Heterogeneity of Bile Pigment Conjugates as Revealed by Chromatography of their Ethyl Anthranilate Azopigments

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1. Azopigments derived from conjugated bile pigments by coupling with the diazonium salt of ethyl anthranilate are analysed conveniently by quantitative t.l.c. or by column chromatography on CM-cellulose. 2. By chromatographic studies combined with a series of chemical tests six groups of azopigments were demonstrable in preparations from bile and from icteric urine of man. Azobilirubin and its β -D-monoglucuronide have hitherto been considered to be the only major derivatives that can be obtained from human bile pigments. In the present work, other azopigments accounted for 30-40% of the total azopigment material, and the amounts of these showed considerable variation among biological fluids. 3. The divergence of the present results from earlier work is probably related to the use of milder diazotization conditions and of chromatographic techniques with a high resolving power. 4. The thin-layer chromatographic systems developed allow rapid and quantitative analysis of azopigments derived from bile pigments.

Work with bilirubin and its conjugates is notoriously difficult. By using the reverse-phase column chromatographic systems of Cole, Lathe & Billing (1954) and of Billing (1955) the so-called pigment-I fraction has been found to change considerably upon rechromatography (Gregory, 1963; Weber, Schalm & Witmans, 1963), although this observation could not be confirmed by Schoenfeld & Bollman (1963). The pigments are sensitive to oxidation when illuminated. Further, different mixtures of azopigments may result whether coupling with *p*-diazobenzenesulphonic acid at acid pH proceeds in the presence or in the absence of ethanol (Pittera & Cassia, 1963). The difficulties involved are also reflected in the continuing efforts to prepare pure conjugated bilirubin (Lucassen, 1961; Jacobsen, 1969; Ostrow & Murphy, 1970), and in the conflicting data about the existence of bilirubin monoglucuronide (Noir, Garay & Royer, 1965; Heirwegh, 1968; Ostrow & Murphy, 1970) and of bilirubin sulphate (With, 1968). Finally, data on the nature of conjugated bilirubin reviewed by With (1968) are difficult to explain in the light of current concepts and suggest a greater degree of heterogeneity than hitherto suspected.

In view of the instability and probable complexity of bile pigments (Tenhunen, 1965) it seemed preferable to characterize them as their stable azopigment derivatives. Most frequently a diazo-reagent containing *p*-diazobenzenesulphonic acid

has been used, and the azopigments separated on paper (Schmid, 1957), on ion-exchange paper (Vegas, 1963) or on thin-layer plates (Tenhunen, 1965). However, neither extraction nor elution from thin-layer plates of the azopigments is quantitative, and the azopigment bands are neither homogeneous nor stable.

The present paper extends previous work with the diazonium salt of ethyl anthranilate (Van Roy & Heirwegh, 1968; Heirwegh, Meuwissen & Jansen, 1969; Black, Billing & Heirwegh, 1970) to the analysis of bile pigments in biological fluids. The coupling reaction, which proceeds at room temperature in aqueous medium at the less acid pH 2.7, offers maximal opportunity to detect acid-labile conjugates. The azopigments are quantitatively extracted into an organic solvent (Van Roy & Heirwegh, 1968; Ostrow & Murphy, 1970) and can be used for photometric determination of total conjugated bilirubin and for quantitative chromatographic analysis of the separated azopigments. In pentan-2-one the separated azopigments are stable for at least 2 months. T.l.c. allows a rapid separation with a high resolution of the azopigments, which can be eluted quantitatively from the plates. Some properties of the azopigments thus isolated have been investigated.

Diazotization of bilirubin leads to cleavage at the central methylene bridge with the concomitant formation of two isomeric dipyrrolic azopigments

differing only in the position of the methyl and vinyl side chains of the dipyrrolyl moiety (for a schematic representation of the reaction see Billing, Cole & Lathe, 1957). As the isomers are not separated by the techniques described in the present work they will be discussed as if they were only one compound and will be denoted by the term 'azobilirubin'. The same term is generally used in the literature to indicate the azopigment mixture obtained from bilirubin with any diazo reagent. Similarly, the azopigment derived from a bilirubin diconjugate containing identical conjugating groups attached to the carboxyethyl groups of bilirubin will consist of two conjugated isomers.

MATERIALS AND METHODS

Chemicals. Pentan-2-one (previously dried on CaSO_4) and chloroform were redistilled before use. D-Glucuronic acid and CM-cellulose (Na^+ form) were obtained from British Drug Houses Ltd. (Poole, Dorset, U.K.). Saccharo-(1 $\rightarrow$ 4)-lactone monohydrate (A grade) and phenolphthalein glucuronide sodium salt (A grade) were purchased from Calbiochem (Los Angeles, Calif., U.S.A.). D-Galacturonic acid monohydrate was from Fluka A.-G. (Buchs, Switzerland). β -Glucuronidase preparations from *Escherichia coli* (type V) and from ox liver (type IIIb) were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other reagents were of reagent grade quality.

Bilirubin (British Drug Houses Ltd.) was repurified by the method of Fog (1964). Bilirubin glucuronide (crude) was prepared from human fistula bile by the method of Lucassen (1961). Azobilirubin was prepared by coupling unconjugated bilirubin in chloroform with diazotized ethyl anthranilate in the presence of propan-1-ol or methanol (Van Roy & Heirwegh, 1968).

Biological materials. Samples of freshly voided urine from patients with post-microsomal liver diseases (Fevery, Claes, Heirwegh & De Groote, 1967) were used immediately after collection. Human fistula bile obtained from cholecystectomized patients was either assayed directly or was used for the preparation of conjugated bilirubin. Bile was obtained from Wistar R-strain male albino rats (300–350 g body wt.) either before or after the intravenous administration of 1.6 mg of bilirubin ('enriched bile'). The rats were fed on RMH-B food (Hope Farms, Woerden, The Netherlands). Except when stated otherwise results in the text refer to the latter 'enriched' preparations.

Coupling of conjugated bilirubin with diazotized ethyl anthranilate. The diazo-reagent was prepared as follows: 0.1 ml of ethyl anthranilate (0.66 mmol), finely suspended in 10 ml of 0.15 M-HCl, is treated with 0.3 ml of NaNO_2 (5 mg/ml). The mixture is kept at room temperature for 5 min, then treated with 0.1 ml of ammonium sulphamate (10 mg/ml) and used 3 min later.

A 1 vol. sample of a bile pigment solution (60 μM or less) is treated in the dark at room temperature with 1 vol. of glycine-HCl buffer (0.4 M-HCl adjusted to pH 2.85 with glycine) and 1 vol. of diazo-reagent. Care is taken to have a final pH between 2.65 and 2.75 and if necessary 1 M-HCl or glycine is added. After 30 min 1 vol. of ascorbic acid

solution (50 mg/ml) is added with mixing to terminate the reaction. With aqueous solution, urine or bile, which contain minimal amounts of protein, complete extraction of azopigments is obtained by vigorous shaking with 3–5 vol. of pentan-2-one-*n*-butyl acetate (17:3, v/v) followed by centrifugation (5–10 min) at 1000 $g_{av.}$

Concentrations of conjugated bilirubin (expressed in bilirubin equiv./l) are determined from the E_{530} values, determined on the organic phases, and ϵ_{530} 44.4×10^3 litre $\text{mol}^{-1} \text{cm}^{-1}$ (Van Roy & Heirwegh, 1968). Blanks are prepared in parallel with the unknowns by omitting NaNO_2 from the reaction mixtures.

Pre-fractionation of azopigments. Extracts of azopigments in pentan-2-one-*n*-butyl acetate (17:3, v/v) (see above) are washed three times with equal volumes of phosphate buffer, pH 7.4 (a mixture of 10.3 ml of 0.5 M- KH_2PO_4 and of 30.0 ml of 0.5 M- Na_2HPO_4 , adjusted to 1 litre with distilled water). The aqueous washings are combined, brought to pH 2.8 with glycine-HCl buffer (0.4 M-HCl adjusted to pH 2 with glycine) and the azopigments re-extracted into pentanone.

Analysis, quantitative determination and separation of azopigments by t.l.c. Pre-coated silica-gel plates (DC-Alufolien 5553/0025; E. Merck A.-G., Darmstadt, Germany) are heated for 30 min at 105°C and stored for 15–30 min in a desiccator over silica gel before use. Azopigment extracts are applied directly to the plates, with an azopigment preparation from human bile as a reference. Chromatograms are developed for 12–15 cm in the dark at room temperature with chloroform-methanol-water (65:25:3, by vol.).

The nomenclature used in the present work to denote the azopigments is based on the progressive appearance of

Fig. 1. T.l.c. of ethyl anthranilate azopigments (derived from human bile) as a function of the distance of migration. The azopigments are denoted by Greek letters. Visual division of β - and γ -azopigments in closely moving sub-groups is indicated by broken lines. The numbers at the bottoms of the chromatographic strips indicate the distance (in cm) between the starting lines (S) and the solvent front (F). Chromatographic solvent: chloroform-methanol-water (65:25:3, by vol.).

spots in the course of t.l.c. Soon after starting development of a complex azopigment mixture, as obtained from human bile, two bands separate. The leading band, which is denoted by the Greek letter α , further splits into three bands (α_0 , α_2 - and α_3 -azopigments) (Fig. 1). The other band separates into three components which are denoted by the letters β , γ and δ . Prolonged development further separates the β - and γ -azopigments into pairs of partially overlapping bands. The corresponding subgroups are denoted by the symbols β_1 and β_2 , and γ_1 and γ_2 respectively. The azopigment mixtures obtained from other sources could then be interpreted in terms of this nomenclature (Fig. 2).

For quantitative determination of the separated azopigments, the coloured bands are scraped from the plates and eluted by shaking with 1 ml of methanol in closed centrifuge tubes. After centrifuging for 3 min at 1000 g_{av} , extinction at 546 nm of the supernatants was measured in micro-cuvettes (light-path 2 cm).

Small amounts of purified azopigments were obtained by preparative t.l.c. The coloured areas are scraped off and the powders transferred to empty chromatographic tubes (internal diameter 6–8 mm) and eluted with methanol or with acetone–water. To minimize destruction of azopigments the methanol elution was performed in cooled tubes with the minimal necessary volume of methanol. Alternatively columns were eluted with acetone–water mixtures containing 0–15% (v/v) of water. Azopigments belonging to the α - β groups are generally eluted with low water concentration (0–2%), whereas higher concentrations are required to elute the more hydro-

philic γ - and δ -azopigments. Water in the eluates is then removed by addition of chloroform or pentan-2-one and more water, until a two-phase system results. The transfer of azopigments (especially the γ - and δ -azopigments) into the organic phases is improved by further addition of glycine buffer.

Organic phases are dried *in vacuo* at room temperature in a rotatory evaporator connected to an oil vacuum pump.

Preparation of azopigments by column chromatography. The organic azopigment extracts obtained either after diazotization of bile pigments or by 'pre-fractionation of azopigments' are stored in glass-stoppered tubes at -20°C for 16–48 h to remove water. When still cold the clear supernatant solutions are decanted from the ice crystals, allowed to warm to room temperature and used for chromatography.

(1) Chromatography on CM-cellulose. Sieved CM-cellulose (100–200 mesh, Na^+ form) (15 g), finely suspended in 40 ml of pentan-2-one, is introduced into a chromatographic column (28 cm $\times$ 1 cm). Immediately after application of 1–5 ml of azopigment extract, elution is begun with pentan-2-one, and 2 ml fractions are collected. After the collection of 18 ml of eluate, the elution is continued with linearly increasing formamide concentrations by using a gradient-mixing device constructed as described by Bock & Ling (1954). The mixing chamber contained 100 ml of pentan-2-one and the other vessel contained 96 ml of pentan-2-one-formamide (9:1, v/v) at the start of gradient formation. Flow rates were maintained at 1–2 ml/min. The E_{546} value was determined on each fraction (Figs. 3–5).

Adequate resolution and complete elution of azopigments requires vigorous drying of solvents and supporting medium. Formamide low in water content is used. Although it is generally sufficient to freeze out water at -20°C , before transferring the pentanone extract on to the CM-cellulose with volumes larger than 4 ml, the last traces

Fig. 2. Thin-layer chromatogram obtained with ethyl anthranilate azopigments derived from rat bile (1 and 2), human bile (3–5) and unconjugated bilirubin (6). Number 5 refers to the azopigment extract obtained from human bile, whereas numbers 3 and 4 show the composition of the derived fractions extracted with phosphate buffer, pH 7.4 (no. 3) or remaining in the initial organic phase (no. 4). The first two tracks were obtained for post-obstructive (1) and normal rat bile (2), respectively. Azopigment fractions are denoted by Greek letters. S and F indicate the starting line and the front. Development was repetitive: 1, chloroform–methanol–water (65:25:3, by vol.) for 10 cm; 2, chloroform–methanol (17:3, v/v) for 10 cm. The relative intensities of the spots are indicated semi-quantitatively by shading.

Fig. 3. Column chromatography on CM-cellulose (Na^+ form) of azopigments derived from icteric urine of man (a case of extrahepatic obstruction).

Fig. 4. Column chromatography on CM-cellulose (Na⁺ form) of azopigments derived from human bile.

Fig. 5. Column chromatography on CM-cellulose (Na⁺ form) of azopigments derived from normal rat bile.

of water are removed by a subsequent cooling to the temperature of acetone saturated with solid CO₂. Before and after sieving the CM-cellulose is stored in open bottles at about 100°C.

Because of variation of the resolution obtained with various batches of CM-cellulose, it is best to use only batches that completely retain all azopigments except azobilirubin when eluted with pure pentan-2-one.

For the most complex elution pattern, i.e. that derived from icteric urine, azopigment peaks were numbered in the

order of their elution from the CM-cellulose columns (Fig. 3). Some peaks were found to be very small or entirely lacking in the elution patterns derived from human bile (Fig. 4) or rat bile (Fig. 5). Synthetic azobilirubin and the α_0 -azopigment are eluted with the first peak. The α_2 - and α_3 -azopigments are eluted between peaks 1 and 2. Peak 2, which contains dominantly the β -azopigments, frequently splits up into 2 overlapping sub-peaks (Fig. 3). Peaks 3 and 4 contain mainly the γ - and δ -azopigments respectively. Peak 5 contains material which on thin-layer plates moves between δ -azopigment and the origin.

The fractions containing the azopigment bands were pooled separately and washed several times with glycine-HCl buffer and once with water to remove formamide; they were then concentrated *in vacuo* to about 5 ml. After freezing to remove water the materials were chromatographed further on silica gel.

(2) Chromatography on silica gel. Columns (25 cm $\times$ 1 cm) were prepared with silica gel (extra pure, particle size 0.05–0.2 mm; E. Merck A.-G.) suspended in chloroform. Azopigments can be adsorbed equally well from pentan-2-one or from chloroform.

Eluates containing azobilirubin (peak 1) were applied to the silica gel columns, washed abundantly with pure chloroform, and then eluted with chloroform-methanol (9:1; v/v). Fractions containing azopigment were pooled, concentrated *in vacuo*, diluted with chloroform and rechromatographed repeatedly.

Eluates containing conjugated azopigment (all peaks except no. 1) were applied to silica gel columns, washed with acetone and eluted at linearly increasing concentrations of water in acetone (maximum concn. 15%, v/v). Fractions containing azopigment were concentrated *in vacuo* to remove acetone, diluted with glycine-HCl buffer, re-extracted into chloroform and rechromatographed.

Rechromatography was repeated until a single spot was obtained by t.l.c. and u.v. spectra became constant.

Photometry and spectrophotometry. An Eppendorf photometer type 1.101M (Netheler und Hinz, Hamburg, Germany) provided with a mercury lamp was used for measurements at 546 and 578 nm. E_{530} values and spectra were obtained at room temperature with a Beckman DB spectrophotometer (Fullerton, Calif., U.S.A.).

Determination of hexuronic acid/azobilirubin ratios. Azopigment fractions were concentrated *in vacuo* and the E_{530} values determined. The concentrations in azobilirubin equiv./l were then calculated from ϵ_{530} 22.2×10^3 litre mol⁻¹ cm⁻¹ (Van Roy & Heirwegh, 1968). Known volumes of the concentrates were further evaporated to dryness. Total hexuronic acid was measured at 578 nm by the naphtharesorcinol method (Fishman & Green, 1955). For each series of determinations a calibration curve was prepared with known solutions of glucuronic acid and of galacturonic acid.

Incubation of azopigments with β -glucuronidase. Dried, purified azopigments were redissolved in 0.05 M-sodium acetate-acetic acid buffer, pH 4.5; 5.2 nmol of azopigment in 0.1 ml of buffer was then incubated at 37°C with β -glucuronidase (0.081 i.u. in 20 μ l of buffer) from bovine liver for 2, 4 and 16 h respectively. Parallel controls were prepared (a) by omission of enzyme, and (b) by addition of 25 μ l of 18 mM-saccharo-(1 $\rightarrow$ 4)-lactone to the complete incubation mixtures (final vol. 150 μ l). As a further

control azobilirubin β -D-monoglucuronide (azopigment δ ; from rat bile) was incubated in parallel with each series of unknowns, with and without added saccharolactone inhibitor. After acidification and extraction the degree of hydrolysis of conjugated to unconjugated azopigment was assessed by t.l.c.

Similarly β -glucuronidase from *E. coli* (0.0068 i.u./test) was used with phosphate buffer, pH 6.8 (10.1), for incubation. In this case the saccharolactone (final concn. 25 mM) was added to controls (b) as the dry powder immediately before starting the incubations.

The activities of the β -glucuronidase preparations were determined at 37°C with phenolphthalein glucuronide as the substrate, as described by Fishman & Bernfeld (1955). The inhibitor concentrations adopted for controls (b) correspond to the 100% inhibition concentrations established with phenolphthalein glucuronide as the substrate.

Formation of methyl esters of the azopigments. Purified azopigments, dissolved in redistilled chloroform, were treated with ethereal diazomethane. At given times (from 30 s to 60 min after starting the reaction) samples were drawn from the reaction mixtures and analysed by t.l.c. to assess the degree of esterification. The reaction was generally complete after 2–30 min. The mixtures were then quickly evaporated to remove ether and excess of diazomethane. Derivatives obtained from azopigments by treatment with diazomethane are denoted by the subscript M, e.g. the azopigment δ yields the derivative δ_M (Table 1).

Alkali-lability of the azopigments. Azopigment with spaces left for the later application of reference materials on a thin-layer plate was developed and then kept overnight at room temperature in a chromatographic tank equilibrated with the vapour produced at 27% (w/v) ammonia (Garay, Noir & Royer, 1965). Excess of ammonia was evaporated in an air stream, and then azobilirubin, un-

treated material and an azopigment reference mixture from human bile were applied. The plate was then developed in the second direction with chloroform-methanol-water (65:25:3, by vol.) or chloroform-methanol (19:1, v/v) or redistilled chloroform.

Purified azopigments applied to a thin-layer plate with spaces left for the application of reference material were exposed to ammonia vapour and subsequently analysed by t.l.c.

Acid-lability of the azopigments. Dried and purified azopigments were treated overnight at 37°C in the dark with aq. 30% (v/v) formic acid. Reaction mixtures were extracted with chloroform or pentan-2-one and the extracts analysed by t.l.c.

Qualitative tests were done essentially as described under 'Alkali-lability of the azopigments', but the chromatographic tanks were equilibrated with the vapour produced by formic acid-water (1:1, v/v).

RESULTS

Chromatography of ethyl anthranilate azopigments

Thin-layer chromatography. T.l.c. of the azopigments derived from human bile showed four main azopigment spots, α , β , γ and δ , when the plates were developed for 2–4 cm (Fig. 1). More prolonged development resolved each spot into closely clustered sub-groups. Rechromatography at right angles to the first direction indicated that each azopigment behaved as a distinct chromatographic entity. When plates were developed with chloroform-methanol (17:3, v/v), which separates the pigments of the α -group well, α_2 and α_3 remained

Table 1. R_F values of ethyl anthranilate azopigments and of the derivatives obtained with diazomethane

R_F values were obtained as outlined in the Materials and Methods section. In the case of the β - and γ -azopigments only the R_F values of the major diazomethane derivative are given. Solvent systems tested were: I, chloroform-methanol (99:1, v/v); II, chloroform-methanol (19:1, v/v); III, chloroform-methanol-formic acid (85:15:2, by vol.), and IV, chloroform-methanol-water (65:25:3, by vol.). For ease of comparison azopigments (α_0 etc.) and the corresponding diazomethane derivatives (α_{0M} etc.) were developed in parallel on the same plates with solvent systems I, II and III. All azopigments were derived from human bile.

Azopigment		Solvent system ...		I		II		III		IV
α_0				0.11		0.52		0.80		0.74
	α_{0M}				0.73		0.77		0.84	
α_2										0.69
α_3										0.60
β_1				0		0.02				0.53
	β_{1M}						0.76			
β_2				0		0		0.54		0.48
	β_{2M}				0.44		0.69		0.83	
γ_1				0		0				0.34
	γ_{1M}				0.01		0.6			
γ_2				0		0		0.33		0.29
	γ_{2M}				0		0.5		0.71	
δ				0		0		0.16		0.16
	δ_M				0		0.06		0.48	

single but a faint azopigment moving close to α_0 was sometimes observed. The azopigments derived from icteric urine generally showed a similar pattern, with relatively large amounts of the components of the β -group.

Occasionally a small reddish spot, called azopigment-x, moving in the β -group or slightly more slowly, was observed. Its colour differed distinctly from the red-violet colours of the other azopigments. It resisted completely treatment by dilute formic acid or ammonia vapours. The presence of traces of ammonia strongly retarded its migration on thin-layer plates. The x-azopigment is more easily isolated after concentrating it previously by extraction into phosphate buffer, pH 7.4 (Fig. 2, no. 3).

In contrast with the patterns obtained with human bile and icteric urine normal and 'enriched' rat bile yielded nearly exclusively azopigments α_0 and δ (Fig. 2), in agreement with previous work (Van Roy & Heirwegh, 1968). Azopigment derived from bile of other strains of rat (Sprague-Dawley and Wistar rats bred in the Royal Free Hospital, London) may show a more complex composition (F. H. Jansen, personal communication). The bile pigment composition of the bile of Wistar R-strain male albino rats has been found to become more complex after previous obstruction of the common bile duct (Fig. 2, cf. tracks 1 and 2) (B. Van Damme, J. Fevery & K. P. M. Heirwegh, unpublished work).

The α_0 -azopigments derived from human and rat bile pigments had the same R_F values as synthetic azobilirubin on t.l.c. in chloroform-methanol-water (65:25:3, by vol.) (Fig. 2) and in chloroform-methanol (19:1, v/v).

Clearly defined spots were obtained by application of 1–90 nmol of azopigment with no significant variation in the proportions of the various azopigment fractions over this range (Fig. 6). This finding illustrates the high sensitivity of the t.l.c. procedure and indicates that quantitative determination of the azopigment composition by methanol elution is possible.

The recovery of the azopigments from the plates was 93–104% of the applied material as confirmed also with [^{14}C]azopigments by Ostrow & Murphy (1970).

Column chromatography. The elution patterns on CM-cellulose columns confirmed the complexity demonstrated by t.l.c. (Figs. 3–5). In human and rat bile and in icteric urine the amounts of azopigments α_2 and α_3 , which move between peaks 1 and 2, are generally quite small. Peak 2, which contains dominantly the β -azopigments, is relatively important in highly icteric urine. Peak 5, which was generally very low, was typically found with icteric urine where it contained 3–5% of total azopigment colour.

Fig. 6. Elution of separated azopigments from thin-layer plates. Varying amounts of total azopigment derived from human bile were separated by t.l.c. Coloured zones were extracted with 1 ml of methanol and the E_{546} values of the azopigment solutions determined (details of the methods are given in the text).

Good reproducibility of the azopigment separation was attained as shown by repeated analyses of the same azopigment extracts. On storage of azopigment extract from icteric urine in pentan-2-one-*n*-butyl acetate (17:3, v/v) at -20°C for 2 months repeated analyses at 10-day intervals showed that no noticeable deterioration of total azopigment colour had occurred. No systematic trends were apparent when the percentages were plotted as a function of time, except for peak 3 which slowly increased from 6.1% (zero time) to 9.3% (after 63 days).

Analytical study of the diazotization reaction

Selectivity of the diazotization reaction. It has been shown previously that in aqueous solution and in media containing protein, the diazonium salt of ethyl anthranilate reacts selectively with conjugated bilirubin at room temperature at pH 2.7 (Van Roy & Heirwegh, 1968). Unconjugated bilirubin (final concn. 200 mg/l) added as an alkaline solution to undiluted normal urine, or to 21-fold diluted human bile, similarly showed less than 1% coupling.

Extraction of azopigments. From the standard reaction system (pH 2.7) azopigments are extracted quantitatively into pentan-2-one-*n*-butyl acetate

(17:3, v/v). Extraction was complete when 1 ml of diluted human bile (14 nmol of bile pigment) was diazotized and extracted with 0.5 ml of pentan-2-one (3–5 ml of extraction solvent are used in the standard procedure).

Re-extraction of azopigment from the organic phases was studied with buffers (I 0.05) ranging over pH 3–8. When the organic phase, containing azopigments derived from human bile, was shaken three times with equal volumes of buffer the content of the δ -azopigment in the combined washings gradually rose to reach 84–93% of total azopigment at pH 7–8 (three experiments). The α -azopigment and azopigment material, which on columns and thin-layer plates moves more slowly than the δ -azopigment, were extracted nearly completely into buffers of pH 6–7. The washed pentan-2-one-*n*-butyl acetate extracts contained three distinct groups of azopigments (α -, β - and γ -groups; see Fig. 2) and relatively small amounts of azopigment δ . The procedure has been found useful to pre-fractionate complex azopigment mixtures.

Coupling as a function of time. At room temperature diazo-coupling with rat bile is complete and stable after 2–4 min. The α_0/δ ratio remained approximately constant when the treatment was prolonged for 15–30 min. In protein-containing systems coupling is slower but was found to be complete at 30 min, when pigment was present that yielded equal amounts of azopigments α_0 and δ (Van Roy & Heirwegh, 1968). Colour development with human bile and with icteric urine is more gradual than with rat bile and reaches stable values only after 30–40 min. T.l.c. analyses of azopigment extracts from human and from rat bile that were diazotized for 2–60 min suggest that bilirubin conjugates which yield δ -azopigment react more rapidly than those pigments which yield the β - and γ -derivatives.

On incubation of purified δ -azopigment preparations from rat and human bile with equal volumes of water, glycine-HCl buffer and diazo reagent at 37°C for 10–90 min a slight but noticeable breakdown to yellow material as indicated by t.l.c. was noticed. The destruction amounted to about 30% of the initial amounts when incubation was prolonged for 24 h. This phenomenon may be related to the destruction of azobilirubin with excess of *p*-diazobenzenesulphonic acid observed by Lucassen (1961), as no noticeable changes occurred when the sodium nitrite was omitted from the diazo-reagent used for the incubations.

Taking account of the observations mentioned above it will be difficult to establish a set of reaction conditions that is generally applicable. As a compromise coupling at room temperature for 30 min, which is complete with δ -azopigment-yielding bilirubin conjugates with minimal destruction of

azopigment, and which is reasonably complete with the other bile pigments, was adopted for further work.

Diazotization of bile pigments as a function of pH, ethanol concentration and ionic strength. Colour formation with rat bile increased from pH 1.4–2.1, to reach constant levels between pH 2.1–4.5 (Fig. 7). At higher pH values colour yields again decreased. The extended plateau contrasts with the sharper maximum found previously with aqueous solutions of purified conjugated bilirubin (Van Roy & Heirwegh, 1968) and with human bile (Fig. 8). At pH values less than 2.1 addition of ethanol considerably increased colour formation, maximal colour yields being the same as with the aqueous system (Fig. 7).

Coupling with human bile showed a similar pH-dependency in aqueous solution (Fig. 8) but maximal colour formation was reached only from pH 2.7–2.8 onwards. By using the same bile pigment

Fig. 7. Diazotization of rat bile in the presence (●) and in the absence of ethanol (○; standard procedure). Samples (0.5 ml) of diluted rat bile containing 36 nmol of bile pigment were treated with 0.5 ml of glycine-HCl buffer (0.4 M-HCl) of various pH values and 0.5 ml of diazo-reagent for 30 min at room temperature. Corresponding reaction mixtures fortified with 1 ml of ethanol were run in parallel (●). After terminating the diazotization with 0.25 ml of ascorbic acid solution (100 mg/ml) 1 ml of ethanol was added to the control reaction mixtures (○) and colour extracted with 3 ml of pentan-2-one-*n*-butyl acetate (17:3, v/v). Determinations of pH were done on mixtures, prepared in parallel, containing diluted rat bile, the appropriate buffer solution, diazo-reagent and eventually ethanol (●).

sample as that employed for Fig. 8 the initial 20-fold dilution was done with either 1M- or 2M-

Fig. 8. Diazotization of human bile in the presence (broken lines) and in the absence of ethanol (solid line). Samples of diluted human bile (0.5 ml) containing 28 nmol of bile pigment were treated as outlined in the legend to Fig. 7. Part of the initial bile sample was stored at -20°C and assayed the next day (first day: ●; second day: ○).

sodium chloride instead of water, and pH-dependency diagrams were established for both dilutions. Between pH 1.1 and 1.6 colour yields were exactly the same as with the aqueous dilutions. However, they were markedly increased between pH 1.6 and 2.4 and reached maximal values from pH 2.4 onwards.

The addition of ethanol to the human bile samples considerably decreased colour formation in three out of four samples tested (Fig. 8). At low pH values diazotization is 'accelerated' as for rat bile. Chromatography showed that in aqueous media at pH values near 2, formation of azopigments β and γ is depressed as compared with their percentages found at pH 2.7, in aqueous medium. The addition of ethanol diminished the percentages of these azopigments at all pH values. Similar inhibitory effects were observed when the ethanol was replaced by acetone.

The overall colour decrease that occurred with some human bile samples is not understood. Colour formation in the presence of ethanol (Fig. 8; broken lines) was less reproducible than in aqueous solution (Fig. 8; solid line). With icteric urine (one sample tested) colour production was also inhibited by ethanol (about 11%). The decreased percentages of the β - and γ -azopigments may be related to a pronounced acid-lability, especially of the β -azopigments, in water-miscible organic solvents containing acid (see below under 'Properties of the ethyl anthranilate azopigments').

Diazo-coupling of mixed bile pigment samples. To investigate if human bile or icteric urine contain

Table 2. *Diazotization of bile pigments from various sources*

Icteric urine and diluted human and rat bile were diazotized either separately or in pre-mixed combinations and the percentage distribution of the azopigments determined by chromatography on CM-cellulose columns (see the text for the methods used). The amounts of bile pigment subjected to the assays are indicated in parentheses after the names of the sources of bile pigments. For the significance of the peak numbers see Figs. 3-5.

Expt. no.	Source of bile pigments	Percentage distribution of azopigments found for							
		Individual samples				Pre-mixed combinations			
1	Human bile (106 nmol)	11.2	6.9	9.7	71.5				
	Rat bile (58 nmol)	28.4	10.0	14.8	46.3				
	Human bile (106 nmol) + rat bile (58 nmol)					Found:	17.8	8.0	13.1 61.1
						Calc.:	17.3	8.0	12.0 62.7
2	Icteric urine (51 nmol)	36.0	26.6	4.1	35.0				
	Rat bile (64 nmol)	44.1	4.4	1.0	50.4				
	Icteric urine (51 nmol) + rat bile (64 nmol)					Found:	43.5	15.4	3.9 37.2
						Calc.:	3.84	16.9	2.7 41.8
3	Icteric urine (84 nmol)	23.2	41.3	7.1	28.4				
	Rat bile (42 nmol)	39.1			61.9				
	Icteric urine (42 nmol) + rat bile (21 nmol)					Found:	29.3	26.4	4.1 40.6
						Calc.:	28.5	27.5	4.7 39.2
	Peak no.	1	2	3	4		1	2	3 4

components that might induce the formation of the β - and γ -azopigments as an artifact during diazotization, rat bile, human bile and icteric urine were treated with diazotized ethyl anthranilate either separately or in pre-mixed combinations: rat bile plus human bile, and rat bile plus icteric urine. The extracts were chromatographed and the percentage distributions determined. As shown in Table 2 the percentages of the mixed samples could be predicted fairly accurately from the proportions found with the individual samples. For the samples examined and under the reaction conditions applied diazo-coupling thus seems to be additive.

Properties of the ethyl anthranilate azopigments

In view of the complexity of the composition of the azopigments of some bile pigment samples, chromatography on CM-cellulose columns or t.l.c. does not offer any easy way to characterize unequivocally azopigments in unknown material. An attempt was therefore made to develop analytical tests that would allow further differentiation of azopigments on a micro-scale. For most tests 20–40 nmol of azopigment is adequate. Smaller samples are sufficient for t.l.c.

Only the already known azopigments, azobilirubin (α_0) and azobilirubin β -D-monoglucuronide (δ), and the dominant additional azopigments, β_1 , β_2 , γ_1 and γ_2 (see, e.g., Figs. 2 and 3) were studied in some detail. To a smaller extent the minor components α_2 , α_3 and x have also been examined.

The extinction spectra of synthetic azobilirubin and of the azopigments α_0 , β_1 , β_2 , γ_1 , γ_2 and δ in chloroform are very similar with two approximately equally intense extinction maxima at 535 ± 1 nm and at 336 ± 1 nm respectively. In all cases a shoulder or barely developed additional maximum was also found near 300 nm. The E_{546} values of purified synthetic azobilirubin in chloroform were found to be 4% higher than in pentan-2-one. No attempt was made to obtain absolute values of the molar extinction coefficients.

Hexuronic acid was detected only in the azopigments β_1 , β_2 , γ_1 , γ_2 and δ (Fig. 9 and Table 3). The colours and visible spectra of the reaction products formed on treating these azopigments or glucuronic acid with naphtharesorcinol were very similar. Indistinguishable colours were obtained from glucuronic acid and from galacturonic acid. It should be noted that naphtharesorcinol reacts with all common hexuronic acids (Marsh, 1966). Therefore, as the nature of the hexuronic acids present in the β - and γ -azopigments is not known, and as the colour yields obtained with different hexuronic acids may differ considerably (see e.g. Fig. 9), it was thought preferable to indicate directly the experimental relationship between colour yields and the

Fig. 9. Colour development of galacturonic acid (solid line no. 1), glucuronic acid (solid line no. 2) and ethyl anthranilate azopigments (experimental points) on treatment with naphtharesorcinol according to the method described by Fishman & Green (1955). Known amounts of the compounds mentioned, in 1 ml of water, were treated with 0.5 ml of a freshly prepared, filtered aqueous solution of naphtharesorcinol (0.4%) and 0.5 ml of 9M- H_2SO_4 . The open tubes containing the reaction mixtures were placed in a boiling water bath for 5 min, then closed and further kept at $100^\circ C$ for 115 min. The tubes were then placed on melting ice for 5 min, and the reaction mixtures treated with 2.5 ml of ethanol. Colour was then extracted with 2 ml of toluene low in S and read at 578 nm. The following azopigments were assayed: α_0 ($\blacklozenge$); α_2 ($\blacksquare$); α_3 ($\square$); x ($\circ$); β_1 ($\blacktriangledown$); β_2 ($\triangledown$); γ_1 ($\blacktriangle$); γ_2 ($\triangle$); δ ($\bullet$).

number of nmol of the test materials used (azopigments and reference hexuronic acids). Calculations based on the results of Fig. 9 show that the hexuronic acid/azobilirubin ratios tend to be slightly higher than 1 when the summation is expressed as glucuronic acid. On four preparations of δ -azopigment derived from rat bile values of the ratio glucuronic acid/azobilirubin were 0.94, 1.00, 0.94 and 1.11.

All azopigments except α_0 and x were attacked by ammonia vapour (Table 3) forming mainly two azopigment derivatives demonstrable on t.l.c. in redistilled chloroform: azobilirubin and its carboxylic acid amide in amounts equal to 10–20% and 80–90% respectively of total azopigment material formed. The formation of these derivatives from azopigments in the course of the ammonia treatment on thin-layer plates indicates that both hydrolysis and ammonolysis occur. The identification of the derivatives is based on the following evidence. In all cases the minor derivatives obtained by the ammonia treatment moved as synthetic

azobilirubin on t.l.c. with chloroform-methanol-water (65:25:3, by vol.) or with chloroform-methanol (19:1, v/v). The major derivative, which was chromatographically identical in all cases, moved as the carboxylic acid amide of azobilirubin obtained from δ -azopigment (from rat bile). The structure of the amide has been established by mass spectrometry (Compennolle, Jansen & Heirwegh, 1970).

The azopigments β_1 , β_2 , γ_1 and γ_2 were very sensitive to acid (Table 3). On incubation at 37°C in formic acid-water (3:7, v/v) they gradually changed into an azopigment (called δ' -azopigment) which in chloroform-methanol-water (65:25:3, by vol.) moved like δ -azopigment. Conversion of the azopigments was generally complete after 30–60 min. In general no further changes occurred when the incubations were prolonged for 25 h and similar treatments did not affect the chromatographic behaviour of azopigments α_0 and δ . In all cases examined azopigment colour recoveries were 84–93% after prolonged contact with dilute formic acid. Exceptionally 24 h treatment produced not more than 5% hydrolysis of the δ - and δ' -azopigments to azobilirubin.

When the pigments, on thin-layer plates, were kept for 13 h at room temperature over formic acid-water (1:1, v/v), azopigments α_0 , α_2 , α_3 and δ were not affected chromatographically. The azopigments β_1 and β_2 had completely disappeared and were largely converted into δ' -azopigment. However, some azobilirubin and trace amounts of other components were also present on the plates after t.l.c. Preparations of γ_1 -azopigment were largely converted into δ' -azopigment (about 75% of total azopigment) with 20–25% of chromatographically unchanged material still present on the plates. Traces of other azopigments were also formed. Apparently either the reaction on thin-layer plates follows a more complex course than in acid solutions or intermediates are more easily detected.

In methanol-12 M-HCl (10:1, v/v) and in methanol-1 M-HCl (10:1, v/v) solutions at 37°C the acid-labile azopigments, and also azopigments α_0 , δ and δ' , were transformed into a final reaction product which had the same R_F values as the synthetic monomethyl ester of azobilirubin, α_{OM} , on t.l.c. with chloroform or with chloroform-methanol (17:3, v/v). The structure of α_{OM} has been studied by mass spectrometry (Compennolle *et al.* 1970). With azopigment α_0 , formation of the methyl ester required 20–40 min in the more acidic system and 1–2 h in the other one. The conversion took more time (12–24 h) in the other cases. By t.l.c. of samples drawn from the reaction mixtures at various times after mixing the reaction components several intermediate reaction steps could be demonstrated. Very rapid conversion of azopigments β_1 and γ_1

into products, which apparently moved as azopigments β_2 and γ_2 respectively, was followed by a series of parallel and consecutive reaction steps in which the following intermediates were shown to occur: (1) δ' -azopigment, (2) the monomethyl esters of the β - and γ -azopigments and of δ' -azopigment, and (3) α_0 -azopigment. Similar changes took place when the methanol was replaced by ethanol.

Incubation of the β - and γ -azopigments for 2 h at 37°C in acetone-0.15 M-HCl (1:1, v/v) produced nearly complete conversion of the β -azopigments into δ' -azopigment. The reaction was partial with azopigment γ_1 and very limited with azopigment γ_2 .

In the initial stages of the work t.l.c. was performed with solvent systems containing formic acid, e.g. chloroform-methanol-formic acid (85:15:2, by vol.). As during t.l.c. partial conversion into δ' -azopigment of conjugated azopigments, especially of the β -group, took place the use of acid solvent systems cannot be recommended.

Among the hexuronic acid-containing azopigments only δ -azopigment was hydrolysed by the β -glucuronidase preparations tested. In general no further changes were observed when the incubations were prolonged from 2 to 24 h (Table 3). It should be noted that δ' -azopigment obtained by partial acid hydrolysis from either β - or γ -azopigments was not attacked at all after a 16 h incubation with β -glucuronidase from bovine liver (two δ' -azopigment preparations tested in each case). The lack of susceptibility cannot be due to a transformation of δ -azopigment into some resistant compound, as δ -preparations were hydrolysed to the same extent either before or after treatment with formic acid (Table 3). Incubation with β -glucuronidase from *E. coli* of δ' -azopigments derived by acid treatment from β - and γ -preparations was also investigated. The degree of hydrolysis, which was 3–5% after 2 h at 37°C, increased after 16 h incubation to 35 and 45% respectively. However, no inhibition of the conversion was obtained by adding saccharo-(1 $\rightarrow$ 4)-lactone to the incubation mixtures. No transformations occurred during the enzyme-free control incubations with buffer. It seems likely therefore that an enzymic reaction not mediated by β -glucuronidase was responsible for the observed processes. Although β -glucosidase activity could be detected in the β -glucuronidase preparations from ox liver, it could not be detected in the preparations of bacterial origin used in the present work.

Hydrolysis was complete with δ -azopigment from rat bile whereas preparations from human bile invariably contained a fraction (5–30%) that resisted prolonged enzymic incubation. It seems likely that the latter preparations contained some δ' -azopigment produced from labile conjugated azopigments in the course of their purification.

Table 3. *Properties of ethyl anthranilate azopigments*

Results were obtained as outlined in the Materials and Methods section. Azopigments were incubated with β -glucuronidase for 2 h at 37°C with and without added saccharo-(1 $\rightarrow$ 4)-lactone, except when the results are provided with an asterisk which indicates that serial incubations for 2, 4 and 16 h were performed. Hydrolysis is expressed as the percentage of azopigment converted into azobilirubin. Inhibition levels are expressed as a percentage of the amount of azobilirubin formed in the absence of saccharolactone inhibitor. Preparations δ^{***} were obtained by treating δ -preparations with formic acid vapour; the corresponding starting preparations, which were studied in parallel with δ^{***} , are denoted by δ^{**} . The number of experiments is indicated in parentheses. For the other tests (two to five experiments in each case) results are denoted by '+' (positive reaction) or '-' (negative reaction).										
Source of azopigment	Material tested	Reactivity towards			Hexuronic acid present	Treatment with β -glucuronidase from				
		Ammonia vapour	Formic acid	Diazo-methane		Bovine liver		<i>E. coli</i>		
						Hydrolysis	Inhibition	Hydrolysis	Inhibition	
Human bile	α_0	-	-	+	-					
	α_2	+	-	-	-			0 (1)		
	α_3	+	-	-	-			0 (2)*		
	β_1	+	+	+	+					
	β_2	+	+	+	+			0 (2)*		
	$\beta_1 + \beta_2$	+	+	+	+	0 (1)		0 (2)		
	γ_1	+	+	+	+	0 (1)		0 (2)*		
	γ_2	+	+	+	+	0 (1)		0 (2)*		
	$\gamma_1 + \gamma_2$					0 (1)		0 (2)		
	δ	+	-	+	+	77-95 (4)*	95-100 (4)*	80-95 (2)	100 (2)	
	δ^{**}					77 (1)	100 (1)			
	δ^{***}					75 (1)	100 (1)			
	Σ									
Rat bile	α_0	-	-	+	-					
	δ	-	-	+	-	96-100 (5)*	97-100 (5)*	90-100 (3)*	100 (3)	
	δ^{**}	+	-	+	+	95 (1)	100 (1)			
	δ^{***}					95 (1)	100 (1)			

Results were obtained as outlined in the Materials and Methods section. Azopigments were incubated with β -glucuronidase for 2 h at 37°C with and without added saccharo-(1 $\rightarrow$ 4)-lactone, except when the results are provided with an asterisk which indicates that serial incubations for 2, 4 and 16 h were performed. Hydrolysis is expressed as the percentage of azopigment converted into azobilirubin. Inhibition levels are expressed as a percentage of the amount of azobilirubin formed in the absence of saccharolactone inhibitor. Preparations δ^{***} were obtained by treating δ -preparations with formic acid vapour; the corresponding starting preparations, which were studied in parallel with δ^{***} , are denoted by δ^{**} . The number of experiments is indicated in parentheses. For the other tests (two to five experiments in each case) results are denoted by '+' (positive reaction) or '—' (negative reaction).

All azopigments except α_2 , α_3 and x reacted with diazomethane and were transformed into pigments of increased R_F (Table 1). None of the derived pigments behaved chromatographically as any one of the parent compounds. The derivatives of the azopigments β_1 , β_2 , γ_1 and γ_2 generally showed two closely moving spots eventually accompanied by traces of other azopigment derivatives. In all cases conversion into the corresponding, characteristic derivatives was nearly instantaneous. By mass spectrometry monomethoxycarbonyl ester structures could be assigned to the derivatives obtained from azopigments α_0 and δ (Compennolle *et al.* 1970).

DISCUSSION

Diazotization of 1 mol of the tetrapyrrolic bile pigments, bilirubin and conjugated bilirubin, leads to the formation of 2 mol of dipyrrolic azopigments (Fischer & Haberland, 1935; Overbeek, Vink & Deenstra, 1955; Brodersen, 1960; Lucassen, 1961; Compennolle *et al.* 1970). According to current concepts only bilirubin and bilirubin β -D-glucuronide are expected to be quantitatively important in bile of higher animals and in icteric urine and serum of man (Schmid & Lester, 1966; Lester & Troxler, 1969). Bilirubin sulphate has been claimed to be detected in small amounts in some bile pigment preparations (Isselbacher & McCarthy, 1958, 1959; Schoenfield, Bollman & Hoffman, 1962; Vegas, 1963; Tenhunen, 1965; Noir, Groszman & De Waltz, 1966; Noir, De Waltz & Garay, 1967) but this has been contested by Watson (1958), Gregory & Watson (1962) and Weber & Schalm (1965). According to Tenhunen (1965) phosphate and taurine conjugates would also occur to minor extents in some cases. Obviously, if this view is correct, two main azopigment derivatives would be formed on diazotization: azobilirubin (α_0 ; also called azopigment-A) derived from bilirubin, and azobilirubin β -D-monoglucuronide (δ -azopigment; also called azopigment-B) originating from bilirubin diglucuronide. The bile pigments could then easily be determined quantitatively and investigated through the derived azopigments. The basic assumption that such an interpretation is valid is present implicitly in the greater part of recent publications on diazo-positive bile pigments.

Evidence is accumulating that at least in some cases the existence of bilirubin β -D-monoglucuronide has to be considered. By using reverse-phase column chromatography Billing *et al.* (1957) isolated a bile pigment (called pigment-I) with the properties of bilirubin monoglucuronide. Since then the existence of this pigment has been doubted as in some cases isolated preparations could be changed into mixtures of more and of less hydrophobic tetrapyrroles by heating (Billing *et al.* 1957; Nosslin,

1960) or by rechromatography (Gregory, 1963; Weber *et al.* 1963). The concept then emerged that pigment-I is a monomolecular complex of bilirubin and bilirubin diglucuronide. Tailing of the preparations in separation columns was considered to indicate dissociation of the complex. Although complex formation might have a role *in vivo* (e.g. Callahan & Schmid, 1969) no direct chemical evidence for its existence *in vitro* has been given. On the contrary, Schoenfield & Bollman (1963) were able to rechromatograph pigment-I isolated from rat bile, and Noir *et al.* (1965) could separate by paper chromatography, not only pigment-I and bilirubin diglucuronide, but also the corresponding conjugates of biliverdin. A pigment with the properties of pigment-I has been extracted into pentan-2-one from human and rat bile, and chromatographed repeatedly on CM-cellulose columns by using pentan-2-one-formamide gradients without obtaining any evidence for its dissociation (our unpublished work). Similarly Ostrow & Murphy (1970) have been able to prepare stable preparations of pigment-I by extraction procedures. Finally kinetic studies support the existence of monoconjugates of bilirubin (Brodersen, 1962; Brodersen, Hermann & Vind, 1963; Van Roy & Heirwegh, 1968). Therefore, as diazotization of monoconjugates of bilirubin (a tetrapyrrole) would produce equal amounts of conjugated and unconjugated azobilirubin, the term azobilirubin for unconjugated azopigment is unsatisfactory. However, as yet a succinct and unequivocal name seems to be lacking.

The great variety of azopigments derived from human bile and from icteric urine contrasts sharply with the current view that only two azopigments, azobilirubin (α_0) and azobilirubin β -D-monoglucuronide (δ), have any quantitative importance. Earlier reported studies with β -glucuronidase are inconclusive. Schmid (1957), who was able to hydrolyse completely preparations of azopigment-B, did not mention any control experiments with specific enzyme inhibitors. In the course of similar work Billing *et al.* (1957) obtained only partial hydrolysis with two out of three enzyme preparations tested, and the enzymic reactions were only partially inhibited by saccharo-(1 $\rightarrow$ 4)-lactone. The latter work is consistent with the complete resistance to attack by β -glucuronidase of azopigments β_1 , β_2 , γ_1 and γ_2 (Table 3), which were the dominant additional azobilirubin derivatives observed in the present work (Figs. 3 and 4). Results obtained with various δ -preparations suggest strongly that, depending on the source of the bile pigments, even such preparations may contain hexuronic acid conjugates different from azobilirubin β -D-monoglucuronide. This may indicate that already in the course of diazotization or chromatographic separation some δ' -azopigment was formed by the removal of

acid-labile conjugating groups. It may be noted that treatment of a purified δ' -preparation with diazomethane yielded a derivative that moved slightly more rapidly than δ_M (prepared from δ -azopigment from rat bile) on t.l.c. with chloroform-methanol-formic acid (85:15:2, by vol.)

The azopigments considered in the present work fall into six main categories, which can be distinguished by their chromatographic behaviour (Table 1 and Figs. 1-5) and by chemical tests (Table 3). The pigments of the β - and γ -groups are most easily differentiated on the basis of their R_F values (Table 1 and Figs. 1 and 2) and their relative susceptibilities to hydrolysis by dilute formic acid, the pigments of the β -group being much more labile than those of the other group. Indeed the pronounced acid-lability of the β -azopigments was very disturbing when attempts at their isolation and purification were made. Great care must be taken in checking the final preparations for acid-stable contaminants that may have accumulated in the course of the operations involved. In contrast, the pigments of the γ -group could be purified with minimal losses.

The question should be raised whether the conjugated azopigments different from δ -azopigment could be artifacts. A distinction should be made between the α_2 - and α_3 -azopigments and the pigments of the β - and the γ -groups. The former azopigments have been shown to be monoglycosides of azobilirubin containing one neutral sugar residue per azobilirubin moiety (our unpublished work). The β - and γ -azopigments, which all contain hexuronic acid (Fig. 9), might originate from either the conjugated dipyrrole moieties of bilirubin mono- or di-glucuronide or from azobilirubin β -D-monoglucuronide. Mixed coupling experiments render the formation of the β - and γ -azopigments as artifacts in the course of diazotization unlikely (Table 2). It seems also very improbable that the β - and γ -azopigments would be either molecular complexes of two or more δ -molecules or of δ -azopigment and some other compounds. Indeed it is difficult to imagine how such complexes could behave as well-defined compounds with two very different chromatographic systems, namely t.l.c. in various solvent systems and column chromatography on CM-cellulose with increasing concentrations of the hydrogen-bond-breaking material, formamide, as the eluent. The rechromatography of pigments on thin-layer plates and the formation of characteristic derivatives by treatment with diazomethane also argue against complex-formation. Further, why would δ' -azopigment formed by partial acid hydrolysis from β - or γ -azopigment resist attack by β -glucuronidase whereas the hydrolysis of δ -preparations after prolonged contact with dilute formic acid is unimpaired?

The evidence available thus strongly suggests that the heterogeneity of bile pigments is far greater than hitherto suspected. Wide variation seems to occur depending on the biological fluid (e.g. urine versus bile) or the species examined (e.g. man versus rat). Work in progress also shows that manipulation of the common bile duct of the rat may influence considerably the bile pigment composition of rat bile (Fig. 2, track 1).

How then can it be explained that the type of heterogeneity most typically revealed by the present work, i.e. by the formation of hexuronic acid-containing azopigments that differ in several respects from azobilirubin- β -D-monoglucuronide, does not seem to have been recognized before? To prepare azopigments for structural work bile pigments have generally been diazotized in strongly acid aqueous medium eventually fortified with ethanol or another water-miscible organic solvent as a 'reaction accelerator'. Under such reaction conditions the β -azopigments, and to a smaller degree the γ -azopigments, are easily converted into δ' -azopigment, which behaves chromatographically very much like azobilirubin β -D-monoglucuronide. The α_2 - and α_3 -azopigments, which were detected only in very small amounts after diazotization of human bile pigments, were difficult to separate from azobilirubin by t.l.c.

As yet insufficient information is available to establish completely the structures of the α_2 -, α_3 -, β - and γ -azopigments.

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