Bilirubin Conjugates in Bile of Man and Rat in the Normal State and in Liver Disease

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ABSTRACT Conjugates of bilirubin were studied in normal bile of man and rat, and in bile of liver patients. In general human bile was obtained by duodenal intubation. In addition T-tube bile was examined in patients operated on for mechanical obstruction. The bile pigment compositions of duodenal and T-tube bile were similar in two patients where comparison was possible. Obstruction of the bile duct in rats was used as an animal model for obstructive jaundice.

Diazotized ethyl anthranilate was used for determination of total conjugated bile pigment and for thin-layer chromatography (t.l.c.) analysis of the derived azopigments. The available t.l.c. procedures are versatile and allow rapid and quantitative analysis. A variety of conjugated azopigments can be distinguished.

With chloroform, negligible amounts of unconjugated bilirubin are extracted from bile of man. Therefore, the percentage of monoconjugated bile pigments present in the initial bile sample can be calculated from the percentage of azodipyrrole found after diazotization.

Normal bile from man and rat yields similar azopigment patterns. The dominant component is azopigment-δ (azodipyrrole β-D-monoglucuronoside). Small amounts of azopigments with complex conjugating structures (γ-azopigments) are present in both cases. Human bile further yields small amounts of azopigments containing xylose or glucose (called azopigments-α_2 and -α_3, respectively). Monoconjugated bilirubin (estimated from the percentage of azodipyrrole) amounts of 22% of total bile pigments in human bile and to 39% in murine bile. In both, the bulk of bile pigment is bilirubin diglucuronoside.

From bile of patients with acquired liver diseases a new azopigment group (β-azopigment) was derived. The γ-azopigment group was increased; the δ-azopigment group (containing azodipyrrole β-D-monoglucuronoside) was decreased. No differentiation was possible between intra- and extrahepatic cholestasis. The percentage of β-azopigment showed a positive correlation with serum bilirubin concentration ($r = 0.6$).

Recovery of the diseases was accompanied by normalization of the azopigment patterns.

In rats, hydrostatic or mechanical obstruction induced increases in β- and γ-azopigments and a decrease in δ-azopigment similar to the changes observed in bile of liver patients. Complete normalization was obtained 6 hr after relieving the hydrostatic obstruction (duration 15–21 hr). In contrast, with man after surgery for extrahepatic obstruction, T-tube bile was not normalized when the T-tube was withdrawn (10 days after operation).

Hydrostatic obstruction in rats provides an easy model when postobstructive bile pigment composition and parameters have to be investigated.

The present investigations stress the importance of the physiopathological state when studying bilirubin conjugation. Hindrance to bile secretion induced heterogeneity of bilirubin conjugates and stimulated the formation of complex structures.

INTRODUCTION

According to past concepts (2, 3) the bile pigment composition of bile and of icteric serum and urine of man and of higher animals is dominated by bilirubin β-D-diglucuronoside. However, recent work with bile samples from normal dog and rat has demonstrated a rather great variety of saccharidic conjugating groups

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Part of the present work has been reported briefly elsewhere (1).

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(4, 5) with marked species variation. Dog bile contained relatively large amounts of β-D-xylopyranoside and β-D-glucopyranoside of bilirubin (5, 6) whereas the β-D-glucuronoside dominated in bile of rat (4, 7). A much more complex composition (4) with existence of disaccharidic conjugating groups (8) was demonstrated for human T-tube bile. In all cases examined the saccharidic conjugating groups were bound in acyl linkage to the propionic acid side chains of bilirubin (4, 7–9). Temporary bile duct obstruction in rats mediated a transition from the initially simple to a more complex composition (1) similar to that found with human T-tube bile (4).

In 1957 Billing, Cole, and Lathe (10) separated conjugated bilirubin into two components called pigment I and pigment II. On the basis of chemical studies a bilirubin monoglucuronoside structure was postulated for pigment I. The alternative, a bilirubin: bilirubin diglucuronoside (1:1) molecular complex was considered possible but less likely. Since the formulation of the monoglucuronoside hypothesis, severe criticism has been raised against it (11–13). This was mainly based on lack of success in the rechromatography of pigment I. The observed heterogeneity of bile pigments present in dog bile and in pathological body fluids of man and rat, and the pronounced lability of some of the conjugating structures (4), largely invalidate the argument. In contrast, the available positive evidence strongly favors the existence of bilirubin β-D-monoglucuronoside in normal murine bile (14, 15). Provided diazotization of bile pigment is complete, estimates of the total amount of monoconjugated bilirubin can be derived from the quantitation of azodipyrrole [1] generated in the coupling reaction (15).

It will be clear that separation of conjugated bile pigments into two fractions, either by chromatography (10) or by simple phase distribution procedures (16, 17), will generally not allow one to appreciate and analyze the full complexity of bile pigment-containing biological samples in various physiopathological states in human. As yet no quantitative technique of evaluation based on direct separation of conjugated bile pigments is available. However, indirect analysis through separation of derived azopigments is possible. For this purpose the diazonium salt of ethyl anthranilate (18) offers definite advantages. The coupling reaction proceeds in aqueous medium at pH 2.7 offering maximal opportunity to detect acid labile structures (4). The reagent shows great selectivity for conjugated bile pigments (4, 18) although completeness of coupling is conserved (4, 5, 18). The latter property may be related to the low water solubility of the derived azopigments.[2] The azopigments are quantitatively extracted into pentan-2-one (4, 15, 18) and can be used for photometric determination of total conjugated bilirubin and for quantitative analysis of the azopigments after their separation by t.l.c. (4, 5).

In the present work the analysis of duodenal bile in normal adults and of duodenal and T-tube bile in patients with liver disease was approached as outlined above. Analyses of duodenal bile samples offer the advantage of general applicability and may yield information about alterations in bile pigment metabolism when no appreciable amounts of conjugated bile pigment are returned to the blood. Model experiments were set up with normal and with bile duct-obstructed rats.

METHODS

Chemicals

Chemicals were as specified elsewhere (4, 5, 18).

Collection of bile of man

Duodenum bile. Normal adults and patients with liver disorders, both in the fasting state, were intubated with a double-lumen Dreilung tube. After passing the pylorus the tip of the tube was placed at the transition of the second and third part of the duodenum. The position was checked by fluoroscopy. Gastric secretions were continuously sucked out. After brief aspiration duodenal fluid was allowed to drain spontaneously by hydrostatic pressure. Usually three samples of so-called A bile were collected. Except when gallstones were present, 40 ml of a concentrated $MgSO_4$ solution (33%, w/v) was then instilled to allow collection of concentrated bile resulting from gallbladder contraction (so-called B bile). Samples of the final C bile (assumed to be composed mainly of hepatic bile) were also obtained. Routinely the first sample (15 ml) of A bile was rejected. Samples were collected in the dark. Further processing was done in dimmed light.

Bile obtained during surgery for cholecystectomy, and by T-tube drainage. In some patients hepatic and/or gallbladder bile was obtained during surgical intervention, or afterwards by T-tube drainage. Samples from the 1st, 4th, and 7th day after the day of intervention were examined. No significant changes were noted between the samples obtained the 1st and the 4th day. Usually T tubes were removed the 9th postoperative day.

Bile samples were either analyzed the day of their collection or were stored frozen overnight before being processed. This treatment did not cause detectable alterations of bile pigment composition. Repeated freezing and thawing (10 times), however, induced partial deconjugation of bile pigments.

Collection of rat bile and induction of cholestasis

Male albino Wistar R\ rats (body weight 250–400 g) having free access to drinking water and food [3] were used.

[1] The term "azodipyrrole" expresses the chemical nature of the azopigment derived from unconjugated bilirubin (or from the nonconjugated dipyrrole moiety of monoconjugated bilirubin) more correctly than "azobilirubin." In view of the heterogeneity of conjugated bile pigments, and more, in particular, of the existence of monoconjugates, the latter expression is objectionable and misleading.

[2] This explanation has been suggested to us by Dr. J. D. Ostrow.

[3] RMH-B food; Hope Farms, Woerden, The Netherlands.

Obstruction experiments of short duration. Under light ether anesthesia a polyethylene (PE_{10}) catheter is brought into the bile duct of a rat and a flat contact thermometer[4] is inserted subcutaneously with the receptor site against the abdominal muscle wall. After suturing the abdomen the animal is placed in a Bollman restraining cage which is then transferred to a thermostated premature infant incubator.[5] The thermostat was usually set at 37.8°C. Body temperature was recorded continuously with a potentiometric recorder[6] and controlled by a system producing alternating currents of moistened air, either warm or cold. The animals had free access to drinking water but were not infused.

Bile samples were collected sequentially for various periods of time until body temperature had reached the preset value. Several control samples were then collected (at least until 4 hr after starting the cannulation) before cholestasis was induced by elevating the free end of the catheter (19–22). In rats, bile flow was counteracted completely by a hydrostatic pressure corresponding to a 23-24 cm bile column. Obstruction was maintained for 3–21 hr. At the end of the obstruction period the catheter was brought down and bile collected for several 10-min periods, followed by a series of longer collection periods. Variants of the procedure are as indicated in the text.

In all experiments with rats, bile was allowed to flow into graduated 10-ml centrifuge tubes with a conical tip, fixed at the upper rim of a small Dewar flask (internal height about 23 cm; internal diameter 7 cm), provided at the bottom with solid CO_2. By roughly regulating the outflow rate of CO_2 vapor from the container the temperature in the tube was kept sufficiently low to cause immediate freezing of any drop of bile falling onto the wall of the tube. Bile samples were processed without delay (if not possible, they were stored frozen overnight).

Long-term obstruction. A polyethylene catheter was introduced into the bile duct of a rat, as outlined above. The tube was cut at a length of 5 cm and sealed by heating. The free end was then inserted subcutaneously and the rat allowed to resume normal activities. After 2 or 4 days the end of the catheter was exteriorized under very light ether anesthesia. The rat was again placed in a restraining cage which was kept in the incubator for 2 hr to allow the animal to reach normal body temperature. The catheter was opened, connected to a sufficient length of polyethylene tubing and bile collection started.

Determination of conjugated bile pigment

Diluted bile was treated with diazotized ethyl anthranilate and the concentration of total conjugated bile pigment determined as described (4).

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

Portions of the extracted azopigments were applied to precoated silica gel plates[7] and developed for 12 cm at room temperature in the dark with chloroform-methanol-water (65:25:3, by volume) (Table I and Fig. 1). With azopigment derived from human bile it was advantageous to dry the plates in an air stream and to apply a second development with chloroform-methanol (17:3, v/v) for 15 cm. The use of the second, less polar solvent system promotes separation of the azopigments of the α-group but has little effect on the R_F values of the more polar components.

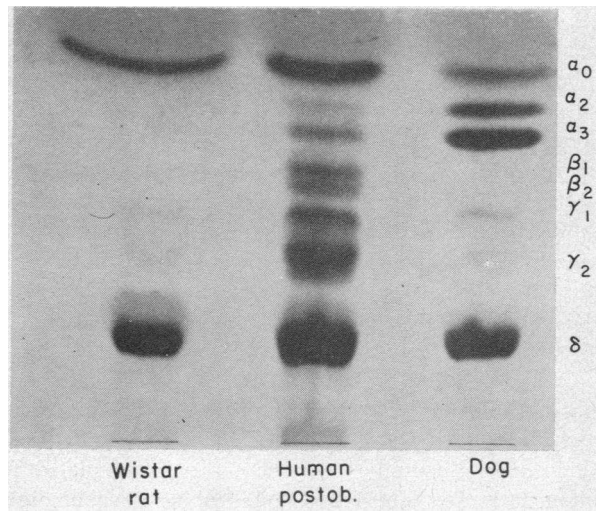

FIGURE 1 t.l.c. of ethyl anthranilate azopigments (dark areas, denoted by Greek letters) derived from normal bile of rat, from postobstructive bile of man, and from normal bile of dog. The start of each track is indicated by a line at the bottom of the figure.

To facilitate the understanding of the present paper, information relevant to the presently used nomenclature has been gathered in Table I. In view of the as yet incomplete knowledge of the structures of several of the azopigments (4–7) a nomenclature based on separation by t.l.c. seems to offer a useful and valid working basis (Table I; Fig. 1). In order to gain acquaintance with the diversity of azopigment derivatives encountered, employment of azopigment mixtures derived from normal bile of dog and rat (4, 5) and from human T-tube bile as references is advised (Tables I and II). Having due regard to the dominant components present in each type of mixture (Table II) unequivocal application of the proposed nomenclature should be easy.

Separation of azopigment-α_0 and -α_2 in samples derived from human duodenum bile was frequently rather poor, the leading spots being laterally elongated and sickle shaped. This caused decreased resolution when the azopigments had to be quantitated by densitometric scanning (5). Considerable improvement was obtained by developing the plates first with chloroform-methanol (17:3, v/v) for 3 cm, followed by chloroform (containing 0.6–1% ethanol) for 18 cm, before application of the solvent systems mentioned above.

Separated azopigments (derived from normal bile) were quantitated by photometric determination of spots eluted with methanol (4). Elution of the spots is quantitative (4). For the more complex mixtures densitometric scanning[8] of the plates (scanning speed 0.5 cm/min) was preferred (5). The instrument calculates the total extinction of the colored

[4] Thermistor 421 and Tele-thermistemp 73 TK; Yellow Springs Instrument Company, Ohio.

[5] Full details can be obtained from Dr. B. Van Damme, Laboratory for Histochemistry and Cytochemistry, Akademisch Ziekenhuis St. Rafaël, B-3000 Leuven, Belgium.

[6] Servogor RE 511; Goerz electro Ges. m.b.H., Wien, Austria.

[7] DC-Kieselgel F254, 5717/0025; E. Merck AG, Darmstadt, Germany.

[8] Flying Spot TLD-100 Densitometer; Vitatron, Dieren, The Netherlands.

TABLE I

Nomenclature, Average R_F Values, and Structural Data of Azopigments Derived from Human Postobstructive Bile (H), and from Normal Bile of Dog (D) and Rat (R)

Name based on t.l.c.*	Average R_F values			Structural data	
	H	D	R	Description of structure	References
Azopigment-α_0	0.84	0.84	0.84	Azodipyrrole (commonly called "azobilirubin")	7
Azopigment-α_1	0.81	0.80		Red color; structure unknown (trace amounts)	
Azopigment-α_2	0.74	0.73		Azodipyrrole β-D-xylopyranoside	5, 6
Azopigment-α_3	0.65	0.63	Trace	Azodipyrrole β-D-glucopyranoside	5, 6
Azopigment-α_4	‡			Red color; structure unknown	
Azopigment-β_1§	0.57			Possibly a group of conjugates (1 mole of hexuronic acid/1 mole of azodipyrrole; acyl glycosides; part of the conjugating structures is very acid labile)	4
Azopigment-β_2§	0.54			Same remarks as for azopigment-β_1	4
Azopigment-γ_1§	0.49	0.47	0.51	Same remarks as for azopigment-β_1 but less acid labile	4
Azopigment-γ_2§	0.40	0.42	0.42	Same remarks as for azopigment-β_1 but less acid labile	4
Azopigment-δ	0.20	0.21	0.21	Azodipyrrole β-D-monoglucuronoside (normal rat bile)	4, 7

* T.l.c. on precoated glass plates (DC-Kieselgel) with chloroform-methanol-water (65:25:3, by volume) as the developing solvent.

‡ Trace amounts when present; was difficult to separate from azopigment-α_3; moved a little more slowly when it appeared.

§ Only the major components are given.

areas and further draws a chromatographic profile (typical examples are shown in reference 5).

Chloroform-extractable bile pigments

The technique of Brodersen and Jacobsen (23) was used. The chloroform phases were separated by centrifugation and read at 454 mμ. Bilirubin concentrations were estimated assuming ϵ_m^{454} 60.7×10^3 liter mole^{-1} cm^{-1} (24). Azopigments for t.l.c. analysis were formed according to procedure A of Van Roy, Meuwissen, De Meuter, and Heirwegh (25). Chloroform extract (1 ml) is mixed with 2 ml of ethanol-acetone (1:1, v/v), containing 30 mg of 2,6-di-tert-butyl-*p*-cresol, and further reacted with 0.5 ml of diazotized *p*-iodoaniline reagent (25) for 60 min at 0°C. After the final addition of 3 ml of ascorbic acid solution (10 mg/ml; solvent: 0.1 M NaCl) the reaction mixture is shaken well. The lower phase, which contains the azopigments is separated by centrifugation. The azopigments were seperated by t.l.c. on precoated silica gel plates[7] with chloroform-methanol-water (65:25:3, by volume) as the solvent system.

Separation and detection of bile salts

Samples of azopigment extract derived from human bile, of pentan-2-one solutions of purified azopigment-α_0, α_3, -β, -γ, and -δ (4) and of solutions of reference compounds in ethanol-water (1:1, v/v) (sodium taurocholate,[9] sodium deoxycholate,[10] or glycocholic acid[11]) were applied to precoated silica gel plates.[12] They were developed at room temperature for 12 cm either with propionic acid-isoamylacetate-water-propan-1-ol (3:4:1:2, by volume) (26) or with chloroform-methanol-water (65:25:3, by volume). A 10% phosphomolybdate solution in ethanol was used as a spray to detect bile salts (26).

TABLE II

Percentage Azopigment Composition of Normal Bile of Man,‡ Rat,§ and Dog‖*

Nature of azopigment	Man	Rat	Dog
α_0	11.1±3.6 (37; 8)¶	19.4±1.0	10.2±2.7**
α_1	Trace	Trace or absent	**
α_2	1.2±0.4 (19; 4)	Trace or absent	9.6±1.9
α_3	3.5±0.8 (19; 4)	Trace or absent	27.6±4.2
$\alpha_2 + \alpha_3$	4.6±1.3 (37; 8)	Trace or absent	
β_1, β_2	Absent	Absent	Trace
γ_1	3.5±1.4 (25; 5)		
γ_2	6.9±2.5 (25; 5)		
$\gamma_1 + \gamma_2$	9.4±3.1 (37; 8)	6.0±1.1	8.6±1.7
δ	75.4±5.7 (37; 8)	72.1±2.3	43.0±3.7

* Individual bile samples were analyzed by t.l.c.; azopigment spots were eluted with methanol and quantitated photometrically; their percentage distribution was calculated; mean values ±SD obtained per azopigment or azopigment group are given.

‡ 37 duodenal bile samples of eight normal adults were analyzed.

§ Bile samples of six normal rats (body temperature 37.8°C) analyzed; bile was collected from 4 to 6 hr after cannulation of the bile duct.

‖ Bile samples from 10 gallbladders of normal dogs (results taken from reference 5).

¶ Numbers between parentheses indicate: first number, number of bile samples analyzed; second number, number of adults from which these samples were obtained.

** Ratio azopigment-α_0: azopigment-α_1, about 4:1 (reference 5).

[9] Taurocholic acid, sodium salt, A grade; Calbiochem, Los Angeles, Calif.

[10] Sodium deoxycholate; British Drug Houses, Ltd., Poole, England.

[11] Glycocholic acid, Grade I; Sigma Chemical Co., St. Louis, Mo.

[12] DC-Alufolien 5553/0025; E. Merck AG., Darmstadt, Germany.

RESULTS

Studies with nonobstructed rats. Rat bile with constant composition and concentration of bile pigments, and constancy of bile flow and biliary bilirubin excretion rate could be obtained 3–4 hr after cannulation (Fig. 2). Initial values (obtained shortly after cannulation) and final, stable values are given in Table III. The changes were most pronounced over the first 2 hr after cannulation with little variation over the next 2 hr (Fig. 2).

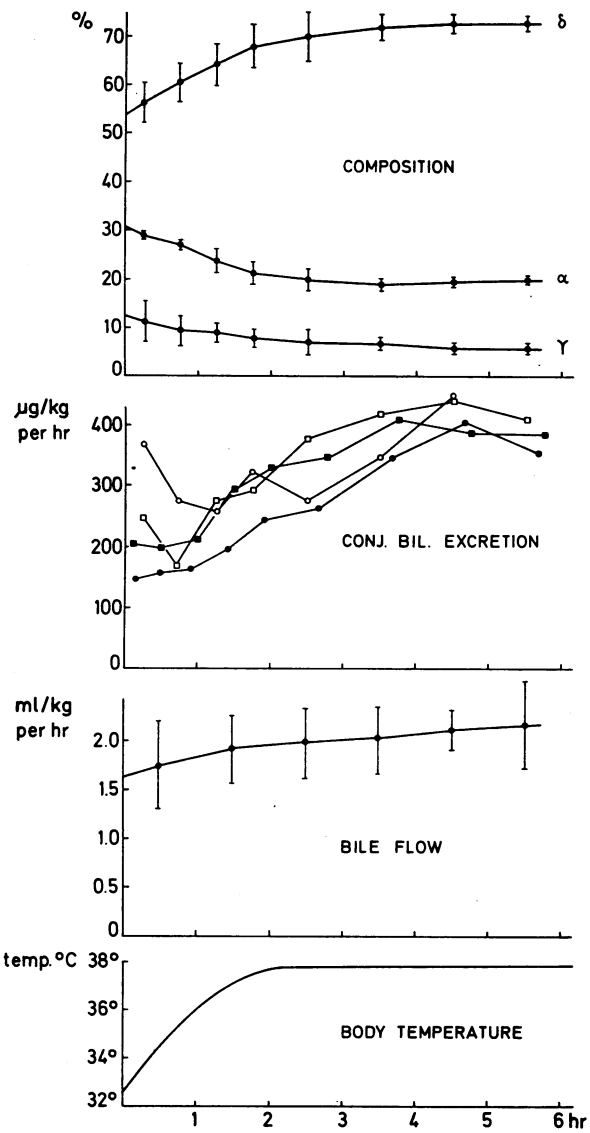

FIGURE 2 Evolution of bile parameters and body temperature of the Wistar rat in a moistened air thermostat at 40°C, after cannulation of the common bile duct under ether anesthesia. Azopigment composition and total conjugated bilirubin were determined on four successive 30-min samples followed by 1 hr samples. Bile flow was determined on successive 1 hr samples. Vertical bars indicate ±1 SD (four animals analyzed). Body temperature was monitored with a contact thermometer as described in the Methods section.

In order to investigate the early postoperative changes attempts were made to separate the effects of ether anesthesia and of lowering the body temperature. Two rats provided with bile fistulae under ether anesthesia were allowed to reach stable postoperative conditions (as monitored by recording the body temperature and by chemical assays on the excreted bile). The rats were then cooled to 33°C over 1 hr and further maintained at that temperature for another hour. No significant changes in either the bile pigment concentration or in its composition were found, but bile flow and concomittantly bilirubin excretion rates decreased roughly twofold.

The effect of ether anesthesia is less easily separated from changes in body temperature. Two rats provided with bile fistulae were allowed to reach stable conditions. They were then kept under ether anesthesia for 30 min. In an attempt to counteract any changes in body temperature (37.8°C at zero time) the environmental temperature was maintained at 40°C. However, a short fall to 35°C in one rat and a minor decrease to 37°C in the other rat could not be prevented. The ether treatment induced prompt decreases of the bile pigment concentration and of the bile flow but these parameters returned immediately to normal when the treatment was stopped. In contrast, a more protracted effect on the bile pigment composition was noted. Compared to control values the percentages of the α_0- and γ-azopigments increased 1.5 to 2-fold during anesthesia with a gradual return to normal when the treatment was stopped.

Studies with obstructed rats. During hydrostatic obstruction the rats were kept in the incubator to facilitate recovery of normal body temperature. In the short-term experiments (3–21 hr hydrostatic obstruction) return to normal body temperature and the establishment of stable values of bile parameters were checked before cholestasis was induced. In the long-term experiments (mechanical obstruction for 2–4 days) recovery is favored by free movement of the animals in their cages.

Hydrostatic obstruction of very short duration (3 hr) in two rats produced no significant changes in the bile pigment compositions of their biles, as compared to control analyses done before inducing cholestasis (Table II). However, more prolonged hydrostatic (15–21 hr) or mechanical obstruction (2–4 days) caused marked increases in the β- and γ-azopigment fractions with concomittant decreases of the other azopigments (Table III). No essential differences appeared between both experimental approaches.

Compared to preobstruction control values both bile flow and bile pigment concentration were increased about threefold immediately after relieving hydrostatic obstruc-

TABLE III

Percentage Azopigment Composition of Rat Bile before and after Obstruction of the Common Bile Duct*

Type of treatment:	No obstruction‡		Short-term obstruction (15–21 hr)			Prolonged obstruction (48 hr)		
	First sample after cannulation	Normal bile (37.8°C)						
Number of rats studied:	3	6		6			3	
Collection period after releasing obstruction, *min*			0–10	10–20	20–30	0–10	10–20	20–30
Percentage azopigment composition:								
Azopigment-α_0	28.4±0.8	19.4±1.0	12.9±5.7	9.9±2.7	10.8±2.3	13.7±2.2	11.8±3.0	12.1±3.5
Azopigments-$\beta + \gamma$			34.2±4.3	31.9±4.6	30.3±4.2	29.3±5.2	28.5±5.3	30.3±6.3
Azopigment-γ	11.0±4.0	6.0±1.1						
Azopigment-δ	57.0±5.0	72.1±2.3	49.4±6.2	54.4±4.0	54.4±4.0	56.4±4.2	58.1±4.4	57.3±4.7

* Individual bile samples were analyzed by quantitative t.l.c. and the percentage azopigment compositions calculated; results are given as mean values ±SD.
‡ The control samples were obtained from the six rats used for the short-term experiments.

tion (15–21 hr) (three experiments). Return to normal required about 6 hr. Full details about these experiments have been given in a preliminary paper (1).

Duodenal intubation in man as a method of obtaining representative bile samples. In view of the greater field of application of the method of assessing changes in bile pigment conjugation and excretion, as compared to analyses on serum or urine, it was of interest to consider in some detail the reliability of the method.

In the preliminary stages of the present work the first 15-ml portions of A bile were also analyzed. Frequently the α_0-percentages were higher than in subsequent samples. In several cases the early samples were slightly acid, showed turbidity and turned greenish before analysis could be begun. Contamination with gastric contents could explain the more acid pH, the tendency to yield biliverdinoid pigments and probably also the increased α_0-fractions (due to partial hydrolysis of conjugated bile pigment). The latter interpretation is supported by the following observations. Normal duodenum bile when acidified to pH 2.5 and incubated at 37°C for 30 min showed an increase of the α_0-fraction from 11.5% to 14.7%. With another sample 120 min incubation under the same conditions increased the α_0-fraction from 13.1% to 20.4%. In view of the observations mentioned above the first 15-ml portions of A bile were systematically rejected for further work.

Serial analyses were done one samples of A, B and C bile (3–7 samples analyzed per subject) obtained from seven normal persons and from six patients with acquired liver disease (acute viral hepatitis, two; toxic hepatitis, one; chronic persistent hepatitis, one; cholelithiasis, one; alcoholic cirrhosis, one). No systematic trends in the azopigment compositions of the different bile samples were apparent. The results demonstrate that if B bile can not be obtained for practical or medical reasons, analysis of A or C bile will yield equivalent information. Instillation of $MgSO_4$ is clearly contraindicated in patients with lithiasis.

In two patients comparison was made between samples of duodenal and hepatic bile. In one patient duodenal bile was collected 7 days before a second sample was obtained during surgery for cholecystectomy. The samples showed nearly the same bile pigment compositions. In the second patient duodenal bile was compared with T-tube bile obtained 30 days later. The compositions were similar but the T-tube bile showed slightly more increased β- and γ-fractions.

Bile pigments in bile of normal and diseased adults. Similar azopigment distributions were found with normal bile of man, dog, and rat (Fig. 1 and Table II). The absence of a β-fraction in bile of man and rat is worthy of note. Chloroform-extractable bile pigment (which

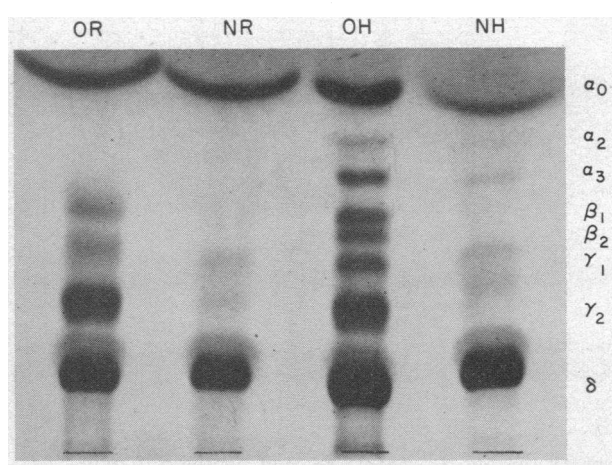

FIGURE 3 Comparative study by t.l.c. of ethyl anthranilate azopigments (denoted by Greek letters) derived from bile of obstructed (O) and nonobstructed (N) man (H) and rat (R). The start of each track is indicated by a line at the bottom of the figure.

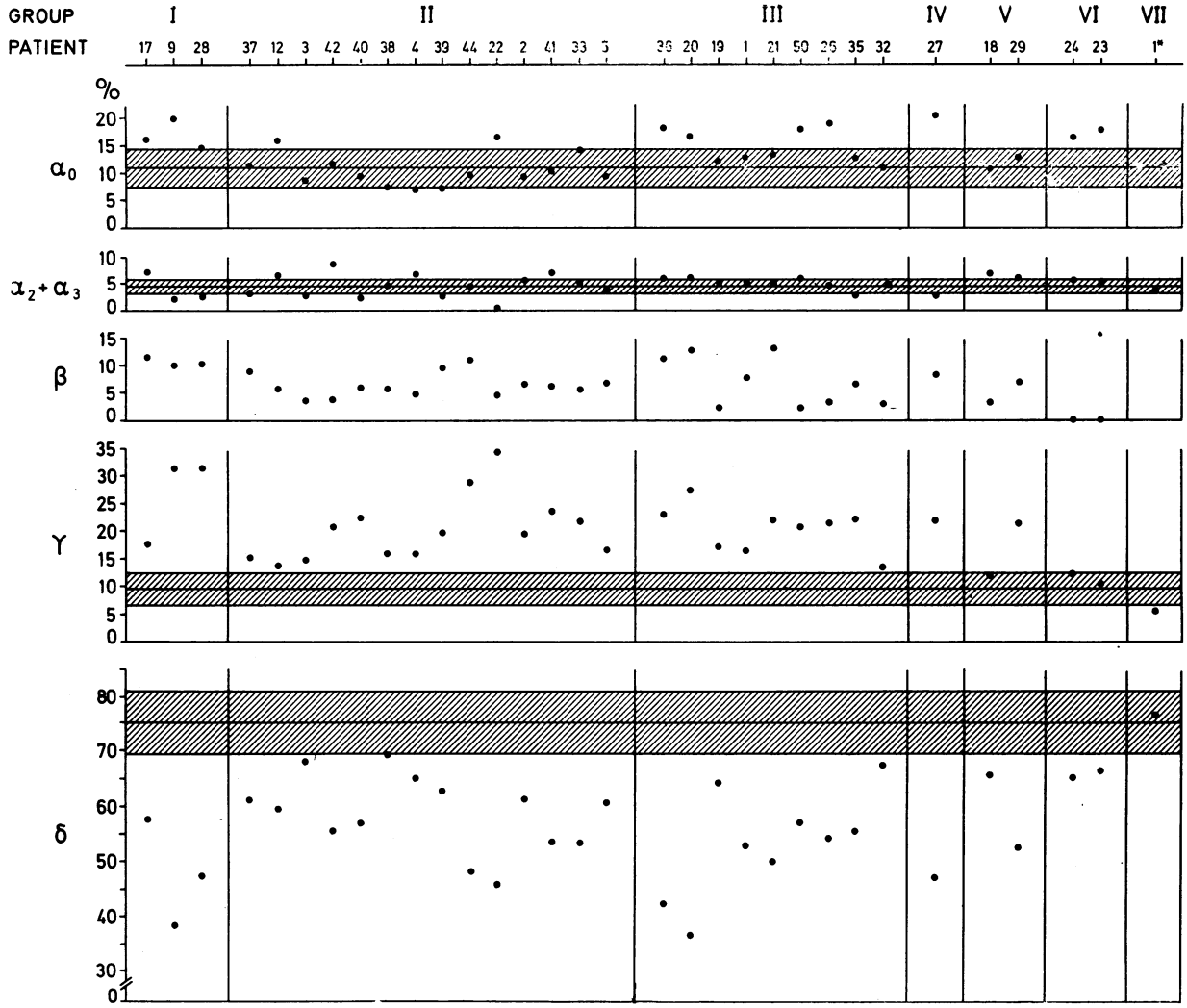

FIGURE 4 Percentage azopigment composition in bile of patients with liver disease. The ordinate of each scattergram indicates the amount of azopigment (or azopigment group) as a percentage of total azopigment obtained with diazotized ethyl anthranilate. Shaded areas denote normal values ±1 SD obtained from eight adults. Groups I and II refer to bile samples from surgical patients (samples obtained on 4th postoperative day); groups III–VII refer to bile obtained by duodenal intubation (mean values of determinations on three samples are given). The numbers at the top of the figure refer to individual patients. Group I, patients with pancreatic or hepatic duct carcinoma; group II, patients with gallstone disease; group III, patients with icteric hepatitis; group IV, a patient with primary biliary cirrhosis; group V, patients with alcoholic liver cirrhosis; group VI, a patient (No. 24) recovering from acute hepatitis and one (No. 23) with chronic persistent hepatitis; patient 1* is patient 1 after complete recovery from acute hepatitis.

should contain the unconjugated bilirubin, if present) varied between 0.14 and 4.48% of total bile pigment (10 samples analyzed). It was partly composed of conjugated bilirubin, as indicated by t.l.c. of diazotized extracts. After correction for extracted conjugated bilirubin (assumed to be composed of monoconjugates only) the chloroform-extracted bilirubin amounted to less than 1% of total bile pigment in nine cases. It was 2.05% for the remaining bile sample.

In patients with liver disorders marked changes in bile pigment composition were noted (Figs. 3 and 4). Acquired liver diseases (hepatitis, cirrhosis, obstructive jaundice, and cholelithiasis) showed the appearance of β-azopigments, increased γ- and decreased δ-fractions. The changes were significant (Fig. 4).

Patients with mechanical obstruction due to pancreatic or hepatic duct carcinoma had deeper jaundice and more pronounced disturbances in bile pigment composition than

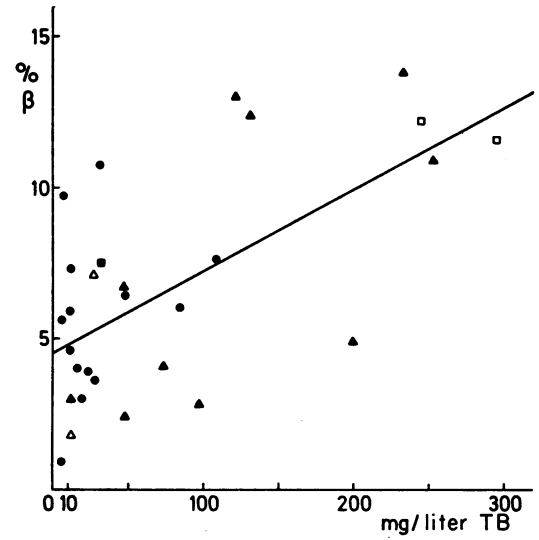

FIGURE 5 Comparison of total serum bilirubin and the percentage of azopigment-β. Values of the concentration of total serum bilirubin (TB) are indicated along the abscissa; the amounts of β-azopigment (expressed as the percentage of total conjugated bilirubin) derived from human bile are given along the ordinate. Patients with icteric hepatitis (▲), cirrhosis (△), lithiasis (●), pancreatic duct carcinoma (□), or primary biliary cirrhosis (■).

the ones with lithiasis. On the present basis patients with intrahepatic cholestasis due to viral or toxic hepatitis could not be distinguished from the ones with mechanical obstruction.

One patient (No. 1 in Fig. 4) was examined during prolonged hepatitis and 1 yr later when all biochemical function tests were normalized (she had been treated by a low dose of steroids and azothioprine). Bile samples obtained during the illness showed increased β- and γ- and decreased δ-fractions, the bile examined after recovery was normal (Table IV). A patient in the recovery phase of acute hepatitis (No. 24 in Fig. 4) showed just a visible trace of β-azopigment but the γ-fraction was still increased and the δ-fraction decreased. Similarly, a patient with chronic persistent hepatitis (No. 23 in Fig. 4) showed only a trace of β-azopigment.

TABLE IV

Percentage Azopigment Composition Obtained from Duodenal Bile Taken from Patient 1 during Prolonged Acute Hepatitis (H) and 1 yr later after Recovery (R), from Patient 24 during Healing of Acute Hepatitis (R), and from Patient 23 during Chronic Persistent Hepatitis (CPH)

Patient	Physiological state	Percentage azopigment composition				
		α_0	$\alpha_2+\alpha_3$	$\beta_1+\beta_2$	$\gamma_1+\gamma_2$	δ
1	H	17.4	4.3	9.1	17.6	51.5
1	R	11.8	7.5	Absent	5.5	75.6
24	R	16.6	6.1	Trace	12.2	65.1
23	CPH	17.6	4.7	Trace	11.1	66.6

Absence of correlation of the presence of β- and γ-azopigments with the presence of cholesterol and bile salts. Cholestasis is known to influence markedly the cholesterol and bile salt compositions because of decreased secretion and increased hepatic synthesis. The presence of β- and γ-azopigments in the chromatograms could therefore be due to interaction with components from bile although this is unlikely in the light of previous work (4–8). As a further test of the previous conclusion that the β- and γ-azopigments are well-defined chemical entities, total azopigment extracts derived from human T-tube bile were developed with chloroform-methanol-water (65:25:3, by volume) on thin-layer plates with sodium taurocholate, sodium deoxycholate, and glycocholic acid as reference compounds. Conjugated bile salts remained near the starting line in the area where the δ-azopigment moved. Unconjugated bile salts moved approximately with the solvent front. Similarly, using solvent system II of Hofman (26) purified preparations of β- and γ-azopigments were shown to be free of bile salts. Bile salts were present in the δ-preparation but in the above-mentioned solvent system they moved independently of the azopigment.

DISCUSSION

Before discussing the relevance of the present work for studies of liver disorders, it seems appropriate to consider in some detail the information which can be derived from the presently used system for analyzing azopigments.

Apart from the azopigments α_0, α_2, and α_3 (Table I) the conjugating groups of the other components have not yet been defined completely (4–7). Azopigment-δ, the dominant component derived from normal human bile (75% of total azopigment color) showed R_F values similar to those found with the δ-azopigment from normal murine bile (Table I). As for the latter substance (4, 7) an azodipyrrole β-D-monoglucuronoside structure has recently been demonstrated.[13] Small amounts of other substances may be present in the δ-azopigment from human T-tube bile as 5–23% of purified preparations resisted attack by β-glucuronidase (4). The rather extreme view has been proposed by Kuenzle (8) that β-glucuronosides of bilirubin do not occur in human T-tube bile. It may be noted, however, that the strength of Kuenzle's work may reside primarily in a brilliant demonstration and structure elucidation of complex disaccharidic conjugating groups. Quantitative recovery appeared to be of less con-

[13] F. Compernolle, G. P. van Hees, and K. P. M. Heirwegh. Mass-spectrometric structure elucidation of human and rat bile azopigments as the acyl glycoside of glucopyranuronic acid. Manuscript in preparation.

cern and the possibility that glucuronosides may have escaped detection can not be excluded.

The situation with respect to the β- and γ-azopigments is also unclear (Table I). They are ester conjugates, are not attacked by β-glucuronidase, and contain 1 mole of hexuronic acid per mole of azodipyrrole (4). Acid lability tests further suggest that in addition they contain residues or groups, bound in an acid labile way to the rest of the azopigments (4). The disaccharidic conjugating structures described by Kuenzle (8) may perhaps be sought among these fractions. The absence of phosphomolybdate-positive material in purified preparations of azopigments-β and -γ, and evidence obtained previously (4–7), indicate that these azopigments are not artifacts in the sense that they would represent molecular complexes with other components. It therefore appears that they represent chemical entities which can be studied in relation to physiological and pathological states.

Apart from the uncertainties affecting the homogeneity and structures of some azopigments, interpretation of the azopigment patterns in terms of the parent conjugated bile pigments is difficult (sometimes even impossible). Indeed, diazo coupling leads to the conversion of the tetrapyrrolic bile pigments into mixtures of dipyrrolic azopigments (4, 7, 15, 27). Except with rather simple mixtures (18, 28), complete and unequivocal reconstruction of the parent bile pigments or evaluation of their quantitative distribution is impossible. Direct separation procedures are needed. Until now no generally applicable solution to this problem has been available.

The presence of azodipyrrole (azopigment-α_0) in mixtures derived from fresh bile reflects with a high degree of probability the existence of monoconjugated bilirubin (4, 15). The percentage of azopigment α_0 multiplied by 2 then represents the percentage of monoconjugated bilirubin in the initial mixture. This leads to estimates of 22%, 16%, and 39% of monoconjugated bilirubin in normal bile of man, dog, and rat, respectively (Table II). High estimates obtained previously for normal rat bile (18) appear to be due, at least in part, to the fact that bile collected during ether anesthesia was used for the determinations. In the case of the rat there is little doubt that mono- and diglucuronosides are indeed the dominant bile pigments in normal bile (15), in agreement with the preponderance of azopigments-α_0 and -δ (Table II) and the assignment of an azodipyrrole β-D-monoglucuronoside structure to the latter pigment (7). With bile of man, chloroform-extractable bile pigment (after correction for extracted conjugates) amounted to less than 1% of total bile pigment. It is likely therefore that with human as with murine bile the azodipyrrole derives from monoconjugated bile pigment.

Normal bile of man yielded a relatively simple azopigment distribution (Table II) with a preponderance of azopigment-δ. Increased heterogeneity and marked quantitative changes were observed in acquired liver pathology (Figs. 3 and 4). In general, a new azopigment group (azopigment-β) appeared, accompanied by increased γ-and decreased δ-azopigment. Similar changes occurred in rat bile when the bile duct was ligated or when bile flow was hindered hydrostatically (Table III). A relationship between pathology and these changes could be demonstrated more directly in three patients. A patient with prolonged acute hepatitis was investigated 1 yr later when all function tests were normalized (Table IV). At that time the bile pigment composition fell within the normal range (Fig. 4). In two patients, one recovering from hepatitis and the other having chronic persistent hepatitis, respectively, the β-azopigment was nearly undetectable, but a slight increase of the γ-azopigment and a decrease of the δ-azopigment were still apparent (Table IV).

In man after release of obstruction, T-tube bile was not normalized within 10 days, at which time the T-tube was usually withdrawn. This is probably related to gradual and slow removal of bilirubin conjugates stored in the liver during cholestasis (29). In rats normalization of bile pigment patterns after hydrostatic (15–21 hr) or mechanical obstruction (2–4 days) required about 6 hr in the former case and was more gradual in the latter (1). During obstruction in rats, bile pigment deposits can neither be demonstrated histochemically (30) nor biochemically.[14]

Some correlation between fraction-β and total bilirubinemia is apparent (Fig. 5). The γ-fraction was consistently increased but its percentage in bile correlated less well with bilirubinemia, suggesting that the β-fraction may be a better index of severe cholestasis. The correlation may improve when data is available in connection with the evolution of liver disease. Indeed, depending on the duration and the stage of the illness, deposition of bile pigment in liver (30, 31) or wash-out may occur proceded by increasing or decreasing bilirubinemia (29).

The altered bilirubin conjugates observed in bile of patients with liver disease may have changed physical properties and could be related to gallstone formation. Bilirubin conjugates, which have an amphipathic structure (32) might play a role in micelle formation. The reported alterations might predispose to secondary gallstone formation. Indeed, increased incidence of pigment-containing gallstones in cirrhotic patients has been documented (33).

Experimental obstruction is the easiest animal model for liver disease. Before such studies can be undertaken validly, adequate normal reference bile must be obtained. Cannulation of the common bile duct under ether anes-

[14] Unpublished work.

thesia produced pronounced changes in bile pigment composition (Table III) and in flow characteristics of bile (Fig. 2). These changes could be traced back, at least in part, to effects of ether per se (increased percentage of monoconjugated bile pigment) and to secundary lowering of body temperature which mainly affected bile flow. Therefore, in establishing normal reference patterns return to normal body conditions has to be checked.

The bile pigment concentration and the flow rate of postobstructive bile in the rat was very high (1) up to 90 min after releasing the obstruction. As a working hypothesis one could suppose that this is due to release of abnormally concentrated bile from a distended bile duct. Reabsorption of water and electrolyes from the bile ducts occurs (34, 35) and distention of the bile duct in rats during obstruction has been demonstrated (20, 21). However, taking into account the values of biliary tree volumes in obstructed rats obtained by Barber-Riley (21), the total fluid volume present in the distended bile ducts of our rats corresponded to less than the bile output over the first 5 min after relieving the obstruction. Therefore release of stored bile cannot be the whole explanation. Removal of noticeable amounts of bile pigments from obstructed liver is also unlikely as no bile pigment deposits are found in liver cells (30). Rapid transit of pigment from the blood to the bile has to be a contributing factor.

Increased heterogeneity of bile pigments (4, 8) thus appears to be related to hindrance of bile flow with decreased biliary elimination of pigments. Even minor hindrance seemed to induce or stimulate mechanisms in the hepatocytes responsible for the attachment to bilirubin of conjugating moieties different from or more complex than β-D-glucuronic acid. Further, excretion of diconjugated bile pigments increased from 61% in normal to 83% in postobstructive bile.

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REFERENCES

1. Van Damme, B., J. Fevery, and K. P. M. Heirwegh. 1971. Altered composition of bilirubin conjugates in rat bile after obstruction of the common bile duct. *Experientia (Basel)*. **27**: 27.
2. Lester, R., and R. F. Troxler. 1969. Recent advances in bile pigment metabolism. *Gastroenterology*. **56**: 143.
3. Fleischner, G., and I. M. Arias. 1970. Recent advances in bilirubin formation, transport, metabolism and excretion. *Am. J. Med.* **49**: 576.
4. Heirwegh, K. P. M., G. P. Van Hees, P. Leroy, F. P. Van Roy, and F. H. Jansen. 1970. Heterogeneity of bile pigment conjugates as revealed by chromatography of their ethyl anthranilate azopigments. *Biochem. J.* **120**: 877.
5. Fevery, J., G. P. Van Hees, P. Leroy, F. Compernolle, and K. P. M. Heirwegh. 1971. Excretion in dog bile of glucose and xylose conjugates of bilirubin. *Biochem. J.* **125**: 803.
6. Compernolle, F., G. P. Van Hees, J. Fevery, and K. P. M. Heirwegh. 1971. Mass-spectrometric structure elucidation of dog bile azopigments as the acyl glycosides of glucopyranose and xylopyranose. *Biochem. J.* **125**: 811.
7. Compernolle, F., F. H. Jansen, and K. P. M. Heirwegh. 1970. Mass-spectrometric study of the azopigments obtained from bile pigments with diazotized ethyl anthranilate. *Biochem. J.* **120**: 891.
8. Kuenzle, C. C. 1970. Bilirubin conjugates of human bile. The excretion of bilirubin as the acyl glycosides of aldobiuronic acid, pseudoalbiuronic acid and hexuronosylhexuronic acid with a branched-chain hexuronic acid as one of the components of the hexuronosylhexuronide. *Biochem. J.* **119**: 411.
9. Schachter, D. 1957. Nature of the glucuronide in the direct-reacting bilirubin. *Science (Wash. D. C.)*. **126**: 507.
10. Billing, B. H., P. G. Cole, and G. H. Lathe. 1957. The excretion of bilirubin as a diglucuronide giving the direct van den Bergh reaction. *Biochem. J.* **65**: 774.
11. Nosslin, B. 1960. The direct diazo reaction of bile pigments in serum. Experimental and clinical studies. *Scand. J. Clin. Lab. Invest. Suppl.* **12**. 26.
12. Gregory, C. H. 1963. Studies of conjugated bilirubin. III. Pigment I, a complex of conjugated and free bilirubin. *J. Lab. Clin. Med.* **61**: 917.
13. Weber, A. Ph., L. Schalm, and J. Witmans. 1963. Bilirubin monoglucuronide (Pigment I): a complex. *Acta Med. Scand.* **173**: 19.
14. Schoenfield, L. J., and J. L. Bollman. 1963. Further studies on the nature and source of the conjugated bile pigments. *Proc. Soc. Exp. Biol. Med.* **112**: 929.
15. Ostrow, J. D., and N. H. Murphy. 1970. Isolation and properties of conjugated bilirubin from bile. *Biochem. J.* **120**: 311.
16. Schachter, D. 1959. Estimation of bilirubin mono- and diglucuronide in the plasma and urine of patients with nonhemolytic jaundice. *J. Lab. Clin. Med.* **53**: 557.
17. Eberlein, W. R. 1960. A simple solvent-partition method for measurement of free and conjugated bilirubin in serum. *Pediatrics*. **25**: 878.
18. Van Roy, F. P., and K. P. M. Heirwegh. 1968. Determination of bilirubin glucuronide and assay of glucuronyltransferase with bilirubin as acceptor. *Biochem. J.* **107**: 507.
19. Erlinger, S. 1968. Les mécanismes de la sécrétion biliaire. *Rev. Int. Hepatol.* **18**: 1.
20. Brauer, R. W., G. F. Leong, and R. J. Holloway. 1954. Mechanics of bile secretion. Effect of perfusion pressure and temperature on bile flow and bile secretion pressure. *Am. J. Physiol.* **177**: 103.
21. Barber-Riley, G. 1963. Measurement of capacity of biliary tree in rats. *Am. J. Physiol.* **205**: 1122.

22. Waldeck, F. 1970. Sekretion und Rückflusz von Galle während und nach chronischer Gallenstauung bei Ratten. *Pfluegers Arch. Gesamte Physiol. Menschen Tiere.* **320:** 300.
23. Brodersen, R., and J. Jacobsen. 1969. Separation and determination of bile pigments. *Methods Biochem. Anal.* **17:** 31.
24. Fog, J. 1964. Bilirubin-purification-purity. *Scand. J. Clin. Lab. Invest.* **16:** 49.
25. Van Roy, F. P., J. A. T. P. Meuwissen, F. De Meuter, and K. P. M. Heirwegh. 1971. Determination of bilirubin in liver homogenates and serum with diazotized *p*-iodoaniline. *Clin. Chim. Acta.* **31:** 109.
26. Hofmann, A. F. 1962. Thin layer adsorption chromatography of free and conjugated bile acids and silicic acid. *J. Lipid Res.* **3:** 127.
27. Lucassen, J. 1961. The Diazo Reaction of Bilirubin and Bilirubin Diglucuronide. Kemink & Zoon, Utrecht, The Netherlands.
28. Heirwegh, K. P. M., J. A. T. P. Meuwissen, and J. Fevery. 1971. Enzymic formation of β-D-monoglucuronide, β-D-monoglucoside, and mixtures of β-D-mono- and dixylosides of bilirubin by microsomal preparations from rat liver. *Biochem. J.* **125:** 28P.
29. Desmet, V. J., A.-M. Bullens, and J. De Groote. 1970. A clinical and histochemical study of cholestasis. *Gut.* **11:** 516.
30. Desmet, V. J., A.-M. Bullens, J. De Groote, and K. P. M. Heirwegh. 1968. A new diazo reagent for specific staining of conjugated bilirubin in tissue sections. *J. Histochem. Cytochem.* **16:** 419.
31. Metge, W. R., C. A. Owen, W. T. Foulk, and H. N. Hoffman. 1964. Bilirubin glucuronyl transferase activity in liver disease. *J. Lab. Clin. Med.* **64:** 89.
32. Hofmann, A. F., and D. M. Small. 1967. Detergent properties of bile salts: correlation with physiological function. *Annu. Rev. Med.* **18:** 333.
33. Bouchier, I. A. D. 1969. Postmortem study of the frequency of gallstones in patients with cirrhosis of the liver. *Gut.* **10:** 705.
34. Barber-Riley, G. 1963. Rat biliary tree during short periods of obstruction of common duct. *Am. J. Physiol.* **205:** 1127.
35. Erlinger, S., and R. Preisig. 1969. Les mécanismes de la cholérèse. *Rev. Fr. Etud. Clin. Biol.* **14:** 117.