaline ethanol (10mg of NaOH/ml). After 1min at room temperature the mixtures were acidified with 6ml of glycine/HCl buffer, pH2.7, and the pigments were extracted into pentan-2-one. The reaction products were chromatographed in benzene/ethyl acetate (9:1, v/v), with the ethyl esters of isomers A and B and their meso derivatives as references. These reference compounds were prepared by treatment of derivative (A+B), the azopigment mixture obtained from bilirubin-IXα, and meso-(A+B) with 3-ethyl-1-*p*-tolyltriazene as described by Compernolle *et al.* (1976) and fully characterized by mass spectrometry (F. Compernolle, N. Blanckaert & K. P. M. Heirwegh, unpublished work). As observed for the methyl esters (Blanckaert *et al.*, 1976*b*), the ethyl esters of azopigments A and B are clearly distinguished from those of meso-A and meso-B in the t.l.c. systems used. Alkaline ethanolysis of the acetates and of the fully acetylated methyl ester derivatives of conjugated azopigments was preferred to alkaline methanolysis, since no side products are formed in the former test. In contrast, alkaline methanolysis produces side products by replacement of the ethyl group in the ethyl anthranilate moiety of the azopigments by a methyl group (Gordon *et al.*, 1974; Heirwegh *et al.*, 1975).

Incubation of azopigments with β-glucuronidase was done as follows: dried purified azopigment (5–10nmol) was first dissolved in 10–20μl of dimethyl sulphoxide and further diluted with 200μl of distilled water; 75μl of the azopigment solution was incubated at 37°C for 1h with 1ml of acetate/acetic acid

buffer, pH5.2, and 130μg of β-glucuronidase in 20μl of water. Parallel controls were prepared by (*a*) omission of enzyme and (*b*) addition of 50mg of saccharo-(1→4)-lactone dissolved in buffer. After acidification with 6ml of glycine/HCl buffer, pH2.7, and extraction into 2ml of pentan-2-one, the degree of conversion of conjugated into unconjugated azopigments was assessed by t.l.c.

Spectral studies of azo derivatives. Characteristic absorption spectra of purified azopigments dissolved in methanol were obtained as described previously (Heirwegh *et al.*, 1975). Mass spectra were obtained on an AEI MS902S mass spectrometer at 180–250°C as required. The electron voltage was 70eV, accelerating voltage 8kV and trap current 500μA. Trimethylsilyl derivatives were prepared by adding 5–10μl of a silylating reagent [a mixture of pyridine and either trimethylsilyl-imidazole, *NO*-bis(trimethylsilyl)acetamide or *NO*-bis(trimethylsilyl)trifluoroacetamide] to the compound applied to the quartz tip of the probe. The silylation reaction was allowed to proceed for 30s to 5min in the direct introduction lock, after which time the excess of reagent was evaporated *in vacuo*. A cyclic butane boronate ester of the methyl ester of dihydroxy compound G_5 was prepared by adding 5μl of a 1% solution of butane boronic acid in pyridine to the azopigment applied on the quartz tip of the probe. After 1min the solvent and excess reagent were evaporated *in vacuo*.

Results and Discussion

Biliary excretion and t.l.c. of bilinoids

Bile from animals kept under dim light. The biliary output of diazo-positive material was 4.9±1.3S.D. ($n=6$)nmol of bilirubin/h per 100g body wt. Bile flow was 254±43S.D. ($n=6$)μl/h per 100g body wt. T.l.c. of the pigments extracted from bile yielded a variety of coloured bands. The yellow components have been numbered 1–14 (Table 1). The diazo-positive bands 1–3 and 5–14 gradually turned green when kept on the plates after removal from the chromatographic tanks. None of the yellow bands showed fluorescence either before or after spraying the thin-layer plates with a saturated solution of zinc acetate in ethanol. Non-yellow pigments migrating between bands 2 and 3, 7 and 8, and 8 and 9 showed fluorescence when illuminated at 254 or 350nm. On illumination at 350nm a colourless component that

Table 1. *Summary of chromatographic, quantitative and structural data on the bilinoid pigments from bile of Gunn rats*
Bile was collected under dim light for 21h (period *a*) from eight Gunn rats. Collection was continued for 25h (period *b*) for three rats that were exposed to light (group A) and for four rats that were further kept under dim light (group B). The pigments were extracted from bile obtained during period *b*, separated by t.l.c. and the relative amounts of the yellow pigments were determined by densitometric scanning at 439nm. The results are given as percentages (±1S.D.) of total yellow colour (Total) and of the sum of the diazo-positive compounds 1–4 and 5–14 (Diazo). The absorption spectra were obtained in methanol solution.

Component No.	R_F	Percentage of yellow colour: Control Diazo	Illuminated Diazo	Illuminated Total	Azo derivatives	Structural data
Rats ...						
No of determinations ...		4	3	3		
1	1.00	31.2±4.9	39.6±5.3	27.9±4.1	(A+B)	Bilirubin-IXα (I)
2	0.94	16.1±1.8	11.7±1.7	8.2±1.2	(J+B), (A+K)	Compounds VI and VII; $\lambda_{max.}$ 446nm
3	0.82	7.9±1.2	4.5±0.4	3.2±0.3	(A+H)	Compound V; $\lambda_{max.}$ 440nm
4	0.80			29.6±1.0		$\lambda_{max.}$ 433nm; pentdyopent-negative‡
5	0.73	6.4±1.0	5.4±1.0	3.8±0.7	(C+D)	Bilirubin-IXβ (II)
6	0.70	3.4±1.3	3.3±0.2	2.3±0.1	(G+C)	Bilirubin-IXδ (IV)
7	0.66	7.4±0.9	8.0±0.2	5.6±0.0	(A+B)*	
8	0.62	10.3±1.6	7.2±1.6	5.0±1.1	(E+F)	Bilirubin-IXγ (III)
9	0.52				(A+B)*	
10	0.44	2.4±0.6	2.5±0.6	1.8±0.4	(A+B)*	
11	0.29	0.23†	3.5±0.4	2.4±0.4	(A+B)*	
12	0.23	2.6±1.4	0.9±0.4	0.6±0.3	(A+B)*	
13	0.18	5.6±3.1	3.4±0.2	2.3±0.1	(A+B)*	
14	0.10	6.7±1.9	10.1±1.7	7.2±1.1	(A+B)*	

* Major derivative.
† Found only in one bile sample.
‡ The pentdyopent reaction was done by the procedure of Bonnett & Stewart (1975).

Table 2. *Composition of azopigments obtained from bile of Gunn rats before and during exposure to light*
The bile samples collected during periods *a* and *b* (see Table 1) were treated separately with diazotized ethyl anthranilate for each rat. The azopigments were separated by t.l.c. and their relative amounts determined by densitometric scanning at 536nm. Results are given as percentages of total azo colour ± S.D.

Rats			Bile collection		Percentage of total azo colour					
No.	Group	Treatment	Period	Duration (h)	G_1+G_2	G_3	G_4	G_5	G_6	G_7
4	A	Dim light	*a*	21	1.8±0.9	58±2	11.7±0.5	3.0±0.4	22±2	3.8±0.6
3		Illuminated	*b*	25	2.1±0.8	61±6	8.8±1.5	3.8±1.9	20±5	5.1±0.8
4	B	Dim light	*a*	21	1.9±0.6	57±3	11.0±1.2	2.9±1.0	23±2	5.0±0.9
4		Dim light	*b*	25	1.4±0.6	57±3	8.6±2.0	6.0±1.2	21±3	6.0±1.1

moved between bands 9 and 10 showed strong reddish fluorescence. These non-yellow pigments have not been further studied.

The yellow bands 1, 5, 6 and 8 could directly be identified with respect to known reference compounds. On rechromatography of methanol eluates of bands 1, 5, 6 and 8 with chloroform/methanol/water (40:9:1, by vol.) the pigments moved respectively as bilirubin-IXα, -IXβ, -IXδ and -IXγ. Identical chromatographic behaviour of band 1 and bilirubin-IXα was further demonstrated with the solvent systems chloroform/acetic acid (99:1, v/v) (McDonagh & Assisi, 1971) and chloroform/methanol (50:1, v/v). The structures were subsequently confirmed by analysis of the azo derivatives of each pigment (see below).

Unconjugated bilirubin-IXα accounted for 31% of excreted diazo-positive bilinoids (Table 1). Assuming that the bilirubin turnover in our Gunn rats is comparable with the value (45nmol/h per 100g body wt.) reported by Robinson (1971) and by Berk *et al.* (1976), the excretion of unconjugated bilirubin-IXα represented only 3–4% of bilirubin production.

Bile from illuminated rats. Bile flow was 245±51 S.D. ($n=4$) μl/h per 100g body wt. and thus comparable with the value for control animals. In comparison with non-illuminated rats, a 2.2-fold increase in the output of diazo-positive material was observed in bile collected after 21–46h of phototherapy. Increases of up to 5-fold have been noted by Ostrow (1971) under similar experimental conditions. At the end of the illumination period serum bilirubin concentrations were the same as for non-illuminated rats. T.l.c. analysis showed similar patterns of yellow bands in bile obtained before and after exposure of Gunn rats to light, except for band 4, which could not be detected in control bile. The latter compound was diazo-negative and accounted for 30% of the yellow colour on the chromatograms (Table 1), and, after drying the plates and exposing them to light, it was transformed immediately into a brown pigment. When only the diazo-positive components were evaluated, densitometric scanning of the yellow bands did not indicate marked differences in composition between the samples obtained before and after phototherapy (Table 1). Only a small increase in the percentage of unconjugated bilirubin-IXα was observed. The similarity of both excretion patterns was confirmed by the almost identical composition of the azopigments prepared directly from the whole bile (Table 2). Therefore a selective enhancement of the excretion of unconjugated bilirubin-IXα by phototherapy did not occur in our Gunn rats. In contrast, Ostrow (1971) and McDonagh (1976) observed the excretion of considerable amounts of unconjugated bilirubin-IXα in their strains of Gunn rats under phototherapy, even at rates comparable with the biliary output of conjugated bilirubin in the bile of normal rats (Ostrow, 1971). Although this discrepancy may be due in part to differences in the extraction and separation procedures, strain differences are probably the major reason. Significant differences in the metabolism of bilirubin-IXα in various strains of normal rats have been demonstrated by Klaassen *et al.* (1969) and are expected for Gunn rats as well.

Structure elucidation of dipyrrolic azo derivatives

The structures of the major diazo-positive bilinoids from Gunn-rat bile were elucidated by identifying the two azodipyrroles formed from each isolated tetrapyrrolic pigment after reaction with diazotized ethyl anthranilate. Since the parent bilinoids are labile and are only present in low concentrations, it was difficult to do a complete structure analysis directly on the azopigments prepared from the separated tetrapyrroles. Therefore an indirect method has been used in which whole bile from Gunn rats was mixed with diazotized ethyl anthranilate and the more stable azodipyrroles were prepared and separated. After purification their structures were determined. These compounds and their derivatives were then used as chromatographic references to identify each pair of azodipyrroles obtained from the separated tetrapyrroles.

(*a*) *T.l.c. of the azo derivatives from whole bile.* Both from light-treated and control Gunn rats seven major azopigment fractions (G_1–G_7) were obtained (Fig. 1; Table 2). Azopigments G_1, G_2, G_3 and G_6 are also obtained from bile of normal rats and dogs. No differences were noted between the azopigments derived from bile of light-treated and control Gunn rats. Therefore no further distinction will be made with regard to their origin.

Since the major azo derivatives from Gunn-rat bile are unconjugated (see below), the azopigments derived from unconjugated bilirubin-IX isomers (Schemes 1 and 2) were used as reference compounds. Azopigments G_1, G_2 and G_6 were homogeneous in all solvent systems used and behaved chromatographically as the reference compounds D, G and C respectively. Azopigment G_3 could be separated into approximately equal amounts of two components identified as A and B. Heterogeneity of component G_4 was demonstrated by chemical modification (see below). Azopigment G_5 did not correspond to any known azodipyrrole. Component G_7 moved approximately as (A+B)-β-D-glucopyranuronoside (Compernolle *et al.*, 1970; Heirwegh *et al.*, 1976); prolonged development with chloroform/methanol/water (10:5:1, by vol.) resolved it into two or three closely moving spots. The R_F value of the upper band was identical with that of the reference glucuronide.

(*b*) *Chemical tests.* None of the major azopigments formed detectable amounts of either amide derivatives when exposed to NH_3 vapour, or methyl esters after treatment with alkaline methanol, indicating the absence of conjugating groups bound in ester linkage.

Azopigments G^1, G_2 and G_6 were unchanged after treatment with acetic anhydride/pyridine. Partial (20–30%) and complete conversion was observed for azopigments G_4 and G_5 respectively, pointing to the presence of one or more hydroxyl and/or amino groups. In each case only one acetylated derivative was obtained. The reactive part of azopigment G_4 is called G_{4a}, the remaining part G_{4b}. All azopigments except G_1 and G_2 reacted with diazomethane. Azopigment G_3 yielded the isomeric monomethyl esters of A and B, and azopigment G_{4b} the isomeric monomethyl esters of pigments E and F. Unmodified pigment G_{4b} could also be separated into the free acids E and F. Methylation of the acetyl derivatives of azopigments G_{4a} and G_5 yielded in each case one more hydrophobic derivative. For azopigment G_5 the same acetylated methyl ester was obtained when it was first methylated, then acetylated. From azopigment G_6 one major and two minor methyl esters were obtained moving respectively as the dimethyl ester and the two monomethyl esters of C. The structures shown in Scheme 2 for azopigments G_1–G_6 agree with the observed esterification and acetylation reactions.

(*c*) *Detection of conjugates.* Special care was taken to determine whether or not any conjugated azo derivative could be detected. After preparative t.l.c. of azopigments obtained from 1 litre of Gunn-rat bile, a faint band behaving chromatographically as (A+B)-β-D-glucopyranoside was observed and further purified by rechromatography. Subsequent treatment with acetic anhydride/pyridine showed the formation of two derivatives, corresponding to both isomeric tetra-acetate derivatives of A and B β-D-glucopyranoside. Alkaline ethanolysis of each of the acetates yielded the expected ethyl esters of A and B. Lack of material prohibited a more rigorous characterization of the conjugating moiety.

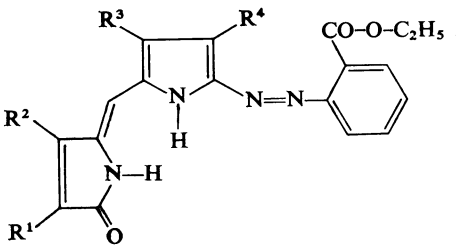
Particular attention was also paid to the upper component of the G_7 fraction, which moved chromatographically as authentic (A+B)-β-D-glucopyranuronoside. After this fraction was isolated from 20ml of Gunn-rat bile, methylation and subsequent acetylation resulted at each step in the formation of derivatives corresponding to those obtained from reference glucuronide after identical treatment. Again alkaline ethanolysis of each isomeric acetate yielded the corresponding ethyl esters of A and B. Further, treatment of the purified azopigment (prepared from 1 litre of Gunn-rat bile) with β-glucuronidase resulted in complete hydrolysis, which was inhibited completely by saccharo-(1→4)-lactone.

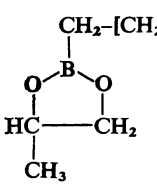
These findings demonstrate the presence of bilirubin-IXα β-D-glucopyranuronoside and probably, of bilirubin-IXα glucoside in bile of homozygous Gunn rats, albeit in minute concentrations.

(*d*) *Mass spectra, absorption spectra and structures proposed.* The structures are shown in Scheme 2. Except for component G_7 all major azo derivatives were examined by spectrophotometry (Table 3) and mass spectrometry (Supplementary Publication SUP 50076). The mass spectra of the derivatives of A, B, C, D, E, F and G have been described previously (Compernolle *et al.*, 1976).

For compounds G_1 and G_2, molecular ions were observed at *m/e* 416; however, fragments caused by thermal degradation of these divinyl-substituted azopigments obscured the fragmentation patterns. Thermal lability was also found for the reference compounds D (identical with G_1) and G (identical with G_2), which are better characterized as the stable tetrahydro derivatives. Further evidence for the identification of compounds G_1 and G_2 as D and G is provided by the identical R_F values and absorption spectra.

Identical mass spectra were found for the methyl esters obtained from compound G_3 and the methyl esters of isomers A and B derived from bilirubin-IXα. In the same way the two methyl esters obtained from compound G_{4b} proved to be identical with the methyl esters of isomers E and F derived from bilirubin-IXγ.



	Azopigment	R^1	R^2	R^3	R^4
G_1	(D)	$CH=CH_2$	CH_3	$CH=CH_2$	CH_3
G_2	(G)	CH_3	$CH=CH_2$	CH_3	$CH=CH_2$
G_3	A	CH_3	$CH=CH_2$	CH_3	$CH_2-CH_2-CO_2H$
	B	$CH=CH_2$	CH_3	CH_3	$CH_2-CH_2-CO_2H$
G_{4a}	K	$CH=CH_2$	CH_3	CH_3	$CHOH-CH_2-CO_2H$
	J	CH_3	$CH=CH_2$	CH_3	$CHOH-CH_2-CO_2H$
	K methyl ester	$CH=CH_2$	CH_3	CH_3	$CHOH-CH_2-CO_2CH_3$
	J methyl ester	CH_3	$CH=CH_2$	CH_3	$CHOH-CH_2-CO_2CH_3$
	K methyl ester trimethylsilyl ether	$CH=CH_2$	CH_3	CH_3	$CHO(CH_3)_3Si-CH_2-CO_2CH_3$
	J methyl ester trimethylsilyl ether	CH_3	$CH=CH_2$	CH_3	$CHO(CH_3)_3Si-CH_2-CO_2CH_3$
	Dihydro K methyl ester	CH_2-CH_3	CH_3	CH_3	$CHOH-CH_2-CO_2CH_3$
	Dihydro J methyl ester	CH_3	CH_2-CH_3	CH_3	$CHOH-CH_2-CO_2CH_3$
	Dihydro K methyl ester trimethylsilyl ether	CH_2-CH_3	CH_3	CH_3	$CHO(CH_3)_3Si-CH_2-CO_2CH_3$
	Dihydro J methyl ester trimethylsilyl ether	CH_3	CH_2-CH_3	CH_3	$CHO(CH_3)_3Si-CH_2-CO_2CH_3$
G_{4b}	F	$CH_2-CH_2-CO_2H$	CH_3	$CH=CH_2$	CH_3
	E	$CH_2-CH_2-CO_2H$	CH_3	CH_3	$CH=CH_2$
G_5	(H)	$CHOH-CH_2OH$	CH_3	CH_3	$CH_2-CH_2-CO_2H$
	H methyl ester	$CHOH-CH_2OH$	CH_3	CH_3	$CH_2-CH_2-CO_2CH_3$
	H methyl ester diacetate	$CHOAc-CH_2OAc$	CH_3	CH_3	$CH_2-CH_2-CO_2CH_3$
	H methyl ester bistrimethylsilyl ether	$CHO(CH_3)_3Si-CH_2O(CH_3)_3Si$	CH_3	CH_3	$CH_2-CH_2-CO_2CH_3$
	H methyl ester cyclic boronate ester		CH_3	CH_3	$CH_2-CH_2-CO_2CH_3$
G_6	(C)		$CH_2-CH_2-CO_2H$	$CH_2-CH_2-CO_2H$	CH_3

Scheme 2. *Structures of the major dipyrrolic azopigments derived from bile of homozygous Gunn rats*

Table 3. *Chromatographic mobilities of and spectral data on the azopigments derived from Gunn-rat bile*
Azopigments were developed on thin-layer plates with (*a*) chloroform/methanol/water (65:25:3, by vol.) over 1–2cm, and subsequently by chloroform/methanol (9:1, v/v) over 18cm, or (*b*) chloroform/methanol (9:1, v/v) over 1–2cm, and benzene/ethyl acetate (9:1, v/v) over 18cm. The absorption spectra were obtained in methanol solution. The letters A and B, and E and F refer to pairs of isomers (see Scheme 2).

Parent chromatographic component	Derivative	R_F value (*a*)	R_F value (*b*)	Absorption spectrum $\lambda_{max.}$ (nm)			Molecular ion (M^+)
G_1		0.93	0.82	285	339	535	416
G_2		0.93	0.69			535	416
G_3		0.64	0.06	287	330	527.5	
	G_3 (A) methyl ester		0.61	292	329	526	476
	G_3 (B) methyl ester		0.67	297	332	529	476
G_{4a}		0.37	0.02	283	330.5	531	
	G_{4a} acetate	0.52	0.04				
	G_{4a} methyl ester	0.75	0.12				492
	G_{4a} methyl ester acetate	0.84	0.38				
	Dihydro G_{4a} methyl ester	0.80	0.35	287	318	521	494
G_{4b}		0.51					
	G_{4b} (E) methyl ester		0.42	290–295	331	530	476
	G_{4b} (F) methyl ester		0.47	284	328	527	476
G_5		0.25	0.00	295	322	521	
	G_5 diacetate	0.49	0.03				
	G_5 methyl ester	0.57	0.03	295	322	521	492 $[M-H_2O]^{+\cdot}$
	G_5 methyl ester diacetate	0.83	0.24				594
G_6		0.21	0.00	293	320	517	
	G_6 dimethyl ester	0.87	0.24	293	319	518	536

The azopigment G_6 yielded a dimethyl ester on treatment with diazomethane, showing a mass spectrum identical with that of the dimethyl ester of compound C derived from either bilirubin-IXβ or -IXδ.

The structure proposed for azopigment G_5 encompasses a $-CHOH-CH_2OH$ side chain located on an exo position (Scheme 2). The presence of a 1,2-dihydroxyethyl instead of a vinyl group was established by mass-spectral analysis (see below). The exo location of the α-diol side chain was inferred from the observation that on isolation and diazo-coupling the parent bile pigment (yellow band 3) yielded the pair of azopigments G_5 and G_3, the latter being composed only of isomer A with the exclusion of isomer B (see below). Isolation of compound A by the diazonium-cleavage reaction is consistent with a IXα but not with a IXβ, IXγ or IXδ basic skeleton for the parent tetrapyrrole. The absence of azodipyrrole B suggests that the dihydroxyethyl side chain is formed via selective oxidation of the exo vinyl group of bilirubin-IXα (oxidation of a vinyl group at the protoporphyrin-IX stage cannot be excluded on the basis of the present evidence, but is expected to be less selective). Thus structure H, a dihydroxy derivative of azopigment B, is assigned to compound G_5.

The mass spectra (Supplementary Publication SUP 50076) of several derivatives of compound G_5 (Scheme 2) clearly demonstrated the presence of the dihydroxyethyl side chain. In the spectrum of the methyl ester of compound G_5 (possessing an esterified propionic acid side chain and an unmodified 1,2-dihydroxyethyl side chain) no molecular ion was observed, but the peak of highest mass (*m/e* 492) corresponded to loss of a molecule of water. A less abundant peak at *m/e* 476 pointed to restoration of a fully conjugated system by elimination of H_2O_2. Both the $[M-H_2O]^{+\cdot}$ and the unusual $[M-H_2O_2]^{+\cdot}$ ions probably had a thermal nature.

The mass spectrum of the methyl ester diacetate of compound G_5 yielded the molecular ion (*m/e* 594) as the base peak. Important peaks corresponded to losses of $-OCH_3$ (*m/e* 563), acetic acid (*m/e* 534), $CH_2-CO_2-CH_3$ or $CO_2-CH_2-CH_3$ (*m/e* 521) and acetic acid and ketene (*m/e* 492). An anthranilate moiety [$C_6H_4(CO_2-CH_2-CH_3)NH^{\cdot}$, 164 atomic mass units] was lost from the molecular ion (*m/e* 430) and from the [*M*−acetic acid]$^{+\cdot}$ and the [*M*−acetic acid−ketene]$^{+\cdot}$ ions (*m/e* 370 and 328).

Clear-cut evidence for the location of the hydroxyl groups came from the mass spectrum of the methyl ester bistrimethylsilyl derivative of azopigment G_5. The molecular ion at *m/e* 654 and ions derived from it by loss of CH_3 (*m/e* 639), trimethylsilanol (*m/e* 564) and combined loss of trimethylsilanol and C_6H_4-$(CO_2-CH_2-CH_3)NH^{\cdot}$ (*m/e* 400) constituted prominent peaks in this spectrum. Structurally most

important is the loss of a Me_3SiOCH_2 group (*m/e* 551) by side-chain cleavage in a position that is alpha to the other trimethylsilyloxy group and allylic to the double bond of the pyrrolenone ring. The ion *m/e* 551 further loses an anthranilyl moiety (*m/e* 387) or an ethyl anthranilate molecule (*m/e* 386). Final proof for the α-diol side chain was provided by its conversion into a cyclic butane boronate ester (Scheme 2), giving rise to molecular ions at *m/e* 576 and 575 (intensity ratio 4:1, one B atom present).

When compared with the corresponding derivatives of dihydroxy compound G_5, derivatives prepared from azopigment G_{4a} proved to be less thermally stable towards the vaporization conditions in the mass spectrometer. The mass spectra of the methyl ester of azopigment G_{4a} and of a trimethylsilyl derivative of this methyl ester were of weak intensity and showed peaks at *m/e* 492 and *m/e* 564 respectively, as the ions of highest mass. These observations indicate the presence of at least one additional oxygen with respect to methyl esters of A and B (M^+ 476). Although the presence of one or more hydroxyl groups was confirmed by the preparation of an acetate from the methyl ester of compound G_{4a}, no useful mass spectrum was obtained from this derivative.

On the assumption that the thermal lability found for the derivatives of compound G_{4a} was due to an unmodified vinyl group (not present in compound G_5), the methyl ester of compound G_{4a} was subjected to brief hydrogenation. Thermally stable dihydro derivatives, yielding characteristic mass spectra were obtained, both as the methyl ester of the dihydro form of compound G_{4a} and as the trimethylsilyl derivative of this methyl ester. The molecular ions in these spectra form abundant peaks (M^+ 494 and M^+ 566 respectively) and, together with associated ions $[M-H_2O]^{+\cdot}$ for the methyl ester and $[M-Me_3SiOH]^{+\cdot}$, $[M-CH_3]^+$ and $[M-Me_3SiOH-CH_3]^+$ for the trimethylsilyl derivative, demonstrate the presence of one hydroxyl group. Fragmentation of the anthranilyl moiety gave rise to further prominent peaks at *m/e* 165, 119 and $[M-164]$ (Compernolle *et al.*, 1976). Cleavage of the propionic acid side chain involved the loss of a CH_2–CO_2–CH_3 radical ($M-73$, and appropriate metastable peaks) giving rise to characteristic peaks at *m/e* 421 for the methyl ester and *m/e* 493 and 403 (further loss of trimethylsilanol) for the trimethylsilyl derivative. The enhanced abundance of these ions (11 and 16% respectively) as compared with the $[M-73]^+$ ions in the spectra of derivatives of dihydroxy compound G_5 (*m/e* 521, 3% for diacetate, *m/e* 581 not observed for bistrimethylsilyl derivative) indicated the methylene group in the α position to the pyrrole ring as the site of attachment of the hydroxyl function for compound G_{4a}.

Confirmation of the latter assignment was found in the absorption spectra (Table 3), which indicate a position that is alpha to the chromophoric system for the hydroxyl group of compound G_{4a}. A general feature of the spectra in Table 3 and previously published spectra (Blanckaert *et al.*, 1976*b*) is the shift to shorter wavelengths observed for the absorption maximum centered around 520–535 nm for compounds possessing no vinyl double bonds. Thus compound C and meso compounds exhibited a blue shift when compared with compounds A and B, whereas compounds with two vinyl double bonds (D and G) showed a red shift.

For compound H, possessing a –CHOH–CH_2OH instead of a vinyl side chain, a bathochromic shift of about 4 nm was observed with respect to the absorption maximum of meso-A and meso-B with $\lambda_{max.}$ 517 nm (Blanckaert *et al.*, 1976*b*) or compound C (Table 3). This shift can be ascribed to the hydroxyl function in the α position of the pyrrolenone ring. A similar shift of 4 nm caused by a hydroxyl group not directly located on a conjugated system was found for the enolized 5-hydroxy-4-methyl-1,3-cyclopentanedione ($\lambda_{max.}$ 241 nm) compared with 4-methyl-1,3-cyclopentanedione ($\lambda_{max.}$ 237 nm) (Vandewalle & Compernolle, 1966; Compernolle & Vandewalle, 1967). A bathochromic shift also is observed for compound G_{4a} compared with compounds A (5 nm) or B (2 nm) and for dihydro compound G_{4a} compared with compounds C, meso-A and meso-B (4 nm). The exact coincidence of the absorption maximum observed for the dihydro form of compound G_{4a} with that found for dihydroxy compound G_5, taken as a reference, indicates the presence of an intact vinyl group for compound G_{4a} and of either a –CH_2OH or a –CHOH–CH_2–CO_2H group, corresponding to oxidation at either a methyl or a methylene α position. A primary hydroxyl function for compound G_{4a} can be ruled out on the basis of the slow silylation reaction observed for dihydro-compound G_{4a} methyl ester. A 1 min treatment with pyridine/*NO*-bis(trimethylsilyl)trifluoroacetamide (1:1, v/v) gave a conversion of about 50% only. An identical treatment of dihydroxy compound H gave about 90% bistrimethylsilyl derivative. It should be noted that, under the reaction conditions used, no silylation of lactam groups occurs, as shown by the unmodified mass spectra observed for the methyl esters of compounds A and B on treatment (3–5 min) with the same silylating reagent. The slow silylation reaction of the hydroxyl group of dihydro compound G_{4a} methyl ester can be explained on the basis of a CHOH–CH_2–CO_2CH_3 side chain. Inspection of structures K and J (Scheme 2) shows that the hydroxyl group can form two hydrogen bridges located on a stable six-membered ring, one involving the CO_2CH_3 ester carbonyl group, and the other an azo nitrogen.

By contrast with the single structure assigned to azopigment G_5 (whose parent bile pigment on diazo-coupling yielded compound H with oxidized exo vinyl group and isomer A with intact endo vinyl group), a mixture of two isomers with interchanged methyl and vinyl groups is postulated for compound G_{4a} (Scheme 2). Indeed, isolation and diazonium cleavage of the parent bile pigment (yellow band 2), besides azopigment G_{4a}, yielded a mixture of isomers A and B.

Structure assignment to the tetrapyrrolic bile pigments

As outlined above, direct determination of the structures of the parent tetrapyrroles is difficult. Identification was done by chromatography of the unknown tetrapyrroles with respect to available reference compounds (see above). Subsequent formation and analysis of the azo derivatives confirmed their identity. This approach could be used for the characterization of the pigments corresponding to bands 1, 5, 6 and 8. In the other cases structure assignment to the tetrapyrroles relied on the identification of the azo derivatives obtained from the isolated bilinoids. In this way the structures of the pigments corresponding to bands 2 and 3 were established.

Properties of diazo-positive bands. Except for band 4 all yellow components reacted with diazotized ethyl anthranilate (Table 1). For pigments 2, 3, 5–8, 11 and 12 colour formation was immediate in aqueous medium. The slow reaction observed for the other pigments necessitated the use of a reaction accelerator to achieve complete conversion.

The azo derivatives obtained from the pigment bands are shown in Table 1. Their chromatographic behaviour both before and after derivative formation and their responses to ammonolysis, alkaline methanolysis, methyl ester formation and acetylation were the same as for the corresponding azopigments prepared from whole Gunn-rat bile (Fig. 1; Table 2). Thus one may safely assume that the structures shown in Scheme 2 apply also to the azopigments obtained from separated bile pigments.

Azo derivatives obtained from isolated bile pigments and proposed structures. The proposed structures of the tetrapyrroles are shown in Scheme 1. As already mentioned direct chromatography of bands 1, 5, 6 and 8 with respect to known reference compounds suggested that they corresponded to bilirubin-IXα, -IXβ, -IXδ and -IXγ respectively. This was confirmed by demonstrating that the azopigments predicted from these structures were indeed obtained when the tetrapyrroles were treated with diazo reagent. Bands 1 and 8 yielded azopigments G_3 (A+B) and G_{4b} (E+F) respectively. Azopigment G_3 could be separated into equal amounts of the isomers A and B. This applied for both the free acids and the methyl esters. In a similar way compound G_{4b} was shown to be composed of azopigments E and F. Band 5 yielded azopigments G_1 (D) and G_6 (C), and band 6 azopigments G_2 (G) and G_6 (C).

From band 3 equal amounts of azopigments G_3 and G_5 were obtained. By t.l.c. of the free acid and the methyl ester, azopigment G_3 was shown to correspond only to isomer A. These observations support the idea that tetrapyrrole V contains a 1,2-dihydroxyethyl side chain replacing the exo vinyl group of bilirubin-IXα. Band 2 yielded approximately equal amounts of azopigments G_3 and G_{4a} and trace amounts of unknown azopigments. Detection of both isomers A and B in azopigment G_3 is consistent with a mixture of the two tetrapyrroles VI and VII.

The four bilirubin-IX isomers (bands 1, 5, 6 and 8), the two monohydroxyl derivatives (band 2) and the dihydroxyl derivative of bilirubin-IXα (band 3) account for 83% of total diazo-positive material in bile of light-treated and control Gunn rats (Table 1). The remaining diazo-positive bands (7 and 9–14) could not be characterized. They yielded azopigment G_3 as the predominant derivative and small amounts of more polar azopigments (G_6 and G_7). In each case, azopigment G_3 consisted of the isomers A and B. These results suggest that the parent bile pigments are modified forms or partially degraded products of bilirubin-IXα.

The structure of the diazo-negative pigment corresponding to band 4 could not be established owing to its extreme lability. In methanol solution it showed an absorption maximum at 433nm. A pigment with similar characteristics has been described by Kapitulnik *et al.* (1973, 1974). The negative diazo reaction and the conversion into a brown pigment on illumination indicate the absence of a biladiene-(a,c) skeleton.

Conjugated bile pigments. As already discussed, analysis of the azo derivatives prepared from 20–1000ml of Gunn-rat bile, without previous isolation of the tetrapyrroles, demonstrated the presence of glycosidic conjugates of bilirubin-IXα with glucuronic acid and also with a hexose, probably glucose. However, obviously because of the minute amounts, the parent conjugated bilirubins could not be detected after t.l.c. of tetrapyrrolic bile pigments.

General comments

Apart from the complete identification of 83% of the diazo-positive bilinoids in Gunn-rat bile a number of other interesting features arise from this work which require comment. The presence of β-D-glycosidic conjugates of bilirubin-IXα is of theoretical importance, since it demonstrates that enzymes catalysing the transfer of sugars to bilirubin-IXα are present and still partially functional in homozygous Gunn rats (jj). This is concluded in spite of the fact that no activity of the UDP-glucuronyltransferase towards bilirubin-IXα could be detected

in tissues *in vitro* from our strain of Gunn rats (Fevery *et al.*, 1972). A similar discrepancy was observed in children with the Crigler–Najjar disease, Type I (Blanckaert *et al.*, 1976*a*). Two possible explanations for these findings have been considered. The bilirubin UDP-glucuronyltransferase may be present but in an abnormal form. Such a possibility is suggested by studies on the glucuronidation of *o*-aminophenol and *p*-nitrophenol which is also partially defective in Gunn rats (Schmid *et al.*, 1958; Stevenson *et al.*, 1968; Zakim *et al.*, 1973). Full restoration of the activity with *o*-aminophenol to normal on the addition of diethylnitrosamine to enzyme preparations (Stevenson *et al.*, 1968), and kinetic studies with *p*-nitrophenol as the acceptor substrate (Nakata *et al.*, 1976) demonstrated that normal amounts of genetically modified enzyme(s) are present in liver. Although the deficiency with bilirubin-IXα as the acceptor substrate is clearly much more pronounced, the present identification of glycosidic conjugates of bilirubin-IXα in Gunn-rat bile suggests that the genetic defect may be similar to the situation established for the two exogenous substrates mentioned. A possible explanation for these findings is that several UDP-glucuronyltransferases share a common defect related either to their primary structures or perhaps to a conformational change induced by abnormal environmental conditions in the microsomal membranes (Nakata *et al.*, 1976). A second possibility could be that bilirubin-IXα is conjugated at a decreased rate by a transferase normally acting on other substrates. To solve these problems, complete purification and characterization of the enzymes involved is required.

The present work provides the first direct demonstration of the existence in a mammal of bilirubin-IXβ and -IXδ. Indirect evidence based on derivative formation has been given by Petryka (1966), O'Carra & Colleran (1970) and Tipton & Gray (1971). The existence of bilirubin-IXγ has not been demonstrated previously in any animal species. The γ-isomer of biliverdin-IX is responsible for the green pigmentation of the caterpillars of the cabbage-white butterfly, *Pieris brassicae* (Rüdiger *et al.*, 1968). The occurrence of all four isomers of bilirubin-IX in bile implies (*a*) that cleavage of the ring structure of protoporphyrin-IX, although predominantly at the α-bridge, also occurs *in vivo* to some extent at the non-α positions, and (*b*) that, in contrast with the findings of Colleran & O'Carra (1970) in guinea pigs, conversion of biliverdin-IXγ into the corresponding rubin must occur *in vivo* in the Gunn rat.

In the present work several hydroxylated bilirubin-IXα derivatives have been demonstrated (Scheme 1). The apparent formation of only one dihydroxyl derivative (tetrapyrrole V) and of two isomeric monohydroxyl derivatives (tetrapyrroles VI and VII) can be rationalized on the following grounds. Oxidation at a vicinal methylene group on one of the two propionic acid side chains, located on the inner pyrrole rings of bilirubin-IXα, must be rather insensitive to remote structural variations (interchanged methyl and vinyl groups on the outer pyrrolenone rings). By contrast, direct oxidation at the site of structural variation, i.e. the vinyl group, is sensitive to the location of that group on either an outer 'exo' position or an inner 'endo' position. Selective acid-catalysed and photo-induced addition of alcohols and thiols to the exo vinyl group of bilirubin-IXα (Manitto & Monti, 1972, 1973) indeed demonstrates such a differentiating influence. However, whether the presently isolated hydroxyl derivatives are derived from hydroxylated protoporphyrin-IX or biliverdin-IXα or are formed by hydroxylation of bilirubin-IXα remains to be established.

In an alternative approach Berry *et al.* (1972) compared the products of the photo-oxidation of bilirubin-IXα *in vitro* with the pigments present in Gunn-rat bile. On the basis of t.l.c. mobilities of the free acids and their methyl esters in different solvent systems, three compounds from Gunn-rat bile were identified with photo-oxidized products of bilirubin-IXα. Proposed structures for two diazo-negative tetrapyrroles encompass an α-diol group located on methine bridge a and on either of the two vicinal ring carbon atoms. Another diazo-positive tetrapyrrole would have an α-diol group located on the central methylene bridge and a neighbouring ring position. Clearly, results obtained with diazo-negative tetrapyrroles cannot correspond to our results, which are concerned with diazo-positive bile pigments only. Further, diazonium cleavage of a tetrapyrrole with oxidized central methylene bridge cannot yield azopigments other than isomers A and B. We conclude therefore that neither of the side-chain-hydroxylated tetrapyrroles isolated by us corresponds to structures proposed by Berry *et al.* (1972). In accord with this conclusion, the absorption spectra (Table 1) of tetrapyrroles V ($\lambda_{max.}$ 440nm), VI and VII ($\lambda_{max.}$ 446nm) confirm the presence of a bilirubin chromophoric system, whereas the absorption spectra reported by Berry *et al.* (1972) indicate a modified chromophore.

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