Conjugated and Unconjugated Bilirubins in Bile of Humans and Rhesus Monkeys

STRUCTURE OF ADULT HUMAN AND RHESUS-MONKEY BILIRUBINS COMPARED WITH DOG BILIRUBINS

By SHULA G. BLUMENTHAL,* DOUGLAS B. TAGGART, RICHARD M. IKEDA
and BORIS H. RUEBNER

Department of Pathology, School of Medicine, University of California, Davis, CA 95616, U.S.A.

and DONALD E. BERGSTROM

Department of Chemistry, University of California, Davis, CA 95616, U.S.A.

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1. Bilirubin-IX α , -IX α diglucuronide, -IX α monoglucuronide, -IX α monoglucoside, -IX α monoxylsode, a bilirubin-IX α diconjugate containing glucose and another unknown compound, and bilirubin-IX β are present in gall-bladder bile of adult human, rhesus monkey and dog. Dog bile normally also contains other bilirubin-IX α diconjugates, i.e. compounds containing two conjugating sugars such as glucuronic acid and glucose, glucuronic acid and xylose and glucose and xylose. 2. Azopigments α F, α 0, α 2, α 3, β x and δ derived from human and rhesus-monkey bilirubins are identical in their chemical composition with those obtained from the dog. 3. Azopigments α F and β x found in diazotized biles of adult humans, rhesus monkeys and dogs are products of unconjugated bilirubin-IX β . 4. Technical modifications of previously published procedures [Heirwegh, Fevery, Michiels, Van Hees & Compennolle, (1975) *Biochem. J.* **145**, 185-199] were introduced which make it possible to separate the bilirubins, diazotize the separated bilirubins, extract the azopigments and chromatograph them in one working day (6-8h).

It is generally recognized that the glucuronides of bilirubin-IX α are the major tetrapyrrole pigments of adult human and dog biles. Structural elucidation has been made possible by chemical and spectroscopic examinations of azodipyrroles derived from the reaction of bilirubins with diazotized ethyl anthranilate (2-ethoxycarbonylbenzenediazonium chloride). In this way the following were shown by Heirwegh *et al.* (1975).

(1) Bilirubin-IX α is present *in vivo* in the unconjugated as well as in conjugated forms. Conjugates of bilirubin in adult dog bile include esters of the following monosaccharides, glucose, glucuronic acid and xylose, formed with the propionic acid side chains on positions 8 and 12 of the tetrapyrrole.

(2) Bilirubin-IX β has been found *in vivo* in the unconjugated form only. The reaction of bilirubin-IX β with diazotized ethyl anthranilate gives azopigments α F and β x in a 1:1 ratio. We have previously reported the presence of azopigment β x in gall-bladder biles and meconiums of newborn humans

and rhesus monkeys (Blumenthal *et al.*, 1976a) and in human foetuses (Blumenthal *et al.*, 1976b). Blanckaert *et al.* (1976b) have reported that biles from humans, dogs, pigs and rats contain up to 5% of their bilirubin in the form of bilirubin-IX β , -IX γ and -IX δ , compounds that previously had not been thought to occur *in vivo* (Schmid & McDonagh, 1975).

By using the methods of Heirwegh *et al.* (1975) with some modifications, we have performed an extensive analysis of human and rhesus-monkey bilirubins in normal adult biles. Bilirubins from dog bile have been well-characterized (Heirwegh *et al.*, 1970, 1975), and have therefore been used as standards.

Materials and Methods

Chemicals

All chemicals were of reagent grade. The chloroform contained approx. 0.6% ethanol as a stabilizer. Anhydrous methanol was obtained by distilling reagent-grade methanol over CaH₂. All aqueous solutions were prepared with twice-glass-distilled

* To whom reprint requests should be sent.

water. Unconjugated bilirubin was obtained from Sigma Chemical Corp. (St. Louis, MO, U.S.A) and kept refrigerated in a desiccator. Sodium methoxide was prepared by the addition of freshly cut sodium metal to anhydrous methanol. Diazomethane was prepared from toluene-*p*-sulphonylmethylnitrosamide (Diazald) by an established procedure (de Boer & Backer, 1963).

Bile samples

Bile samples were collected from the gall bladders of five healthy adult dogs immediately after death. Bile samples from rhesus monkeys (*Macaca mulatta*) were obtained from three animals without liver disease immediately after death. Human bile was obtained from the gall bladders of ten adults at autopsy. Autopsies were performed 8–12h after death. All the patients had died of either a heart attack or stroke. Two bile samples were collected, with informed consent, from healthy adult volunteers by a duodenal tube. A series of four bile samples was collected from a T-tube in the common bile duct of a patient suffering from calculous common bile-duct obstruction. The protocols for these studies were approved by the Committee on Research Involving Human Subjects. All bile samples were collected in dark containers, frozen immediately, and kept at -75°C until used. A small sample from every bile collected was analysed for diazo-positive bilirubins as soon as it arrived from the hospital. Bilirubins were separated by t.l.c. 8–24h after collection. Separation of azopigments from larger samples (4–6ml) were carried out within 1 week of collecting any sample.

Methods

All experimental procedures were performed in semi-darkness and at room temperature (24°C) except where stated otherwise. Chromatographic separations of bilirubins and storage of azodipyrroles were done under argon. Other chromatographic separations were done in glass chambers without exchange of air. All chromatographic separations were done on glass plates (20cm $\times$ 20cm) pre-coated with silica gel G60 (E. Merck, Darmstadt,

Germany). Chloroform, redistilled over P_2O_5 , containing 0.1% of methanol and kept at 4°C was used for chromatography. Centrifugations were usually done with an IEC laboratory bench centrifuge at 3000–3500 rev./min (r_{av} 12 cm) for 10 min. The glycine/HCl buffer was prepared by adjusting a 0.4M-HCl solution to pH 2.7 with solid glycine.

Extraction of bile pigments and separation by t.l.c.

All extraction procedures were done in the dark and on ice. Solutions and solvents were precooled. Centrifuge tubes and rotors were cooled to 4°C and the tubes kept on ice.

Extractions of bilirubins were performed as described by Heirwegh *et al.* (1975). The resulting chloroform extract was concentrated under vacuum by using a water bath at 35°C . The concentrated extract was applied as a streak (19cm long) on silica-gel G thin-layer plates. The solvent was evaporated off by using a stream of air. The plates were developed with solvent system III (see Table 1 for a list of the solvent systems used). The plates were then dried, photographed and R_F values of the separated bilirubins recorded. The bilirubin bands were scraped into 10.0ml centrifuge tubes.

Formation and separation of azopigments

(a) To every tube, 1 ml of ethyl anthranilate diazo reagent (Heirwegh *et al.*, 1975) was added. The tubes were shaken well and kept at 27°C for 3–5 min, after which the coupling reaction was terminated by the addition of 5.0ml of freshly prepared ascorbic acid solution (50mg/ml). The mixtures were shaken well, centrifuged (1000 g, 10 min), and the clear supernatant was decanted. The pigmented silica gels and methanol were mixed vigorously with a vortex mixer, centrifuged and the fluid was decanted. The silica gels were vortex-mixed with methanol/butyl acetate (1:1, v/v), centrifuged and the fluid was decanted. If necessary a third extraction with methanol was done. All decanted fluids were pooled. Then glycine/HCl buffer (pH 2.7)/water (1:1, v/v) was added to every tube. The butyl acetate layer containing the azopigments was pipetted off after a 5 min centrifugation.

Table 1. List of solvent systems used for t.l.c. separations
All t.l.c. separations were carried out on glass plates (20cm $\times$ 20cm) pre-coated with silica gel G60.

Number of system	Solvents	Proportions (by vol.)	Length of development (cm)
I	Chloroform/methanol	17:3	2.0–5.0
II	Chloroform/methanol/water	65:25:3	17.0–18.0
III	Chloroform/methanol/water*	24:14:3	17.0–18.0
IV	Benzene/ethyl acetate	37:3	17.0–18.0
V	Chloroform/methanol	9:1	1.0
VI	Benzene/ethyl acetate	9:1	16.5–17.0
VII	Propan-1-ol/water	17:3	15.0–16.0

* 10 mg of 2,6-di-*t*-butyl-*p*-cresol added/ml.

(b) Bilirubins were eluted from the silica-gel powders with methanol. Then 0.5–1 ml of ethyl anthranilate diazo reagent was added to each eluate. The coupling reaction proceeded at room temperature and in the dark for 30 min. Then 0.5 ml of butyl acetate was added, followed by 2–5 ml of ascorbic acid (10 mg/ml) to separate the butyl acetate from the mixture. The butyl acetate phase containing the azodipyrroles was pipetted off after a 5 min centrifugation at 1000 g. The azodipyrroles of the various bilirubins were separated by t.l.c. with solvent system I for 3–4 cm, followed by solvent system II. Usually the entire procedure was completed the same day. In some instances the butyl acetate extracts were stored at -75°C under argon. To obtain adequate amounts of azopigments $\alpha 1$, $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$, diazotizations with ethyl anthranilate were performed on the original biles of those specimens that contained significant quantities of bilirubins giving rise to these azopigments. The azopigments $\alpha 1$, $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ were scraped off the t.l.c. plates, eluted with methanol and separated by t.l.c. as described above.

Absorption spectra of azodipyrroles

Azopigments purified by t.l.c. were scraped off the plates and eluted with methanol. They were partitioned into *n*-butyl acetate as described above. After the azopigments were chromatographed and eluted a second time, their u.v.–visible spectra (in methanol) were recorded with a Cary 17 spectrophotometer.

Ammonolysis of azopigments

Purified preparations of azopigments $\alpha 2$, $\alpha 3$ and δ were spotted on t.l.c. plates at 2.5 cm intervals. The spotted plates were placed for 5.0–6.0 h in a closed tank saturated with ammonia vapour. The ammonia was then blown off the plates with an air stream. Untreated azodipyrroles were spotted adjacent to the corresponding ammonolysed azodipyrrole. Markers of ammonolysed azopigments δ (prepared as described above), $\alpha 0$ and $\alpha 0\text{M}$ (prepared as described below) were spotted after the plates were removed from the ammonia tank. All the plates were developed in solvent systems I and II.

Methanolysis of azopigments and analysis of the derived methyl esters of the azodipyrroles

n-Butyl acetate solutions of purified conjugated azopigments (0.3–0.5 ml) were transferred to 12.0 ml stoppered glass tubes. Then 1 ml of 0.013 M-sodium methoxide (Gordon *et al.*, 1974) in dry distilled methanol was added to each tube. Tubes were closed and kept for 2 h in the dark at room temperature. The reaction mixtures were neutralized with sufficient glycine/HCl buffer to give two phases. The pigmented butyl acetate layer was pipetted off and spotted on t.l.c. plates. Markers of untreated pure azopigments

were spotted next to every treated azopigment. Plates were developed with solvent systems I and II.

Treatment with diazomethane

For this 0.5 ml of methanolic eluate of purified azopigments αF , $\alpha 0$ and βx were transferred to stoppered glass tubes. A few drops of diazomethane in diethyl ether (de Boer & Backer, 1963) were added. The reaction took place for 1 min at room temperature and in the dark. The reaction was terminated with an excess of 0.1 M-acetic acid. The methylated azopigment solutions were spotted on t.l.c. plates and untreated αF , $\alpha 0$ and βx azopigments were used as markers. Plates were developed with solvent systems I and II.

Alkaline hydrolysis of azopigments

Methanolic eluates of the purified conjugated azopigments were transferred into stoppered glass tubes, and 1 ml of 0.1 M-NaOH was added to each tube. The stoppered tubes were heated at 60 – 70°C for 1 h. The reaction mixtures were cooled, and neutralized with 0.1 M-HCl. The azopigments were spotted on t.l.c. plates. Markers of pure untreated azopigments were spotted adjacent to every treated azopigment. The plates were developed with solvent systems I and II as described before.

Mass spectra of azopigments

Derivatives of various azopigments were subjected to mass spectrometry after preparation from natural or chemically prepared bilirubins. After chromatographic purification, the azopigment derivatives were eluted from the silica gel with methanol. The methanol was removed and the pigments were dried and placed in capillary tubes for spectral determination. Spectra were recorded with a model 3200 Finnigan mass spectrometer controlled by the model 6000 data system and operated in electron-impact mode at 70 eV.

Preparation of bilirubins -IX β and -IX δ from haemin

Biliverdin-IX β and -IX δ were prepared and purified by the procedure of Bonnett & McDonagh (1973). The entire procedure was done in dim light. A sample of commercial haemin (25 mg) was dissolved in 400 ml of water/pyridine (3:1, v/v) and the solution was flushed with O_2 for 15 min. The temperature was maintained at 37°C , while ascorbic acid (300 mg in 5.0 ml of water) was added. After 3 min the mixture was poured into 100 ml of ice-cold chloroform. This solution and all others to follow were flushed with argon and the procedures were carried out in an argon atmosphere. The chloroform was separated and extracted with a fresh 30 ml portion of chloroform. The combined chloroform solution was dried over Na_2SO_4 and concentrated to near-dryness. The resulting green paste was dissolved in 1.3 ml of chloro-

form and washed with three 7.5 ml portions of light petroleum (b.p. 30–60°C) and dried under a stream of argon. The resulting green solid was dissolved in 6.3 ml of methanol, treated with 0.5 ml of 2.0M-NaOH for 1.0 min and then with 6.3 ml of 14% (w/v) BF_3 in methanol for 15 min under reflux. The solution was left overnight at room temperature in the dark and then diluted with 40 ml of water. The biliverdin dimethyl esters were extracted with 50 ml of chloroform. The chloroform was then washed with 2×30 ml of water and dried over Na_2SO_4 . The pigments were concentrated almost to dryness and dissolved in 20 ml of benzene/chloroform (9:1, v/v), which was separated on a column (1 cm $\times$ 20 cm) of deactivated alumina. The blue-green pigment was eluted with benzene/chloroform (9:1, v/v) and concentrated to dryness. The four isomers were separated by t.l.c. on silica-gel plates with chloroform/acetone (9:1, v/v). The biliverdin dimethyl esters were immediately treated with NaBH_4 in 1.0 ml of methanol for about 2.0 min (Blanckaert *et al.*, 1976a). Then 1 ml of chloroform and 4.0 ml of ice-cold glycine/HCl (containing 10 mg of ascorbic acid/ml) were added. After mixing the solution, the chloroform layer was separated and washed with 3×4.0 ml of water. The resulting bilirubin dimethyl ester solution was concentrated and the residue was dissolved in 2.0 ml of methanol. To this solution 1.0 ml of 1M-NaOH, 20 mg of ascorbic acid and a trace of EDTA were added. The mixture was heated for 30 min at 37°C under argon, then neutralized with acetic acid; next 2 ml of chloroform and 4.0 ml of $(\text{NH}_4)_2\text{SO}_4$ -saturated glycine/HCl buffer were added. The aqueous phase was separated and re-extracted with 1.0 ml of chloroform. The final bilirubin product was stored in chloroform under argon at -78°C .

Azopigments αF and βx

Azopigments αF and βx were prepared by treatment of bilirubin-IX β with the ethyl anthranilate diazo reagent (see part b of 'Formation and analysis of azopigments' above). Azopigment βx was purified by t.l.c. solvent systems I and II. Azopigment αF was purified by repeated t.l.c. (two or three times) in solvent system V for 1.0 cm, followed by solvent system IV for 17–18 cm.

Preparation of azopigment meso αF

Bilirubin-IX β and -IX δ prepared from haemin and dog bilirubin, assumed to be bilirubin-IX β on the basis of chromatographic data, were hydrogenated and diazotized. Hydrogenation was performed in the dark by the procedure of Blanckaert *et al.* (1976a) with one important modification. Since our hydrogenations were performed on a micro scale, we used approx. 0.1% Pd on CaCO_3 as catalyst, prepared by the method of Combe *et al.* (1967). The hydrogenated bilirubin solutions were neutralized with acetic acid

and extracted with chloroform in the presence of glycine/HCl buffer. The chloroform solution was washed twice with water and immediately treated with ethyl anthranilate diazo reagent in the presence of acetone/ethanol (1:1, v/v) as an accelerator. The azopigments were separated on t.l.c. with solvent system V, eluted with methanol and separated on t.l.c. a second time with benzene. The purified meso αF azopigments were scraped into bottom-sealed glass capillaries and subjected to mass spectroscopy.

Determination of the conjugating sugars

For this 0.2–0.5 ml of purified azopigments $\alpha 2$, $\alpha 3$ and δ were treated with sodium methoxide as described above. Then 2 h later 1.0 g of resin (Amberlite CG-50; polymethylacrylic type; Mallinckrodt AR chromatographic grade; 100–200 mesh) was added and the mixture stirred. Then 1–2 ml of methanol was added to obtain a homogeneous dispersion. The mixture was centrifuged and the methanol separated. The resin was washed twice with 1.0–1.5 ml of methanol. The combined methanol supernatant was evaporated under vacuum. Water/butan-1-ol (1:1, v/v) was then added and mixed. The mixture was centrifuged and the pigmented butanol layer removed. The water layer was washed again with butanol until all the pigment was removed. The water extract was concentrated under vacuum. The conjugating carbohydrates in the water were spotted on t.l.c. plates. Markers of methoxide-treated glucuronic acid, glucose and xylose were spotted on every plate. Plates were developed with solvent system VII. Dried plates were sprayed with 2% (v/v) aniline and 2% (v/v) chloroacetic acid in ethyl acetate, followed by heating for 2–5 min at 95°C. Carbohydrates gave a distinctive brown colour; 2 μg of any sugar resulted in a detectable colour spot on the plates.

Results

Extraction and separation of bile pigments

The methods used for these studies did not give quantitative results. Some of the reasons for this are as follows: the chloroform extracts contained only a part of the bilirubins present in the biles. The remainder was retained in the methanol/water phase and in the sediment. Some changes and losses of bilirubins also occurred during the procedures. The fact that we obtained small amounts of unconjugated bilirubin in all chloroform extracts of adult biles is evidence that some of the carbohydrates of the unconjugated bilirubin were hydrolysed and gave rise to unconjugated bilirubin.

Fig. 1 shows a t.l.c. separation of a chloroform extract of dog bile pigments. The dog bile extracts gave 12–14 yellow bands. Only ten of them were considered to represent significant quantities of bilirubin. Human and rhesus-monkey biles gave nine

Fig. 1. Chromatographic separation of bilirubins extracted from dog bile

Bilirubins extracted from normal adult dog bile were separated by t.l.c. in solvent system III (to 17–18 cm). Bands were numbered from the application line (AL) to the solvent front (SF). The photograph depicts half a plate. (Bands usually run with a 'trough' in the middle of the plate.)

or ten yellow bands, six of which were considered to represent bilirubins in significant amounts. The ten identified bilirubin bands present in dog bile are given in Table 2, and their structures are shown in Scheme 1.

The relative amount of band 8 and 9 in dog biles was fairly high, but low in human and rhesus-monkey bile. The amount of bilirubin-IX α (unconjugated) in band 9 must represent a fraction of bilirubin that was deconjugated during the isolation procedure, because unconjugated bilirubins could not be demonstrated in the same bile samples by using specific methods to detect unconjugated bilirubin (Feverly *et al.*, 1972).

Reaction of bilirubins with diazotized ethyl anthranilate

The diazotization was carried out as described by Heirwegh *et al.* (1975) but modified by extracting the azopigment from the silica gel with methanol/butyl acetate rather than formamide. After formamide, almost every bilirubin band showed unconjugated azopigments. Methanol/butyl acetate extracted as much of the azopigments as did formamide,

but less unconjugated azopigments were formed by using methanol/butyl acetate instead of formamide. This was observed in bands 1, 2, 3, 7, 8 and 9 (Table 2). We therefore concluded that methanol/butyl acetate gave more reliable results. Eventually we found that the best results were obtained when the bilirubins were extracted from the silica gel before diazo coupling (see method *b* under 'Formation and analysis of azopigments' above).

Fig. 2 shows the azopigments derived from the various bilirubins chromatographically separated from dog bile (see Fig. 1). The list of bilirubins in Table 2 was derived from the pattern of azopigments present on the t.l.c. plate shown in Fig. 2. The first bilirubin band produced azopigment δ . The second bilirubin band produced azopigments δ and $\alpha 3$ in a ratio of 1:1. The third bilirubin band produced azopigments δ , $\alpha 2$ and $\alpha 0$. This band probably represents two bilirubins. There is a small amount of the bilirubin that gave rise to azopigments δ and $\alpha 3$, and a larger amount of the same bilirubin that was present in the next bilirubin band, i.e. δ and $\alpha 0$. The fourth bilirubin band produced azopigments δ and $\alpha 0$ in a ratio of 1:1. The fifth bilirubin band produced azopigments $\gamma 1$ and $\alpha 3$. There is somewhat more of azopigment $\gamma 1$ than of $\alpha 3$. The sixth bilirubin band contained two bilirubins; one, bilirubin-IX β , produced azopigments βx and αF , and the other bilirubin produced azopigments $\alpha 3$ and $\alpha 2$. The ratio of azopigments βx and αF is 1:1 and the ratio of azopigments $\alpha 3$ and $\alpha 2$ is 1:1. The seventh bilirubin band produced azopigments $\alpha 3$ and $\alpha 0$ in a ratio of 1:1. The eighth bilirubin band produced azopigments $\alpha 2$ and $\alpha 0$. Azopigment $\alpha 0$ from the ninth bilirubin band was present also in the eighth bilirubin band as well as in the ninth bilirubin band. R_F values of unconjugated azopigments from dog bile are given in Table 4 and those of conjugated azopigments are given in Table 5.

U.v.-visible spectra of azopigments

U.v.-visible spectra of azopigments obtained from human, rhesus-monkey and dog biles were recorded. The spectra of azodipyrroles αF , $\alpha 0$ and βx from dog bile are given in Fig. 3(a). The spectra of the corresponding azopigments purified from diazotized ethyl anthranilate-treated rhesus-monkey biles (Fig. 3b) and human biles were identical. For these and other azopigments the λ_{max} and the absorbance at the higher wavelength maximum relative to that at the lower wavelength maximum (symbolized by R) are given in Table 3.

Both λ_{max} and R were nearly identical for azopigments $\alpha 0$, $\alpha 2$, $\alpha 3$ and δ . The spectrum of azopigment $\alpha 3$ showed the same λ_{max} values (532 and 333 nm) as that of $\alpha 0$, but R was significantly greater. The λ_{max} values of azopigment αF were shifted to

(11) Bilirubin-IX β

(1) Bilirubin-IX α $R^1, R^2 = H$

(2) Bilirubin-IX α diglucuronide (GlcUA, GlcUA)

(3) Bilirubin-IX α monoglucuronide (GlcUA)

(4) Bilirubin-IX α diglucoside (Glc, Glc)

(5) Bilirubin-IX α monoglucoside (Glc)

(6) Bilirubin-IX α dixyloside (Xyl, Xyl)

(7) Bilirubin-IX α monoxxyloside (Xyl)

(8) Bilirubin-IX α monoglucuronide, monoglucoside (GlcUA, Glc)

(9) Bilirubin-IX α monoglucuronide, monoxxyloside (GlcUA, Xyl)

(10) Bilirubin-IX α monoglucoside, monoxxyloside (Glc, Xyl)

$R^1, R^2 =$

(β -D-glucopyranuronosyl)

$R^1, R^2 = H, \beta$ -D-glucopyranuronosyl

$R^1, R^2 =$

(β -D-glucopyranosyl)

$R^1, R^2 = H, \beta$ -D-glucopyranosyl

$R^1, R^2 =$

(β -D-xylopyranosyl)

$R^1, R^2 = H, \beta$ -D-xylopyranosyl

$R^1, R^2 = \beta$ -D-glucopyranuronosyl and β -D-glucopyranosyl

$R^1, R^2 = \beta$ -D-glucopyranuronosyl and β -D-xylopyranosyl

$R^1, R^2 = \beta$ -D-glucopyranosyl and β -D-xylopyranosyl

Scheme 1. Structures of bilirubin-IX α , bilirubin-IX β and bilirubin-IX α conjugates
Bilirubins 3, 5 and 7–10 may be mixtures of isomers where R^1 and R^2 are either of the groups indicated.

Table 2. Bilirubin in adult-rhesus-monkey bile as separated on t.l.c. with system III

See Figs. 1 and 2. T.l.c. separations were carried out as described in Table 1 and in the text. γ_1 , Unknown molecule linked with an ester bond to one carboxyl group of bilirubin-IX α .

Band no.	Colour	R_F	Bilirubin	No. in Scheme 1
1*	Yellow	0.35	IX α GlcUA, GlcUA	2
2	Yellow	0.52	IX α GlcUA, Glc	8
3	Faint yellow	0.61	IX α GlcUA, Xyl	9
4*	Yellow	0.66	IX α GlcUA	3
5*	Yellow+pink	0.69	IX α γ_1 , Glc	
6†	Light yellow	0.76	IX β unconjugated	11
7	Vivid yellow	0.80	IX α Glc, Xyl	10
8*	Yellow	0.85	IX α Glc	5
9*	Light yellow	0.88	IX α Xyl	7
10*	Yellow+dark yellow	0.95-0.99	IX α	1

* Present in all human and rhesus-monkey biles.

† Present in some human and rhesus-monkey biles.

Fig. 2. T.l.c. of azopigments obtained by treatment of isolated bile pigments from normal dog bile with diazotized ethyl anthranilate

The reference numbers indicated below the application line (AL) denote the parent bilirubin band in Fig. 1. The Greek letters are symbols for the different azopigments found in biles of dog and other animals. Abbreviation: SF, solvent front.

Table 3. Properties of various azodipyrroles obtained from bilirubins separated from human, rhesus-monkey and dog biles

U.v.-visible spectra of azodipyrroles in methanol were recorded with a Cary 17 spectrophotometer.

$$R = A_{333-338}/A_{520-540}$$

Azodi-pyrrole	$\lambda_{max.}$ (nm)	R		
		Human	Monkey	Dog
αF	537, 340		1.15	
$\alpha 0$	530, 335		1.13	1.13
$\alpha 2$	530, 334		1.12	1.05
$\alpha 3$	530, 334		1.77	1.04
βx	520, 324	0.97	1.00	
$\gamma 3$	532, 333	1.34	1.37	1.28
δ	532, 333	1.04	1.13	1.04

ment $\alpha 0$. In all cases the spectra of the various azopigments prepared from the bilirubins separated from human and rhesus-monkey biles were identical with the same azopigments from the dog biles.

Ammonolysis of azopigments

The following purified unconjugated and conjugated azopigments from human, rhesus-monkey and dog biles were subjected to ammonolysis: αF , $\alpha 0$, $\alpha 1$, $\alpha 2$, $\alpha 3$, βx and δ . All azopigments except αF , $\alpha 0$ and βx yielded azodipyrrole amide and various amounts of azodipyrrole acid. This reaction has been shown previously (Heirwegh *et al.*, 1975; Gordon *et al.*, 1974) to cleave the propionate ester bond of conjugated azodipyrroles to give the corresponding amides. Where there are no ester linkages, no change in chromatographic properties was observed. Azopigments αF , $\alpha 0$ and βx did not change. R_F values of acids, amides and methyl esters of azodipyrroles αF , $\alpha 0$ and βx are given in Table 4.

Methanolysis of azopigments

The following purified unconjugated and con-

longer wavelengths (537 and 340nm) and those of azopigment βx were shifted to shorter wavelengths (520 and 324nm) with respect to the $\lambda_{max.}$ for azopig-

Fig. 3. Characteristic absorption spectra of azopigyrroles (in methanol) obtained from bilirubins separated from (a) dog bile and (b) rhesus-monkey bile

—, Azopigment αF ; ----, azopigment $\alpha 0$; ····, azopigment βx .

jutated azopigments from human, rhesus-monkey and dog biles were subjected to methanolysis by treatment with 0.013M-sodium methoxide in methanol: αF , $\alpha 0$, $\alpha 1$, $\alpha 2$, $\alpha 3$, βx and δ . All azopigments except αF , $\alpha 0$ and βx yielded azopigyrrole methyl esters. Azopigments αF , $\alpha 0$ and βx were therefore methylated with diazomethane. Azopigyrrole $\alpha 0$ reacted to give azopyrrole methyl ester (13, Scheme 2). Azopigment βx gave azopigyrrole dimethyl ester (18, Scheme 2). The R_F value of the dimethyl ester was 0.20-0.21 in solvent system V followed by solvent system IV. Azopigyrrole βx remained at the origin in these systems (Table 4). Azopigment αF

Fig. 4. Characteristic absorption spectra (a) of azopigyrroles βx (in methanol) obtained from dog and synthetic bilirubin-IX β , and their dimethyl esters, and (b) of natural azopigyrroles αF and meso αF

(a) —, Azopigment βx from dog bilirubin-IX β ; ····, azopigment βx from synthetic bilirubin-IX β and -IX δ ; ----, dimethyl ester of dog azopigment βx ; — —, dimethyl ester of synthetic azopigment βx . (b) —, azopigment αF from dog bilirubin-IX β ; ····, azopigment αF obtained from synthetic bilirubin-IX β ; ----, azopigment meso αF from dog bilirubin-IX β ; — —, azopigment meso αF from synthetic bilirubin-IX β .

(12) Azodipyrrole $\alpha 0$; $R^3 = \text{H}$

(13) Azodipyrrole $\alpha 0\text{M}$; $R^3 = \text{CH}_3$

(14) Azodipyrrole δ ; $R^3 =$

(15) Azodipyrrole $\alpha 2$; $R^3 =$

(16) Azodipyrrole $\alpha 3$; $R^3 =$

Scheme 2. Azodipyrroles derived from bilirubin-IX α and conjugated bilirubin-IX α

Each of the azodipyrroles 12–16 may be a mixture of isomers with respect to R^1 and R^2 .

did not change after methylation. The spectra of purified azopigments βx and βx dimethyl ester are shown in Fig. 4(a). Their λ_{max} and R values differed slightly but reproducibly. This was true for both azopigment βx and its dimethyl esters obtained from natural bilirubin-IX β and synthetic bilirubin-IX β . R_F values of azodipyrrole methyl and dimethyl esters are given in Table 4.

Alkaline hydrolysis of azopigments

Azopigments $\alpha 0$, $\alpha 2$, $\alpha 3$ and δ were hydrolysed with aq. 0.1M-NaOH. Both ester groups of these molecules were cleaved, and on neutralization

yielded carboxylic acids. Thus the final product was identical with the product of bilirubin-IX α diazotized with the diazonium salt of anthranilic acid. Similarly hydrolysis of azopigments αF and βx resulted in reaction at the ester attached to the benzene ring to yield a carboxylic acid. These compounds were chemically prepared by the reaction of bilirubin-IX β with the diazonium salt of anthranilic acid.

Mass spectra

Mass-spectral data for azopigment *meso* αF and the dimethyl ester of azopigment βx derived from

Table 4. R_F values of ethyl anthranilate azopigments obtained from unconjugated bilirubins-IX α and -IX β separated from dog bile and from synthetic bilirubin-IX isomers

The values given in parentheses are R_F values given by Heirwegh *et al.* (1975). T.l.c. separations were carried out as described in Table 1 and in the text.

Parent tetrapyrrole	Azopigment	R_F value in:			
		Solvent systems I+II		Solvent systems IV+V	
		Natural	Synthetic	Natural	Synthetic
Bilirubin-IX α	Azodipyrrole ($\alpha 0$)	0.74	0.74		
	Azodipyrrole methyl ester	0.98		0.51	0.51 (0.57, 0.63)
	Azodipyrrole amide	0.90	0.90		
Bilirubin-IX β	Azodipyrrole βx	0.40	0.46	0.00	
	Azodipyrrole dimethyl ester	0.98		0.20–0.21	0.20–0.21 (0.21)
	Azodipyrrole αF	0.96		0.83	0.83
	Azodipyrrole <i>meso</i> αF	0.96		0.74	0.74
Bilirubin-IX δ	Azodipyrrole βx	0.40		0.00	
	Azodipyrrole dimethyl ester	0.98		0.20–0.21	(0.21)
	Azodipyrrole αF	0.96		0.68	0.68
	Azodipyrrole <i>meso</i> αF	0.98		0.62	0.62

Table 5. R_F values of ethyl anthranilate azopigments obtained from conjugated bilirubins-IX α and other bilirubin-IX isomers separated from dog bile

Plates were developed with chloroform/methanol (17:3, v/v) (5.0 cm) followed by chloroform/methanol/water (65:25:3, by vol.) (16–17 cm). T.l.c. separations were carried out as described in Table 1 and in the text.

Parent tetrapyrrole	Azopigment	R_F value
Bilirubin-IX α	$\alpha 0$	0.80
	$\alpha 2$	0.74
	$\alpha 3$	0.66
	δ	0.20
Unknown (probably bilirubin-IX α)	$\alpha 1$	0.80
	$\gamma 1$	0.40
	$\gamma 2$	0.32
	$\gamma 3$	0.29
	$\gamma 4$	0.25

natural and synthetic bilirubins are shown in Table 6. The mass-spectral data clearly support the contention that azopigment *meso* αF from dog bile results from bilirubin-IX β . Mass-spectral data for βx dimethyl ester from dog bile and from bilirubin-IX β synthesized from haemin are presented in Table 6 and indicate that azopigment βx could result from bilirubin-IX β .

Finally, although not presented in Table 6, mass-spectral data were obtained for azopigment $\alpha 0M$ derived from azopigments $\alpha 0$ and δ prepared from human bilirubins, and these were found to be identical.

It should be noted that the mass spectra of all the

azopigment derivatives are dominated by fragments of lower mass. This result is a consequence of the instrumentation available to handle samples of approx. 1 μg .

*Properties of azopigment αF and azopigment *meso* αF from bilirubins-IX β and -IX δ*

R_F values of αF and *meso* αF azopigments obtained from dog bilirubin-IX β and chemically prepared bilirubin-IX β and -IX δ are given in Table 4. It is noteworthy that we were able to distinguish by t.l.c. azopigment αF obtained from bilirubin-IX β and azopigment αF obtained from bilirubin-IX δ . In fact, the material that we tentatively identified as αF was shown to be derived from bilirubin-IX β , as shown by the t.l.c. comparison in Table 4. Azopigment αF and azopigment *meso* αF were prepared on a larger scale by using the same methods used for small-scale experiments. The u.v.-visible spectra of αF and *meso* αF azopigments from natural bilirubin-IX β and synthetic bilirubins-IX β and -IX δ are given in Fig. 4(b).

The difference in colour between azopigment *meso* αF and azopigment αF was clearly discernible. Azopigment *meso* αF obtained from *meso*-bilirubin-IX β (natural and synthetic) was pink (λ_{max} 520 and 320 nm), whereas azopigment αF from bilirubin-IX β (natural and synthetic) and bilirubin-IX δ (synthetic) was bluish-purple (λ_{max} 537.5 and 340 nm).

Mass spectra of purified azopigment *meso* αF obtained from natural bilirubin-IX β and synthetic bilirubins-IX β and -IX δ are given in Table 6.

Analysis of the conjugating groups

R_F values for glucose, xylose and glucuronic acid

Table 6. Mass spectra of azopigment *meso* α F and of β x dimethyl ester prepared from dog bile and from commercial haemin
Values given in parentheses are relative intensities.

<i>meso</i> α F (dog)	<i>meso</i> α F (20, Scheme 3)	<i>meso</i> α F (22, Scheme 3)	β x-dimethyl ester (dog)	β x-dimethyl ester (synthetic)
420 (16)	420 (40)	420 (1)	536 (8)	536 (7)
405 (1)			463 (4)	463 (4)
391 (1)	391 (1)		313 (3)	313 (7)
375 (1)			299 (2)	299 (7)
374 (1)	374 (1)		285 (2)	285 (4)
347 (7)	347 (15)		236 (6)	236 (12)
256 (5)	256 (10)	256 (2)	165 (7)	165 (21)
165 (2)	165 (24)	165 (5)	119 (18)	119 (58)
120 (15)	120 (51)	120 (8)	97 (41)	97 (45)
119 (20)	119 (57)	119 (12)	83 (51)	83 (50)
43 (100)	43 (100)	43 (100)	69 (42)	69 (46)
			57 (67)	57 (66)
			43 (100)	43 (100)

Table 7. R_F values of commercially obtained sugars and sugars from human, rhesus-monkey and dog azopigments α 2, α 3 and δ in solvent system VII

T.l.c. separations were carried out as described in Table 1 and in the text.

Sugar	R_F value	
	Untreated	Methoxide-treated
Glucuronic acid	0.17	0.49
D-Glucose	0.42	0.43
D-Xylose	0.55	0.39–0.40
From azopigment α 2 sugars		0.40
From azopigment α 3 sugars		0.43
From azopigment δ sugars		0.49

and for these sugars treated with sodium methoxide are given in Table 7. There was a marked change in R_F values of sodium methoxide-treated glucuronic acid and xylose, but almost no change in the R_F value of treated glucose. Chromatographic separation of the sugars cleaved from azopigment α 2 prepared from human, rhesus-monkey and dog biles gave xylose. The sugar cleaved from azopigments α 3 prepared from human, rhesus-monkey and dog biles was glucose, and the sugar cleaved from azopigment δ prepared from human, rhesus-monkey and dog bile was glucuronic acid.

Discussion

The present results substantiate the conclusion that most of the bilirubins present in the biles of humans, rhesus monkeys and dogs are conjugates of bilirubin-IX α . This agrees with results of previous workers (Gordon *et al.*, 1976; Heirwegh *et al.*, 1975; Noir, 1976). However, we found that band-6 bilirubin in dog biles and band-4 bilirubins in human and

rhesus-monkey biles were bilirubin-IX β . We have not found any evidence for the presence of bilirubins-IX γ and -IX δ in any of the biles analysed. From our studies of synthetic bilirubin-IX β and -IX δ , we have established that there is a definite difference in R_F values between azopigments α F 19 and 21 (Scheme 3) and between *meso* α F azopigments 20 and 22 derived from bilirubin-IX β and -IX δ respectively. The R_F values of azopigments β x and dimethyl β x of both bilirubin-IX β and -IX δ were identical. We are certain that the azopigment α F obtained from human, rhesus-monkey and dog bilirubins is derived from bilirubin-IX β . Both synthetic and natural bilirubin-IX β yielded azopigments α F and β x in equal proportions. The colour of azopigment α F is violet, that of azopigment *meso* α F is pink. This change in colour was associated with a shift of the $\lambda_{\max}$ of azopigment *meso* α F from 537 to 518 nm and from 340 to 320 nm (Figs. 3a and 3b). These spectra are identical with those reported by Blanckaert *et al.* (1976a) for azopigments derived from synthetic bilirubins. There also was a change in the relative absorbance of the peaks: $A_{537.5}/A_{340}$ of azopigment α F was 0.91, and A_{518}/A_{320} of azopigment *meso* α F was 0.71. Azodipyrrole β x from bile and from synthetic bilirubin-IX β or -IX δ showed the same characteristics. Methylation with diazomethane yielded the same compound, a dimethyl ester of the azodipyrrole (18, Scheme 3). A final proof of their identity was the mass-spectral fragmentation pattern. Those of azopigment β x dimethyl ester derived from dog bile and of this ester from synthetic bilirubin-IX β were identical (Table 6). Azopigment *meso* α F derived from dog bile was found to be identical with azopigment *meso* α F derived from synthetic bilirubin-IX β , but unlike azopigment α F derived from bilirubin-IX δ . One characteristic difference between *meso* α F azopigments derived from bilirubin-IX β and

(17) Azodipyrrole βx	$R^1, R^4 = \text{CH}_3$	$R^2, R^3 = \text{CH}_2\text{CH}_2\text{CO}_2\text{H}$
(18) Azodipyrrole βx , dimethyl ester	$R^1, R^4 = \text{CH}_3$	$R^2, R^3 = \text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_3$
(19) Azodipyrrole αF	$R^1, R^3 = \text{CH}=\text{CH}_2$	$R^2, R^4 = \text{CH}_3$
(20) Azodipyrrole <i>meso</i> αF	$R^1, R^3 = \text{CH}_2=\text{CH}_3$	$R^2, R^4 = \text{CH}_3$
(21) Azodipyrrole αF isomer	$R^1, R^3 = \text{CH}_3$	$R^2, R^4 = \text{CH}=\text{CH}_2$
(22) Azodipyrrole <i>meso</i> αF isomer	$R^1, R^3 = \text{CH}_3$	$R^2, R^4 = \text{CH}_2\text{CH}_3$

Scheme 3. Azodipyrroles derived from bilirubin-IX β and bilirubin-IX δ

-IX δ consists in the differing intensity of the fragment of m/e 256, as previously observed (Compennolle *et al.*, 1976).

Azopigments $\alpha 0$, $\alpha 2$, $\alpha 3$ and δ were previously isolated from dog bile and identified as the following azodipyrroles: azodipyrrole (Compennolle *et al.*, 1970, 1971; Jansen & Stoll, 1971), azodipyrrole β -D-xylopyranoside, azodipyrrole β -D-glucopyranoside (Compennolle *et al.*, 1971; Fevery *et al.*, 1971) and azodipyrrole β -D-glucopyranuronoside (Heirwegh *et al.*, 1970; Compennolle *et al.*, 1970). The corresponding azopigments obtained from human and rhesus-monkey bilirubins proved to be identical in all respects, with those obtained from dog bile, i.e. the ring and side-chain structure, including the ester linkage to sugars.

The bilirubins from which these azopigments were derived may be mono- or di-conjugates of bilirubin-IX α , where the two carbohydrates may be identical or different. Dog biles contained various conjugates of bilirubin-IX α , which gave rise to one or two different azopigments (see Table 2). Primate biles contained only some of the bilirubin-IX α conjugates. Usually they lacked those bilirubins that have two different sugars attached to the same bilirubin, i.e. bilirubin-IX α (glucuronic acid, glucose) (8, Scheme 1); -IX α (glucuronic acid, xylose) (9, Scheme 1); and -IX α (glucose, xylose) (10, Scheme 1).

The presence of two different sugar molecules attached to the propionic side chains of the same bilirubin-IX α molecule (glucose and glucuronic acid, glucose and xylose, or xylose and glucuronic acid) may be explained by the suggestion that two transferases are involved in the esterification of bilirubin-IX α , and one sugar molecule is attached at a time (Jansen

et al., 1976). If this mechanism resembles that reported for diglucuronides (Chowdury *et al.*, 1976), then one molecule of glucuronic acid is attached to bilirubin-IX α in the endoplasmic reticulum by microsomal glucuronyltransferase. Subsequently a second molecule of sugar (glucuronic acid, glucose or xylose) is attached by a transferase (Vaisman *et al.*, 1976) present in the canalicular plasma membrane of the liver cell. The presence of one molecule of glucuronic acid, glucose or xylose may be explained by esterification of bilirubin-IX α that has not been esterified by the microsomal glucuronyltransferase. The presence of bilirubin-IX α diglucoside, dixyloside and/or glucoside/xyloside in the dog biles may be due to esterification of a bilirubin molecule by two enzyme molecules at the canalicular plasma membrane of the liver cell. This hypothesis is supported to some extent by work on the hepatic storage and intracellular binding of conjugated bilirubin (Wolkoff *et al.*, 1976). Mixed diconjugates of bilirubin, i.e. bilirubin diesters of xylose and glucose (10, Scheme 1), xylose and glucuronic acid (9, Scheme 1) and glucose and glucuronic acid (8, Scheme 1), have been reported for dog bile by Heirwegh *et al.* (1975) (components 13, 18 and 19). We did not obtain any of these bilirubins from human or rhesus-monkey biles, but did confirm their presence in dog biles. We also did not achieve full separation of some of the bilirubin bands. This is clearly shown in Fig. 2, where band 3 is a mixture of azopigments derived from two bilirubins, bilirubin monoxylsides and bilirubin monoglucuronides, and band 6+7 (Fig. 2) is a mixture of bilirubin-IX β and bilirubin-IX α glucoside/xyloside. These results are due to the tailing of the various bilirubins present in the extract.

The azopigments produced from the various bilirubins make it possible to determine which bilirubins were present in the various yellow bands on the t.l.c. plates prepared from bile extractions. Every molecule of bilirubin is converted into two molecules of azopigment. These azopigments were readily separated on t.l.c. plates. The bilirubins were identified from the azopigments obtained (Heirwegh *et al.*, 1975).

A number of unknown azopigments have been reported in human, rhesus-monkey and dog biles. These azopigments are considered to be conjugated, but their chemical composition is not yet established (Heirwegh *et al.*, 1970). Of those unknown azopigments, $\alpha 1$, $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ were selected. They were purified from the biles of those humans, rhesus monkeys and dogs that contained relatively large amounts of the bilirubins from which these azopigments are obtained. The u.v.-visible spectra of the five unidentified azopigments were recorded ($\lambda_{\max}$ values are given in Table 8). Only azopigment $\alpha 1$ had $\lambda_{\max}$ at different wavelengths than the other α - and γ -type azopigments. All the unidentified azopigments showed a $A_{333-343}/A_{524-535}$ of 1.2–1.6, larger than those of azopigments αF , $\alpha 0$, $\alpha 2$, $\alpha 3$ and δ (Tables 3 and 8). Treatment of azopigments $\alpha 1$, $\alpha 2$, $\alpha 3$, $\gamma 1$, $\gamma 2$, $\gamma 3$, $\gamma 4$ and δ obtained from bile pigments of human, rhesus-monkey and dog biles with sodium methoxide produced azodipyrrole methyl ester (13, Scheme 2), indicating that the conjugated groups were linked by an ester bond to the terminal carbon atom of the propionic acid on ring B and/or C of the bilirubin. The five unidentified azopigments were conjugates and their conjugating moiety was linked by an ester bond to the dipyrroles obtained from the parent bilirubins. The tetrapyrrole of all five azopigments was bilirubin-IX α . The conjugating moieties have not yet been identified.

The t.l.c. patterns of azodipyrroles obtained from biles diazotized before chromatographic separation were identical with those diazotized after separation. Comparison of a sample produced by direct diazo-

tization of bile and a sample produced by mixing azopigments obtained from the separated bilirubins showed the same t.l.c. patterns. Our previous results with t.l.c. obtained from biles diazotized before separation (Blumenthal *et al.*, 1976a) are consistent with these results. The chloroform extracts of human, rhesus-monkey and dog biles contained about one-third of the total bilirubins present in the biles. Half of the bilirubin glucuronide in the chloroform extract was in the form of diglucuronide, the other half in monoglucuronide form. The relative proportions of the various bilirubins in the biles of adult individuals differed in different individuals of the same species and sometimes in samples taken at different times from the same individual. Some of the minor bilirubin types were present in some individuals or absent from others. Therefore it is difficult to make a clear-cut distinction between normal and abnormal bile samples, although it certainly is true that biliary obstruction and other hepatic diseases will enhance the formation of bilirubin-IX β (yielding azopigments αF and βx) and bilirubins yielding the γ s and other β azopigments (Fevery *et al.*, 1972).

Our results have established that azopigments αF and βx found *in vivo* are products of bilirubin-IX β and not of bilirubin-IX δ ; this is consistent with experiments showing that cleavage of haemoglobin haem by a non-enzymic reaction forms only bilirubins-IX α and -IX β (O'Carra & Colleran, 1969). We have also confirmed and extended previous studies on the principal conjugates of bilirubin-IX α in human adult bile and have made the first systematic comparison of human and rhesus-monkey bile with respect to these components.

Note Added in Proof (Received 30 September 1977)

The structures of azopigments $\gamma 2$, $\gamma 3$, $\gamma 4$, and δ have recently been established by F. Compennolle, N. Blanckaert & K. P. M. Heirwegh (personal communication).

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Table 8. *Properties of the unidentified azodipyrroles prepared from bilirubins separated from the bile of a patient suffering from biliary obstruction*

U.v.-visible spectra in methanol were recorded with a Cary 17 spectrophotometer. $R = A_{335-347}/A_{524-533}$.

Azodipyrrole	$\lambda_{\max}$ (nm)	R
$\alpha 1$	524, 343	1.23
$\gamma 1$	532, 333	1.62
$\gamma 2$	533, 334	1.18
$\gamma 3$	530, 333	1.33
$\gamma 4$	530, 335	1.22

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