Bilirubin Conjugates in Bile of Man, Rat and Dog

SEMI-QUANTITATIVE ANALYSIS OF BILE COMPOSITION BY THIN-LAYER CHROMATOGRAPHY

By BEATRIZ A. NOIR

*Department of Gastroenterology, Policlínico Professor Dr. A. Posadas,
Haedo, Argentina*

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1. Conjugated bile pigments, separated in two fractions by semi-quantitative t.l.c. performed on silicic acid with phenol/water as the developing solvent, were treated with diazotized ethyl anthranilate. Resulting dipyrrolyazo derivatives were analysed by quantitative t.l.c. 2. The tentative structure elucidation of tetrapyrrolic bilirubin conjugates and semi-quantitative evaluation of rat bile, post-obstructive human bile and dog bile composition is presented. 3. Homogeneous and mixed hexuronic acid diesters of bilirubin containing glucuronic acid constitute 51 % of the total conjugates in normal rat bile, 45 % of those in human post-obstructive bile and 38 % of those in obstructed rat biles. 4. Monoconjugated bilirubin amounts to 33 % of total conjugated bile pigments in normal rat bile, and 17 and 14 % in post-obstructive hepatic human bile and gall-bladder bile of dog respectively. After loading with unconjugated bilirubin a greater amount of monoconjugates (56%) occur in the rat bile, whereas bilirubin diglucuronide excretion is decreased (34%). 5. In gall-bladder bile of normal dog, 40 % of glucose-containing diconjugates, 32 % of homogeneous and/or mixed hexuronic acid (mainly glucuronic acid) diesters of bilirubin and 14 % of xylose-containing diconjugates are estimated. 6. Increased amounts of bilirubin conjugates, including some with unidentified uronic acid groups, were observed in cholestatic rat biles and quantities of conjugates with glucuronic acid were decreased.

A major fraction of the bilirubin excreted in human and rat bile is a β -D-diglucuronoside; however, the postulated existence (Billing *et al.*, 1957) of a mono-glucuronoside has been confirmed (Ostrow & Murphy, 1970; Jansen & Billing, 1971). In addition, variable amounts of pigment appear to be conjugated with other sugar moieties (Kuenzle, 1970; Comper-nolle *et al.*, 1971) and evidence for sulphate conjugation has also been obtained (Noir *et al.*, 1970; Noir & Nanet, 1974).

The coupling reaction between diazonium salts and bilirubin (conjugated and unconjugated) results in the formation of two molecules of dipyrrolic azo-pigments for each molecule of bile pigment. The coupling of monoconjugates causes cleavage at the central methylene bridge, with formation of equi-molecular quantities of unconjugated azodipyrroles and azodipyrroles still having one conjugating group of the original tetrapyrrole. The first of these fractions will be a mixture of isomers formed in consequence of the unsymmetrical order of β side chains in bilirubin IX α . The fraction of conjugated azodipyrroles will be complex for the same reason and because of variation of conjugating groups (Scheme 1). The re-

action with bilirubin diconjugates produces two azodipyrroles with the original conjugating group(s) intact. A ratio R (unconjugated azodipyrrole/mono-conjugated azodipyrrole) of 1 will then be obtained from monoconjugated bilirubin, and a ratio of 0 for diconjugated pigments.

Qualitative and quantitative analyses of these stable reddish-violet azo derivatives have been useful for elucidation of the structure of conjugated bile pigments present in biological fluids (Billing *et al.*, 1957; Talafant, 1956; Schmid, 1957; Heirwegh *et al.*, 1973). More recent work indicates the presence of a great variety of tetrapyrrole conjugates in bile. The most definitive method for the structural study of these would seem to be initial isolation of the individual pigments followed by analysis of their azo derivatives.

A simple and rapid t.l.c. method for the separation of unconjugated bilirubin, bilirubin monoconjugates and diconjugates on silicic acid in a phenol/water solvent system has been described by Thompson & Hofmann (1971). In the present study we used this procedure to separate conjugated bilirubin of normal rat and dog biles into two fractions. These were then

Scheme 1. Diazo coupling of mono- and di-conjugates of bilirubin

Me, $-\text{CH}_3$; V, $-\text{CH}=\text{CH}_2$; P, $-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}$; P*, $-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{X}$, where X is a conjugating group: (I) and (II), monoconjugated bilirubin; (III), unconjugated bilirubin; (IV), unconjugated azodipyrroles [vinyl (a) and isovinyl (b) isomers]; (V), conjugated azodipyrroles [vinyl (a) and isovinyl (b) isomers]. For further explanation see the text.

further analysed by separation of the derived ethyl anthranilate azopigments. Chromatographic evidence is presented to confirm that the 'diconjugated bilirubin fraction' contains variable amounts of highly polar bilirubin diconjugates containing only uronic acids as conjugating groups. By contrast, the less-polar component, 'monoconjugated bilirubin fraction', is a mixture of bilirubin monoconjugates and mixed diconjugates of xylose or glucose and uronic acids. Analyses of post-obstructive human and rat biles and studies of the changes occurring after administration of a loading dose of unconjugated bilirubin to normal rats are also presented.

Materials and Methods

All chemicals used were of analytical grade. Individual samples of human fistula bile were obtained 2–9 days postoperatively from a T-tube placed in the common bile duct of 15 cholecystectomized patients at operation. The examination of the biles was performed with the patients' informed consent. Gall-bladder bile was obtained from nine mongrel dogs. The bilirubin contents of all these biles were determined by the method of Malloy & Evelyn (1937).

Wistar rats weighing 250–350 g were lightly anaesthetized with ether and the common duct was cannulated with polyethylene tubing approx. 2 cm distal to the hepatic-duct bifurcation to avoid obstructing pancreatic drainage. The prepared animals were placed in restraining cages at room temperature (25–28°C), and bile was collected in ice-refrigerated tubes screened from light. All the studies were performed on individual bile samples collected 2 h after finishing the cannulation. This delay allowed the animal to attain normal body temperature (Fevery *et al.*, 1972c). Water was allowed *ad libitum*. Three groups of rats were studied as follows. (1) Bile of 20 animals was collected during the subsequent 2–5 h period. (2) Bile of eight rats was collected during the first 1 h period after administration, via the femoral vein, of 2 mg of bilirubin (BDH Ltd., Poole, Dorset, U.K.) per 100 g body wt. For injection the bilirubin was dissolved in 0.5 ml of 0.1 M-NaOH, 1 ml of 0.2 M-sodium phosphate buffer, pH 7.4 (Sørensen, 1909) and 0.5 ml of normal rat serum. (3) To induce cholestasis in a third group of six animals the bile tube was sealed by heating, secured subcutaneously and the animal allowed to resume normal activities. Then 48 h after obstruction the animals were again anaesthetized and the bile tube exteriorized. After the abdomen had been sutured, the rat was returned to the restraining cage and, after 2 h, the bile tube was opened. Three consecutive 1 h samples of bile were then collected.

Biles were analysed immediately after collection or, occasionally, after storage at –15°C for a maximum of 48 h.

Separation of bile pigments by t.l.c., and preparation of azopigments

Bile samples were applied to thin-layer plates (Noir & Nanet, 1974) (silica gel H; E. Merck, Darmstadt, Germany) in 15 cm-long bands and dried in a stream of cold air. The plates were developed (2 h) in the dark with phenol/water (41:9, v/v) as solvent system (Thompson & Hofmann, 1971). Two yellow regions of the chromatograms containing (a) the less rapidly moving bilirubin conjugates (more-polar fraction), and (b) the less-polar conjugates, were immediately scraped off, transferred to centrifuge tubes and washed free of phenol by twice shaking vigorously with diethyl ether. Washings were separated by short centrifugation at 1000g_{av.}. More-polar and less-polar bile pigments absorbed in the sedimented silica-gel particles were then extracted into 5–10 ml quantities of water or aq. 30% (v/v) ethanol, and treated immediately with diazotized ethyl anthranilate as described by Heirwegh *et al.* (1970). Alternatively, the eluted bile pigments were treated with diazotized sulphanilic acid (Noir *et al.*, 1970). After 20 min the coupling reaction was terminated by adding ascorbic acid. Quantitative extraction of ethyl anthranilate and sulphanilic acid azopigments was performed by repeated washing with pentan-2-one/*n*-butyl acetate (17:3, v/v) or butan-1-ol after addition of (NH₄)₂SO₄. The complete procedure, including extraction, washing and coupling of bile pigments, was completed within 5–8 min in dim light at room temperature.

On the assumption that the molar extinction coefficients of unconjugated azodipyrrole and conjugated azopigments are equal, the E_{530} of the azopigment-containing pentanone layers or the E_{545} of the azopigment-containing butanol layers was determined by using a Beckman DU spectrophotometer. Relative amounts of both conjugated fractions were thus determined. Repeated t.l.c. of the same bile followed by coupling with both ethyl anthranilate or sulphanilic acid diazo reagents, and quantitative estimation of the two conjugated fractions, showed reproducibility to be within ±6% error.

Quantitative analysis by t.l.c. of azopigments derived from conjugated bile pigments

The ethyl anthranilate azopigment mixtures from both conjugated fractions were subjected to t.l.c. on silica gel H. The plates were first subjected to a run at room temperature in the dark with chloroform/methanol/water (65:25:3, by vol.) followed, after drying the plate, by development in the same direction with chloroform/methanol (17:3, v/v). Red-violet dipyrrole azopigments were distinguished according to the notation of Heirwegh *et al.* (1970). Preparations of total azopigment obtained from dog bile and post-obstructive human bile were applied as markers. Structurally characterized preparations

(Compennolle *et al.*, 1970, 1971; Heirwegh *et al.*, 1970; Jansen & Stoll, 1971; Fevery *et al.*, 1971, 1972b) of unconjugated azodipyrrole (α -azopigment), azodipyrrole β -D-glucopyranuronic acid (δ -azopigment from rat bile or from enzyme incubation mixtures), azodipyrrole β -D-glucopyranose (α -azopigment from dog bile) and azodipyrrole β -D-xylopyranose (α -azopigment from dog bile) were also used. Synthetic bilirubin disulphate, prepared and purified as described by Noir *et al.* (1970), was converted into the azo derivative and also used as reference material for identification of alkali-stable azopigments after treatment of the chromatograms with ammonia (Noir & Nanet, 1974).

For quantitative determination of the separated azopigments, the coloured bands were scraped off the plates and transferred to 10 ml closed centrifuge tubes. The silica-gel powders with the adsorbed azopigment(s) were shaken vigorously with 1 ml of 60 mM-HCl in 50% (v/v) methanol, 1 ml of pentan-2-one/*n*-butyl acetate (17:3, v/v) and 1.5 ml of water. After centrifuging for 5 min at 1000 g_{av} , the E_{530} of the upper layer was measured in micro-cuvettes (light-path 1 mm) with pentanone solvent in the reference cuvette.

For the study of monoconjugated bilirubin, dilute bile samples were coupled with diazotized ethyl anthranilate, as described by Heirwegh *et al.* (1970). The azopigments were then extracted, chromatographed and quantified as described above.

Mean values are given with standard deviations calculated for small populations. Significance of differences between means was calculated by Student's *t* test. A *P* value of less than 0.05 was considered significant.

Results and Discussion

Comments on the separation procedure for bile pigments and assignment of structure to the more-polar and less-polar bilirubin conjugates

In the present study, the method of Thompson & Hofmann (1971) for direct separation of unconjugated bilirubin, bilirubin monoconjugates and diconjugates was applied for the investigation of the composition of different bile samples. This simple and rapid procedure avoids unsatisfactory extraction of bile pigments from bile, is reproducible and is applicable to small quantities of material. Identification and determination of the dipyrrolic derivatives obtained by diazo coupling provided much interesting information. T.l.c. of bile pigments leads to the isolation of two predominant yellow bands which have been described as 'diconjugated bilirubin' (R_F 0.24) and 'monoconjugated bilirubin' (R_F 0.38). On the basis of their relative chromatographic mobilities we prefer to call these respectively 'more-polar' and 'less-polar' conjugated bilirubin fractions. Relative

amounts of both fractions were determined spectrophotometrically after elution and diazo coupling. Trace amounts of free bilirubin or biliverdin occasionally observed were not extracted. The quantitative differences found in the bile specimens examined (Tables 1 and 2) accorded with the visual inspection of the chromatograms.

Comparing the values obtained for normal rats, the percentage of the less-polar bilirubin conjugates increases about 1.5-fold after loading the animals with unconjugated bilirubin. By contrast, a significant decrease is observed immediately after releasing a long-term (48 h) obstruction, and no essential differences occur 2–3 h after bile collection is re-started. Return to normal percentages of both conjugated bile-pigment fractions usually requires more than 10 h. More-polar bilirubin conjugates predominate in post-obstructive human biles, whereas the opposite situation obtains in gall-bladder bile of dog.

Coupling of the more-hydrophilic pigments from rat bile, either before or after intravenous administration of bilirubin, and from gall-bladder bile of dog yields one major fraction, azodipyrrole β -D-glucopyranuronic acid (Heirwegh *et al.*, 1970; Compennolle *et al.*, 1970), which accounts for about 85% (range 79–96%) of the total azopigments present (Table 1). The remaining fraction is shown to be β - + γ -azopigments. Diazotized preparations of the same fraction from both post-obstructive human and rat biles afford greater percentages of the components of the β - + γ group (range 20–53% of total azo colour) with concomitant decreases of the δ -azopigment. The changes observed in obstructed rats are significant as compared with control analyses carried out before induction of cholestasis. Azopigments of the α group are systematically absent from preparations of bilirubin conjugates of high polarity, or do not exceed 5% of total azo colour.

The fact that the so-called 'diconjugated bilirubin' yields almost exclusively hexuronic acid-containing conjugated azodipyrroles (up to 99% of the total azopigment derived from the more-polar fraction) can be interpreted as indicating that t.l.c. separation by using a phenol/water solvent system is valuable for isolation of highly polar bile pigments in a reasonably pure state. It therefore seems possible to calculate that the more-polar diconjugates of bilirubin in rat bile, both before or after loading with unconjugated bilirubin, and in gall-bladder bile of dog consist of about 85% of homogeneous (δ - δ) and/or mixed (δ - β , δ - γ) diesters of bilirubin containing glucuronic acid, and 10–15% of other diconjugates containing unidentified uronic acid residues (β - β , γ - γ , β - γ). The terminology for the bile pigments is that used by Heirwegh *et al.* (1975). Mechanical bile-duct obstruction (48 h) in rats causes a significant increase in the diesters of bilirubin containing unidentified uronic acid groups, with simultaneous decrease in the

Table 1. Determination of the ethyl anthranilate azopigments derived from the more-polar bilirubin conjugates

Individual bile samples (0.2–0.9 ml) containing 0.1–0.5 μ mol of total bilirubin were subjected to t.l.c. as described by Thompson & Hofmann (1971). Conjugated bile pigments separated in two fractions were eluted and treated with diazotized ethyl anthranilate. The relative proportion of more-polar conjugates and of the dipyrrolyazo derivatives obtained from the more-polar fraction of each bile is determined as described in the text. The Greek-letter notation refers to the different azopigment groups isolated by t.l.c. from ethyl anthranilate azopigments derived from natural bile pigments (Heirweh *et al.*, 1970). Azopigment α_0 is identical with the ethyl anthranilate azobilirubin prepared from bilirubin; α_2 - and α_3 -azopigments were shown to correspond to azobilirubin β -D-monoxypyranoside and azobilirubin β -D-monoglucopyranoside respectively. β - and γ -azopigments are uronic acid-containing acyl glycosides of ethyl anthranilate azobilirubin. δ -azopigment has been shown to be the β -D-monoglucuronide of ethyl anthranilate azobilirubin. Numbers in parentheses indicate the number of patients or animals studied in each group. Mean values $\pm$ S.D. for separate analysis of each bile specimen are given. * $P < 0.0005$ as compared with normal-rat-bile data. $R' = \%(\beta + \gamma + \delta)$.

Source	More-polar conjugates (% of total conjugates eluted from the plate)	Ethyl anthranilate azo derivatives (% of total azopigment colour eluted from the plate)					R'
		α_0	α_2	α_3	$(\beta + \gamma)$	δ	
Human bile, T-tube drainage (15)	67.2 $\pm$ 5.1	1.7 $\pm$ 0.8	0.9 $\pm$ 0.5	1.1 $\pm$ 0.7	32.4 $\pm$ 7.9	63.9 $\pm$ 10.7	96.3 $\pm$ 1.3
Gall-bladder bile of dog (9)	34.3 $\pm$ 4.2	1.0 $\pm$ 0.6	absent	0.5 $\pm$ 0.3	15.3 $\pm$ 2.8	83.2 $\pm$ 3.0	98.5 $\pm$ 1.9
Normal rat bile (20)	58.2 $\pm$ 1.9	1.9 $\pm$ 0.6	0.9 $\pm$ 0.4	1.6 $\pm$ 0.8	10.6 $\pm$ 3.6	85.0 $\pm$ 4.8	95.6 $\pm$ 1.8
Rat bile after intravenous bilirubin load (8)	39.3 $\pm$ 5.9*	1.7 $\pm$ 0.4	absent	1.1 $\pm$ 0.8	10.8 $\pm$ 3.9	86.4 $\pm$ 3.6	97.2 $\pm$ 0.6
Rat bile collected after releasing a 48 h obstruction (6)							
First hour	74.3 $\pm$ 0.9*	1.6 $\pm$ 0.3	1.2 $\pm$ 0.3	1.8 $\pm$ 0.6	41.5 $\pm$ 6.7*	53.9 $\pm$ 6.1*	95.4 $\pm$ 0.8
Second hour	71.3 $\pm$ 2.6*	1.5 $\pm$ 0.2	0.9 $\pm$ 0.2	1.1 $\pm$ 0.1	47.6 $\pm$ 3.7*	48.9 $\pm$ 3.4*	96.5 $\pm$ 0.6
Third hour	70.5 $\pm$ 3.1*	1.5 $\pm$ 0.1	0.5 $\pm$ 0.2	0.8 $\pm$ 0.6	40.6 $\pm$ 6.9*	56.6 $\pm$ 7.5*	97.2 $\pm$ 0.8

glucuronosides. Post-obstructive human bile also yields large amounts of those not yet fully identified saccharide diconjugates.

Less-polar bilirubin conjugates show characteristic patterns of azopigments for each of the species examined (Table 2). In normal rat bile, azopigment α_0 , and δ , in approximately equal proportions, amount to about 80% of total azo colour, the remaining colour being distributed among azopigments α_2 , α_3 , β and γ . Bile samples obtained after loading with bilirubin show marked increased α_0 - and decreased α_2 - and α_3 -azopigments, as compared with normal values. No essential differences appear in the $(\beta + \gamma)$ - or δ -azopigment fractions. Therefore the ratio R (unconjugated azodipyrrole/conjugated azodipyrroles) increases significantly in the bile of the overloaded rats, whereas the ratio R' [$\% \alpha$ -group/ $\%(\beta + \gamma + \delta)$] is not changed. By contrast, obstruction causes marked increases in the $(\beta + \gamma)$ -azopigment fraction with concomitant decreases of the δ fraction, but no changes in the composition of the α group. The values of R and R' do not change after cholestasis.

The less-hydrophilic conjugates from post-obstructive human bile yield predominant amounts of δ -azopigment. Other azopigments are also observed in proportions which decrease in the following order: α_0 , $\beta + \gamma$, α_3 and α_2 . Preparations from dog bile yield two major fractions in approximately equal amounts, δ and α_3 , which together account for 70% of the total azopigment colour. The remaining 30% consists of smaller although still considerable amounts of the other azopigments. Comparing the values obtained for normal rat bile, the ratio R decreases in human and dog less-polar bile pigments to values of 0.33 and 0.1 respectively.

Small amounts of the alkali-stable ethyl anthranilate azo derivative of bilirubin sulphate (Noir & Nanet, 1974) are detected in δ -azopigment preparations obtained from both fractions of conjugated bilirubin isolated from all the bile specimens examined.

The ratio of unconjugated azodipyrrole/azodipyrrole monoconjugate (R) found in each of the preparations examined from the less-polar conjugated bilirubin is never exactly 1:1 as would be expected if the fraction was composed only of monoconjugates; the conjugated azodipyrrole group is always more abundant. T.l.c. in silicic acid separates bilirubin conjugates completely from unconjugated pigment. All the α_0 -azopigment formed from the less-polar fraction must therefore be derived exclusively from monoconjugated pigment. The percentage of total monoconjugates in the mixtures may therefore be calculated by multiplying the percentage of unconjugated azodipyrrole by two. Our values thus demonstrate that monoconjugated bilirubin accounts for 75–82% of total less-polar bile pigments from rat bile, both before and after cholestasis, and 92% after loading with unconjugated bilirubin. The less-

Table 2. Determination of the ethyl anthranilate azopigments derived from the less-polar bilirubin conjugates

Experimental conditions were as outlined in the legend to Table 1. The relative proportion of less-polar conjugates, and of the azo derivatives prepared from the less-polar fraction of each bile is given. Values are means $\pm$ S.D. * $P < 0.0005$ as compared with normal rat bile data. $R = \% \alpha_0 / 100 - \% \alpha_0$. $R' = [\% \alpha\text{-group}] / \% (\beta + \gamma + \delta)$.

Source	Less-polar conjugates (% of total conjugates eluted from the plate)	Ethyl anthranilate azo derivatives (% of total azopigment colour eluted from the plate)						R	R'
		α_0	α_2	α_3	$(\beta + \gamma)$	δ			
Human bile, T-tube drainage (15)	32.8 $\pm$ 5.1	26.2 $\pm$ 3.2	7.9 $\pm$ 2.0	12.8 $\pm$ 1.6	21.5 $\pm$ 5.8	31.6 $\pm$ 6.6		0.33 $\pm$ 0.05	0.90 $\pm$ 0.07
Gall-bladder bile of dog (9)	65.7 $\pm$ 4.2	11.1 $\pm$ 2.6	10.9 $\pm$ 3.3	30.1 $\pm$ 1.7	10.1 $\pm$ 2.8	37.8 $\pm$ 4.0		0.10 $\pm$ 0.04	1.1 $\pm$ 0.07
Normal rat bile (20)	41.8 $\pm$ 1.9	39.3 $\pm$ 3.0	4.9 $\pm$ 1.8	6.8 $\pm$ 1.2	9.5 $\pm$ 4.8	39.5 $\pm$ 5.9		0.62 $\pm$ 0.09	1.0 $\pm$ 0.07
Rat bile after intravenous bilirubin load (8)	60.7 $\pm$ 5.9*	46.0 $\pm$ 2.7*	2.0 $\pm$ 0.5*	3.7 $\pm$ 1.4*	9.1 $\pm$ 4.0	39.2 $\pm$ 4.4		0.90 $\pm$ 0.03*	1.07 $\pm$ 0.06
Rat bile collected after releasing a 48 h obstruction (6)									
First hour	25.7 $\pm$ 0.9*	37.5 $\pm$ 2.6	3.5 $\pm$ 0.5	6.6 $\pm$ 0.9	22.7 $\pm$ 2.1*	29.7 $\pm$ 1.6*		0.60 $\pm$ 0.05	1.0 $\pm$ 0.1
Second hour	28.7 $\pm$ 2.6*	39.0 $\pm$ 0.5	2.4 $\pm$ 0.6	6.3 $\pm$ 1.2	27.4 $\pm$ 0.8*	24.9 $\pm$ 2.2*		0.64 $\pm$ 0.02	0.96 $\pm$ 0.08
Third hour	29.5 $\pm$ 3.1*	40.9 $\pm$ 3.7	2.9 $\pm$ 0.6	6.6 $\pm$ 2.0	21.7 $\pm$ 1.5*	27.9 $\pm$ 2.2*		0.70 $\pm$ 0.1	1.0 $\pm$ 0.1

polar conjugated bilirubins in human bile and in dog gall-bladder bile were shown to contain respectively not more than 50 and 22% of monoconjugates.

Clearly the less-polar fraction of bile pigment conjugates contains compounds other than monoconjugates, and analysis of azopigment fractions may be used to characterize these.

Ethyl anthranilate-conjugated azodipyrroles are not artifacts of the dipyrrole moieties from conjugated bile pigments (Heirwegh *et al.*, 1970; Fevery *et al.*, 1971), thus their structures must reflect pre-existing structures in the parent bile pigments. The chromatographic mobilities of the ethyl anthranilate azo derivatives separated by t.l.c. in the sequence of solvents outlined above, indicate that the water solubility of the conjugated azodipyrroles decreases in the following order: δ -, γ -, β -, α_3 -, and α_2 -azopigments. Unconjugated azodipyrrole, the least polar azopigment, runs closely with α_3 -azopigment and specially so with α_2 -azopigment. This suggests a proportionately low polarity for all the tetrapyrroles from which they are derived.

Ester monoconjugates of bilirubin (α_0 - α_2 , α_0 - α_3 , α_0 - β , α_0 - γ , α_0 - δ) and diconjugates containing carbohydrates (α_2 - α_2 , α_2 - α_3 , α_3 - α_3) or mixed carbohydrate/uronic acid conjugates (α_2 - β , α_2 - γ , α_2 - δ , α_3 - β , α_3 - γ , α_3 - δ) would exhibit closely similar chromatographic behaviour in the phenol/water system. Conversely high polarity of δ -, γ - and β -azopigments indicates that bilirubin conjugated uniquely with hexuronic acids would be well separated from the less-polar fraction. Accordingly azo derivatives from 'diconjugated bilirubin' were found to contain no α -group pigments.

The presence, in the low-polarity chromatographic band, of monoconjugates and xylose or glucose diconjugates is apparent from the ratio (R) of α -azodipyrroles to hexuronic acid azodipyrroles. This ratio is always unity, irrespective of the source of the bile. Compatible with this is the finding that the less-polar conjugates in human, dog and rat biles contained respectively 16%, 22% and less than 10% of a mixture of monoxylsides (α_0 - α_2), dixylsides (α_2 - α_2) and monoxylsides monohexuronic (α_2 - β , α_2 - γ , α_2 - δ) diesters of bilirubin. Glucosidic mono- and diesters of bilirubin amount to less than 15% in less-polar conjugates of rat bile, 25% for post-obstructive human bile and 60% in dog bile.

Semi-quantitative evaluation of bile composition

The t.l.c. system of Thompson & Hofmann (1971) incompletely separates bilirubin mono- and diesters. Nevertheless, as an adjunct to the present study it allowed a more detailed analysis of natural bile pigment conjugates than hitherto possible. Thus it was possible to calculate concentrations of individual pigments by multiplying the percentage of mono-

and di-conjugates, as determined from t.l.c. of their azo derivatives, by the total amount of intact bilirubin conjugate in each chromatographic fraction from the original bile. Losses during chromatography, azo coupling and removal of fraction from plates averaged 40% with a range of 20–50%. The present discussion assumes this loss to be equally distributed between all the compounds concerned.

With this reservation, normal rat biles were shown to contain 51% of homogeneous glucuronic acid or mixed hexuronic acid diesters of bilirubin and predominantly the diglucuronide. Other pigments were bilirubin monoconjugates (33%), diconjugates (10%) containing xylose (4%) or glucose (6%), and disaccharidic esters containing unidentified uronic acids (6%). The amount of less-polar conjugated bilirubin increases significantly after loading rats with bilirubin. The bile-pigment fractions then contain 56% of ester monoconjugates and 34% diglucuronides together with diconjugate containing xylose (2%), glucose (5%) and unidentified disaccharides (4%). Post-obstructive rat bile contains 38% of diglucuronide, 22% of monoconjugates, and 6% of diconjugates containing xylose (2%) and glucose (4%). The ester diconjugates containing unidentified uronic acids are increased to 32%.

In dog bile most of the conjugated bilirubin occurs as diesters; these are mixed diconjugates containing glucose (40%) or xylose (14%), about 27% of diglucuronoside, together with 5% of disaccharides containing unidentified uronic acids. Monoconjugates amount to only 14% of the total. With post-obstructive human bile, where highly polar conjugates are predominant, bilirubin diglucuronoside amounts approximately to 45% of total bile pigment, diconjugates of unknown uronic acids to 22%, monoconjugates to 17% and diconjugated esters containing xylose (5%) or glucose (9%) to 14%.

Kuenzle (1970) has proposed that the major bilirubin conjugates of human T-tube bile bilirubin occur not as glucuronides but as acyl glycosides of acidic disaccharides. Results reported in the present paper are not incompatible with the presence of bilirubin diglucuronide with an hexuronosylhexuronide (azopigment B₃) in the chromatographic region of the more-hydrophilic bile pigment when separated by the system of Thompson & Hofmann (1971). However, aldobiouronides (azopigment B₄) and pseudoaldobiouronic acids (azopigment B₆) appear absent from this fraction, since on azo coupling it afforded less than 3% of azo derivative conjugated with carbohydrate.

In the present study the biliary excretion of monoconjugated bilirubin was calculated as double the percentage of α -azopigment (azo derivative given by coupling with diazoethylantranilate) (Ostrow & Murphy, 1970). Monoconjugates were thus shown to constitute $37.9 \pm 1.3\%$ of pigment in normal rat bile,

$60.8 \pm 6.4\%$ of that in bile from bilirubin-loaded rats, $16.5 \pm 1.8\%$ for dog gall-bladder bile and $23.8 \pm 5.4\%$ for human 'obstructed' bile.

Van Roy & Heirwegh (1968) found that monoglucuronide in bile from anaesthetized rats previously injected with bilirubin (470 μ g per 100g body wt.) accounts for 73% of the colour developed on azo coupling. Fevery *et al.* (1972c), however, using unloaded rats, obtained a value of 39% for this fraction. The present findings for rats recovered from anaesthesia suggest that the different results of the earlier studies depend on bilirubin loading rather than on effects of anaesthetic, as was suggested by Fevery *et al.* (1972b).

It was proposed (Fevery *et al.*, 1972b) that biosynthesis of bilirubin glycosides occurs over at least two enzymically catalysed reaction steps: bilirubin $\rightarrow$ bilirubin monoglycoside $\rightarrow$ bilirubin diglycoside. Our present findings suggest that high hepatic concentration of substrate may influence the regulation *in vivo* of the whole conjugation process, by stimulating the first step and/or by inhibiting the second. In incubation mixtures containing rat liver microsomal preparations, unconjugated bilirubin and UDP-xylose, dixyloside formation is stimulated at low concentrations of the pigment and high concentrations of the sugar source (Fevery *et al.*, 1972a). Thus increased requirements for UDP-glucuronic acid and/or for some factor essential for diglucuronoside formation in rat liver (Fevery *et al.*, 1972b) might also limit the rate of secretion of bilirubin glucuronide into the bile of pigment-loaded rats.

Markedly increased amounts of β - and γ -azopigments were observed in cholestatic human and rat biles (Van Damme *et al.*, 1971; Fevery *et al.*, 1972c). The latter authors, considering the values for total azodipyrrole, reported that excretion of diconjugated bile pigments increased from 61% in normal to 83% in post-obstructive rat bile. The present findings demonstrate, however, that only ester diconjugates containing unidentified uronic acid are increased in obstructed rats, whereas both mono- and diglucuronides appear to be simultaneously decreased.

Bile samples from one patient with obstructive jaundice caused by gallstone disease were examined within 10 days after relief of the obstruction (Fig. 1). The proportion of less-polar bilirubin conjugates, detected in the common-duct cholestatic bile obtained during surgery, decreases slowly in the post-operative days (line B), with a simultaneous increase of the more-polar fraction (line A). The presence of a large amount of less-polar conjugates in the bile immediately before relief of the obstruction could be due to preferential synthesis of monoconjugates and/or carbohydrate-containing diconjugates, to preferential renal excretion of more-polar diconjugates or to their hydrolysis by hepatic β -glucuronidase during

Fig. 1. Pigment composition of the bile of one patient with cholelithiasis

Bile samples are collected before (BS) and after (PS) surgical relief of the mechanical obstruction. After t.l.c. with phenol/water as developing solvent, the conjugated bilirubin fractions were quantified as described in the text. The lower ordinate indicates the amount of total more-polar fraction (line A) and less-polar fraction (line B), as a percentage of total conjugated pigment. The amount of hexuronic acid diesters of bilirubin containing either glucuronic acid (line A₁), or unidentified uronic acid groups (line A₂) are calculated from quantitative t.l.c. of azopigments derived from the more-polar conjugates as described in the text. Monoglucuronide and mixed diconjugates of bilirubin-containing carbohydrates and glucuronic acid (line B₁), and mono- and mixed di-conjugates containing unidentified uronic acids (line B₂) are calculated as a percentage of total conjugated bile pigments from the quantitative t.l.c. of azopigments from the less-polar conjugates. The bilirubin content in each bile sample is indicated in the upper diagram.

stasis. Release, during the first post-operative days, of abnormal pigments stored in the liver seems considerable, since the day-3 samples show the highest bilirubin concentration. After 5 days the glucuronide conjugation increases (Fig. 1, lines A₁ and B₁), whereas excretion of abnormal mono- and di-conju-

Fig. 2. Comparison of the biliary excretion of bilirubin monoconjugates and total less-polar conjugates

The proportion of monoconjugated bilirubin, calculated as twice the total unconjugated azodipyrrrole, correlated well with the proportion of less-polar conjugates isolated by the method of Thompson & Hofmann (1971) from normal rat bile (○) ($r=0.66$; $P<0.005$), bile from bilirubin-loaded rats (●) ($r=0.99$, $P<0.01$), and post-obstructive human bile (△) ($r=0.66$, $P<0.01$). No correlation was demonstrated for gall-bladder bile of dog (▲).

gates is diminished (Fig. 1, lines A₂ and B₂). The presence in cholestatic bile of the unidentified, β -glucuronidase-resistant hexuronic acid-containing bile pigments which yield β - and γ -azopigments is not yet understood.

Much azobilirubin β -D-monoxyside and azobilirubin β -D-monoglucoside (30–40% of total azopigment colour) are obtained from dog bile (Fevry *et al.*, 1971). Our results show that these azopigments are derived from ester diconjugates containing glucose or xylose as major components (about 54% of total bile pigment) of gall-bladder bile of dog. These pigments belong to the rather heterogeneous less-polar fraction, and small amounts of monoglycosides might also exist in normal dog bile. Also the bilirubin diconjugates could be homogeneous or mixed diglycosides and/or mixed diesters containing carbohydrates and uronic acids. Heirwegh *et al.* (1975) reported semi-quantitative studies of dog gall-bladder bile. From densitometry tracings of t.l.c. chromatograms they found the most important bilirubin conjugates to be monoglucose, monoglucuronide (α_3 - δ), the diglucuronide (δ - δ), the monoglucuronide (α_0 - δ) and the monoxylucose monoglucuronide (α_2 - δ). These were accompanied by small quantities of the monoglucoside (α_0 - α_3), the monoxylucose, monoglucose diester (α_2 - α_3), the dixylside (α_2 - α_2) and the

diglucoside ($\bar{\alpha}_3$ - $\bar{\alpha}_3$). An alkali-stable δ -type pigment probably derived from the bilirubin sulphate of Noir *et al.* (1970) was also found in the azo derivatives of the bilirubin diglucuronoside fraction.

Purification of bilirubin hexuronic acid diconjugates is achieved most efficiently with the phenol/water system of Thompson & Hofmann (1971), whereas better resolution of less-hydrophilic conjugates is obtained with the system of Heirwegh *et al.* (1975). Both methods yield similar information about dog bile. Further, the best correlation ($r = 0.99$) between the proportion of monoconjugated bilirubin (as twice the total unconjugated azodipyrrole) and the proportion of less-polar conjugate isolated by the method of Thompson & Hofmann (1971) is found for bilirubin-loaded rats. The low-polarity fraction from the bile of these animals consists almost entirely of monoconjugates. On the other hand the same fraction from dog gall-bladder bile contains only 22% of monoconjugate and therefore affords the most significant discordance ($r = -0.22$).

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