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The Isolation and Characterization of Bilirubin Diglucuronide, the Major Bilirubin Conjugate in Dog and Human Bile

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The chemical structure of the major conjugate of bilirubin was unequivocally elucidated by structural analysis. The conjugated bilirubins were first separated from the lipid components of human duodenal aspirates or dog gall-bladder bile, and then resolved by t.l.c. into a series of tetrapyrroles. The major tetrapyrrole was then converted into its more stable dipyrrolic azo derivative for further analysis. The conjugated moiety of the azopigment was characterized after methanolysis with sodium methoxide. This reaction yields two types of product, those soluble in water and those soluble in organic solvents. The organic-soluble fraction was shown by t.l.c. and mass spectrometry to contain the methyl esters of the dipyrrolic azo derivatives of bilirubin. The water-soluble materials were analysed by enzymic procedures, t.l.c., n.m.r. spectrometry and combined g.l.c. and mass spectrometry. This analysis showed that the only water-soluble product resulting from the methanolysis was glucuronic acid. The structure was identical with that of pure standards, on both mass spectrometry and n.m.r. spectroscopy. No contaminating moieties were found. Quantitative measurement indicated that the glucuronic acid had been released in a 1:1 molar ratio with the resulting methyl esters of the dipyrrolic azo derivatives of bilirubin. This unequivocally establishes bilirubin diglucuronide as the major pigment present in bile. Past problems with identification of bilirubin diglucuronide were shown to originate from procedures which resulted in incomplete separation and isolation of the azopigments of the conjugated bilirubins, owing to contamination by biliary lipids.

Billing *et al.* (1957) and Schmid (1957) reported that bilirubin, the major end product of haem catabolism, is secreted in the form of bilirubin diglucuronide. This problem has been re-examined with more precise analytical techniques (Heirwegh *et al.*, 1970; Kuenzle, 1970; Gordon *et al.*, 1974), and the results obtained indicate that bilirubin appears in bile conjugated to a number of compounds. Heirwegh and his group have separated a number of conjugated azo derivatives from dog gall-bladder bile, and have identified a series of monosaccharidic moieties as the conjugating groups (Heirwegh *et al.*, 1970; Fevery *et al.*, 1971; Heirwegh *et al.*, 1975). They have again suggested that glucuronic acid is the conjugating moiety in the major azo derivative. Kuenzle (1970), on the other hand, has been unable to detect simple monosaccharides, but instead, starting from pooled human fistula bile, obtained after operation, has isolated in low yield a set of conjugates in which bilirubin is linked to glucuronic acid-containing disaccharides. These two sets of findings have been difficult to reconcile.

We present here a new examination of this problem. We have now unequivocally identified bilirubin diglucuronide as the major tetrapyrrole in human duodenal aspirates and in dog bile. We have used the methodological approach of isolating the tetrapyrrole, and then forming and identifying its stable dipyrrolic azo derivative. The key to our success in isolating and identifying this conjugate appears to have been the use of a technique which removes over 90% of biliary lipids, in the initial stage of the isolation. As a result, a large number of masking interactions have been avoided in subsequent manipulations. Purification techniques have been more productive, and we have been able to collect enough material to characterize our final product unambiguously, by using n.m.r. spectrometry and combined g.l.c. and mass spectrometry.

Materials and Methods

Bile obtained from the gall bladders of young mongrel dogs under light pentobarbital anaesthesia was stored in the dark at −10°C until processed,

generally for a period of less than 1 week. Human bile was obtained by duodenal aspiration. This source material was processed immediately.

Isolation of bile pigments from bile

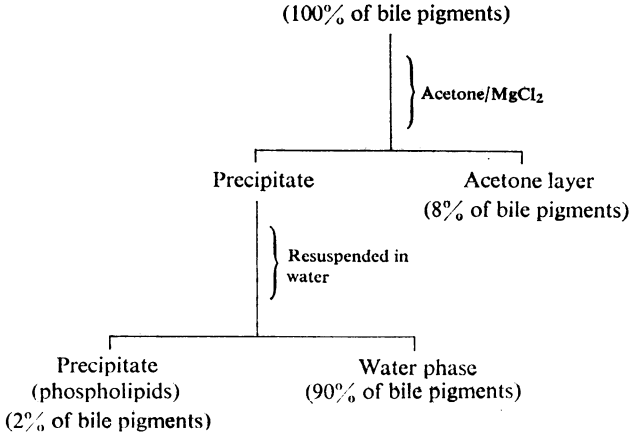
Investigations of the chemical structure of the bile pigments have classically been made after bilirubin and its conjugates have been converted into their more stable dipyrrolic azo derivatives (Billing *et al.*, 1957; Schmid, 1957; Kuenzle, 1970; Heirwegh *et al.*, 1970; Fevery *et al.*, 1971; Gordon *et al.*, 1974; Heirwegh *et al.*, 1975). In our initial studies we also used this approach. The bile pigments were cleaved and coupled with ethyl anthranilate and separated into a series of azo pigments by t.l.c. (Gordon *et al.*, 1974; Plate 1). The system used was similar to that described by Heirwegh *et al.* (1970). The major azopigment detected was the azopigment δ. After methanolysis of this azopigment, approximately equimolar amounts of glucuronic acid and azopigment α_0' (the family of methylated azobilirubins) were formed. Glucose was also detected, however, but in amounts too small for it to be considered to be part of an appropriate disaccharide. The azopigment δ was therefore also subjected to mild acid hydrolysis (0.1 M-HCl for 0.5 h at 20°C). The reaction did not go to completion, but yielded, as products, azopigment α_0 and a small amount of azopigment α_3. This again suggested that azopigment α_3 had arisen from hydrolysis of a more complex compound or that it was running as a contaminant in this preparation of azopigment δ. Re-examination of our original chromatograms for contaminants showed that phosphatidylcholine and taurocholate were, indeed, associated with azopigment δ (Plate 1).

We therefore reconsidered the rationale underlying our initial procedure for the isolation of the pigments. The bilirubin in bile is fairly tightly associated with the micellar phase of this secretion. Verschure & Mijnlieff (1956) and Juniper (1965) have emphasized that, when the micelles in bile are separated in an ultracentrifugal field, the total amount of bilirubin moves with the micelles. It was thought necessary to remove these materials before separating the bile pigments. We therefore devised a procedure which removed over 90% of the phospholipids in the first step and still preserved recovery of the bile pigments in high yield (see Scheme 1).

The phospholipids and bile pigments were precipitated from bile by using acetone saturated with $MgCl_2$ (1.0 vol. of bile: 25 vol. of acetone saturated with $MgCl_2,6H_2O$). During this precipitation the samples were protected from light, and were kept at −10°C for 18 h. The acetone layer was removed and discarded. The precipitate, which contained the phospholipids and bile pigments, was resuspended in water (1 vol. precipitate: 10 ml of water), and the insoluble phospholipids were removed by centrifugation at 8000*g* for 20 min. This precipitate was then washed twice with a small volume of distilled water. The water phases, which contained the bile pigments, were combined, concentrated and used for analyses (Scheme 1 indicates the distribution of the bile pigments in this procedure).

Separation of the bile pigments found in bile

Our aim was to characterize the major bilirubin conjugate present in bile. The preponderant dipyrrolic azopigment δ is formed by the schism



Scheme 1. *Distribution of bile pigments in the initial separation procedure*

See the Materials and Methods section for details of procedures.

into half-molecules of the bilirubin tetrapyrroles containing the appropriate conjugates. The nature of the major parent molecule of the azopigment δ can thus be elucidated only by its separation from bile. We therefore devised a method for separating the parent bilirubin tetrapyrroles present in bile, and focused our attention on the major component in this group.

The initial procedure, outlined above, for separating phosphatidylcholine and bile salts from the bile pigments, was carried out. The combined aqueous phase obtained from this was extracted three times with an equal volume of chloroform containing 10mM-tetraheptylammonium chloride (compound 9505; Distillation Products Industries, Rochester, NY, U.S.A.) (Hofmann, 1967). The latter quantitatively transfers the bile pigments to the chloroform phase by an anionic-exchange reaction. The organic phases were combined and dried in a rotary flash evaporator. The residue was dissolved in a small volume of chloroform/methanol (1:1, v/v) and spotted on silica-gel H plates (E.M. 5763; 0.250mm × 20cm). The chromatogram was developed twice in the solvent system propan-1-ol/water (17:2, v/v), and five families of yellow bands were thus made visible (Plate 2). Only the most prominent component of the lower family A was analysed at this time. It was extracted from the silica gel with 50% (v/v) methanol, and the ethyl anthranilate azopigments (Gordon *et al.*, 1974) were formed. Chromatographic analyses showed that the azopigment δ was the only derivative formed as a result of this reaction. These findings indicate that in this case the dipyrrolic azopigment δ arises from each half of the molecule.

Separation and purification of the azopigment δ

The product azopigment δ was then spotted on silica-gel H thin-layer plates (E.M. 5763; 0.250mm × 20cm × 20cm; Brinkman Co., Rexdale, Ont., Canada). The chromatogram was developed twice in the solvent system chloroform/methanol (17:3, v/v) at 20°C in the dark. This step was designed to separate any contaminating azopigments α_0 and α_3 from the azopigment δ. Traces of cholesterol were removed by developing the chromatogram twice in diethyl ether. Any contaminating azopigments γ and β were separated from azopigment δ by developing the chromatogram twice in the solvent system chloroform/methanol/water (65:25:2, by vol.). Azopigment δ was extracted from the silica gel with butan-1-ol, and the extract was evaporated to dryness in a rotary flash evaporator. It was then dissolved in a small volume of chloroform/methanol (1:1, v/v), spotted on an Anasil H plate (0.250mm × 20cm × 20cm; Analabs, North Haven, CT, U.S.A.), and a chromatogram was developed in the solvent system propan-1-ol/water (17:2, v/v). In this system azopigment δ appeared pure (Fig. 1). The last traces of phosphatidylcholine and taurocholate had been separated from the azopigment. The azopigment was then extracted from the silica gel with butan-1-ol, evaporated to dryness, and stored in the dark under N_2. It is referred to below as purified azopigment δ.

Chemical analysis of purified azopigment δ

The fundamental approach underlying the procedures utilized to analyse and characterize purified azopigment δ is one in which attempts are made to hydrolyse the conjugating moiety from the compound, and the products are then characterized and quantified. The various methods used to carry this out are detailed below.

Acid and alkaline hydrolysis. Azopigment δ (100–200μg) was dissolved in 0.1ml of methanol. Acid hydrolysis with 0.1M-HCl was carried out in one of two ways: at room temperature (25°C) for 0.5h, or at 60°C in a sealed vessel for 1h. Alkaline hydrolysis with 0.1M-NaOH was also carried out under the same conditions. The reactions were stopped by the addition of butan-1-ol, and the product azopigment was separated from azopigment δ by t.l.c. as described above. The amount of each azopigment present was quantified as previously described (Gordon *et al.*, 1974). After acid hydrolysis the combined water phases were freeze-dried, and after alkaline hydrolysis Na^+ was removed by treating the sample with resin (Resyn 101 H^+) before the water phase was freeze-dried. The composition of the resulting material was determined by t.l.c. and classical chemical analyses.

Hydrolysis by NH_3 vapour. The procedure followed was essentially the same as that outlined by Fevery

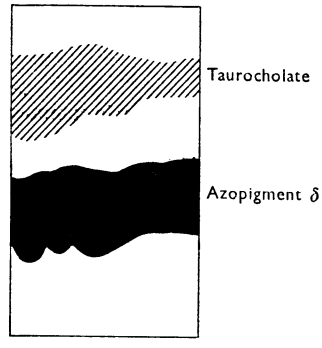


Fig. 1. *Sketch of thin-layer chromatogram of azopigment δ showing its final separation from the small amount of remaining taurocholate*

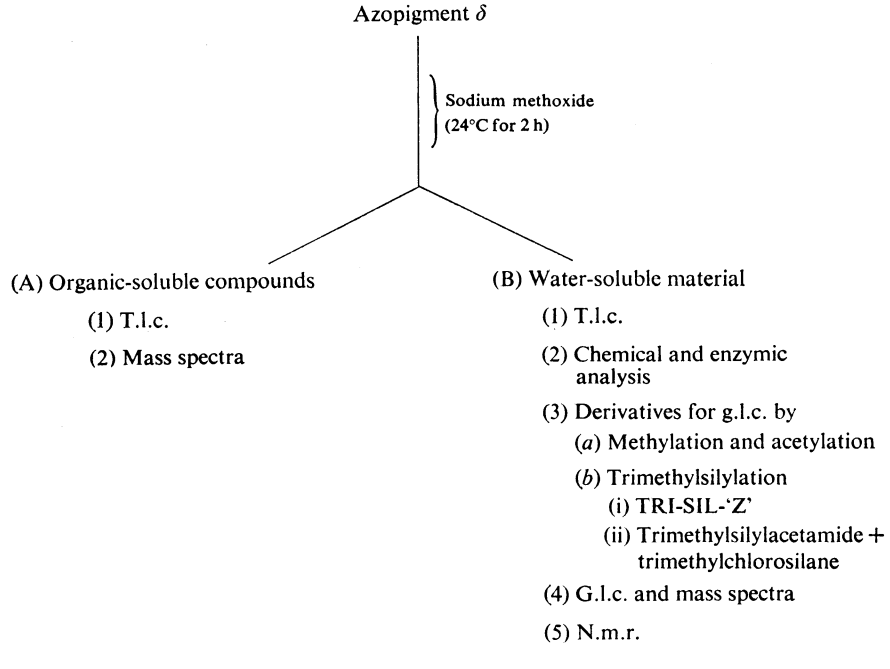
Azopigment δ was reddish-purple, and, after exposure to iodine vapour, the taurocholate became visible as a yellow band. The chromatographic steps are given in the text.

et al. (1971). Azopigment δ and standards (glucose, ribose, xylose, glucuronic acid and D-glucurono-lactone) were spotted on pre-coated silica-gel H plates (E.M. 5763; 0.250mm×20cm×20cm) and exposed to NH_3 vapour (27%, w/v) for 18h in the dark at room temperature. After removal of the NH_3 vapour, the chromatogram was developed in the solvent system chloroform/methanol (17:3, v/v). In this system, the non-polar azopigments run to the top of the plate. The chromatogram was then developed in the solvent system propan-1-ol/water (17:3, v/v), air-dried, sprayed with aniline diphenylamine and heated at 110°C for 15min. Blue spots on the chromatogram indicated the presence of reducing carbohydrates.

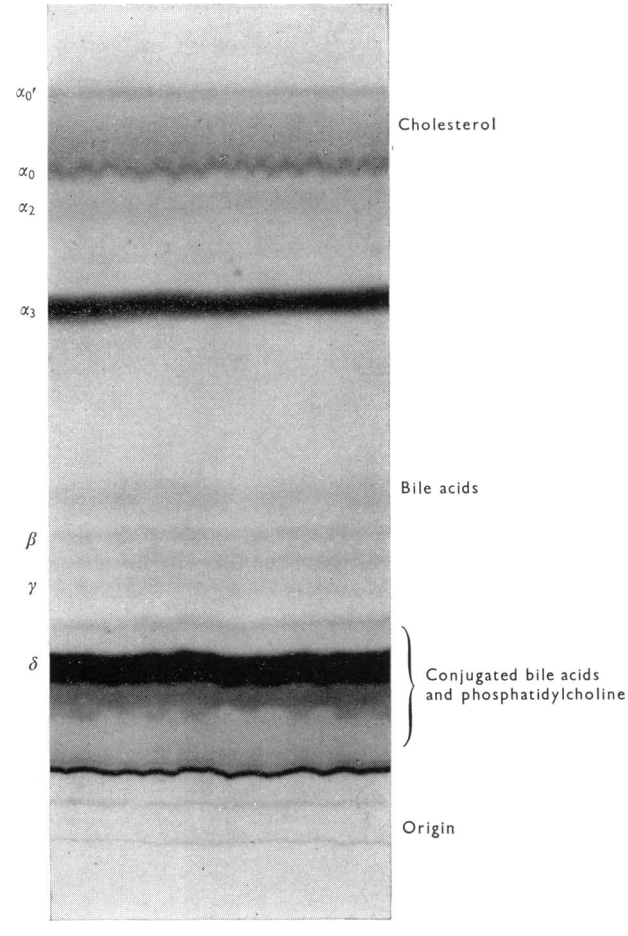
Enzymic hydrolysis. The effects of β-glucuronidase (EC 3.2.1.31), β-glucosidase (EC 3.2.1.21) (Sigma Chemical Co., St. Louis, MO, U.S.A.) and Mylase P (*Aspergillus oryzae*; EC 3.4.24.4) (Nutritional Biochemical Co., Cleveland, OH, U.S.A.) on azopigment δ were determined, by use of methods previously described (Fevery *et al.*, 1971; Gordon *et al.*, 1974). Azopigment δ (150–300μg) was dissolved in 0.1ml of methanol, and 2.0ml of sodium acetate buffer (0.05M, pH5) was added. The reaction was initiated by the addition of β-glucosidase (0.5mg of emulsin from almonds), or of β-glucuronidase (bovine liver; 0.2mg/ml), or of Mylase P (1.0mg/ml). After incubation at 37°C for 120min the reaction was stopped by the addition of butan-1-ol and resin (Resyn 101 H^+). The azopigments were removed from the water phases by repeated extraction with butan-1-ol. The azopigments formed during these reactions were characterized by t.l.c. and then quantified as described previously (Gordon *et al.*, 1974). The water phases were analysed for the presence of carbohydrates and uronic acids.

Methanolysis. Methanolysis of the azopigment (200–500μg) was accomplished by using sodium methoxide, as described previously (Gordon *et al.*, 1974). The reaction was carried out in a sealed vessel at room temperature for 2h, and stopped by the addition of approx. 500mg of resin (Resyn 101 H^+). The resin was removed by filtration, and was repeatedly washed with small volumes of methanol. The combined washes were dried in a rotary flash evaporator, and the residue was redissolved in water/butan-1-ol (1:1, v/v). The two phases were separated by centrifugation. The organic phase was removed and the water phase was re-extracted with butan-1-ol until colourless. The organic phases were pooled and back-washed three times with water. Total azopigments were quantified spectrophotometrically. The water and organic phases were then evaporated to dryness separately. The analyses conducted on these two phases are summarized in Scheme 2.

Fraction A: organic-soluble compounds. The degree of methanolysis was indicated by the



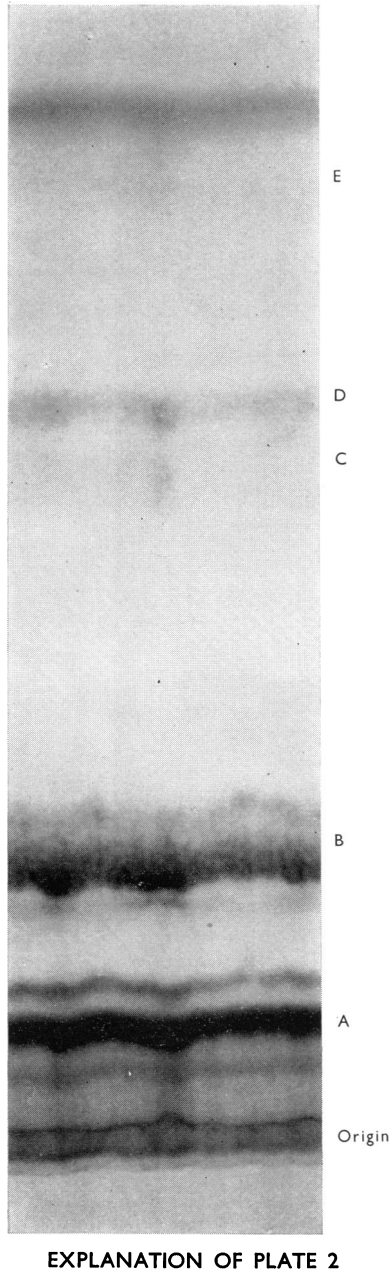
Scheme 2. *Procedures used to identify the products of the methanolysis of azopigment* δ



EXPLANATION OF PLATE 1

Thin-layer chromatogram of the ethyl anthranilate azopigments formed from the pigments in dog gall-bladder bile, showing the presence and position of contaminating materials

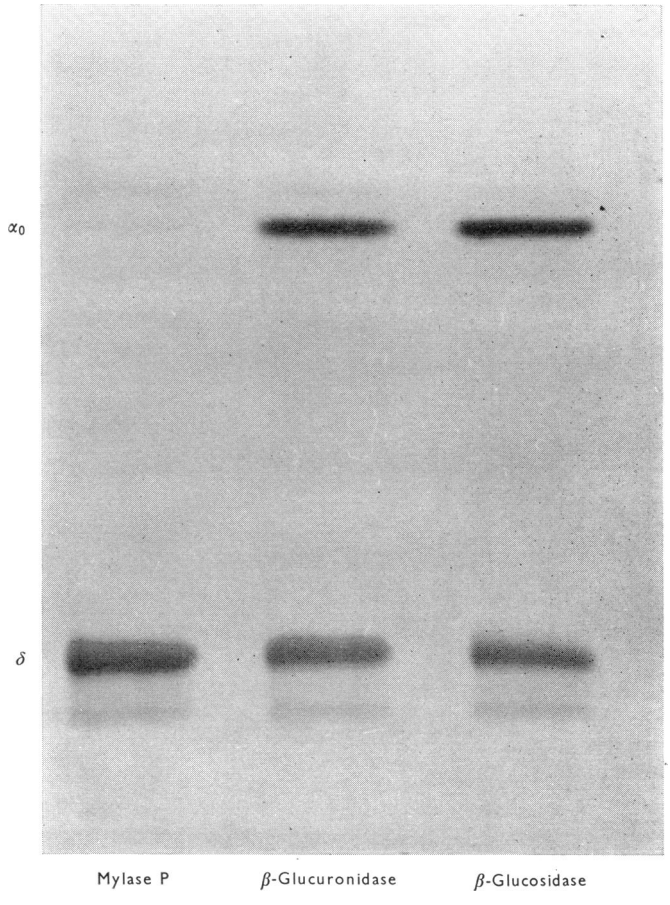
The azopigments were reddish-purple and the biliary lipids yellow, after exposure to iodine vapour.



EXPLANATION OF PLATE 2

Thin-layer chromatogram of bilirubin and its conjugates

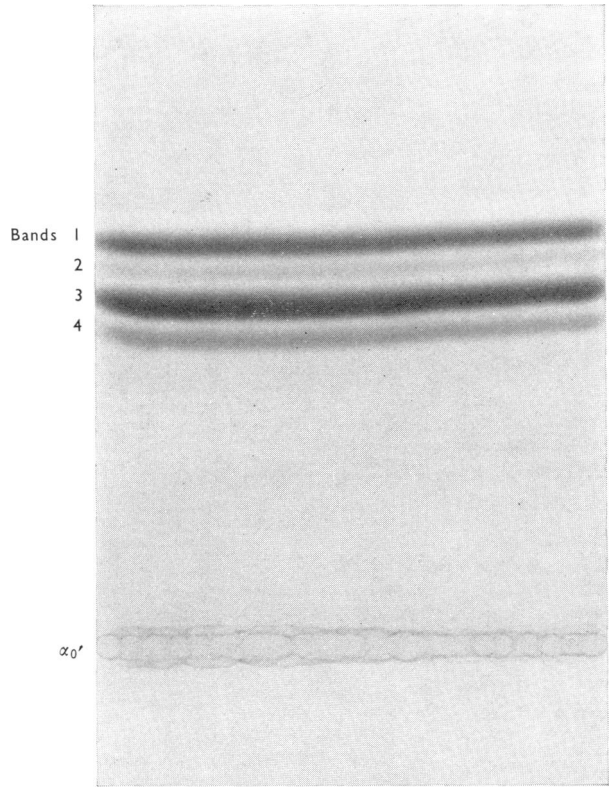
Five families of yellow compounds were detected, and are denoted A, B, C, D and E. This chromatogram was developed twice in a solvent system of propan-1-ol/water (17:2, v/v).



EXPLANATION OF PLATE 3

Dipyrrolic azopigments formed by treatment of azopigment δ with Mylase P, β-glucuronidase and β-glucosidase

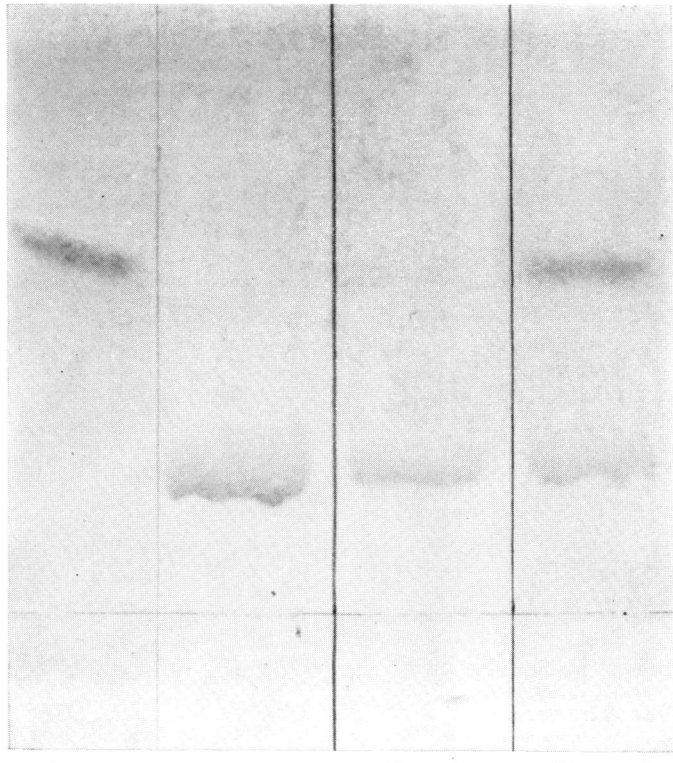
This chromatogram was developed in the solvent system chloroform/methanol/water (65:25:3, by vol.). See the text for further details.



EXPLANATION OF PLATE 4

Thin-layer chromatogram of the methyl esters formed on methanolysis of azopigment δ, *showing the separation of azopigment* α_0' *into multiple bands*

This chromatogram was developed ten times in the solvent system chloroform/benzene (1:1, v/v).



EXPLANATION OF PLATE 5

Thin-layer chromatogram of the compounds in the water-soluble fraction B after methanolysis of azopigment δ

This chromatogram was developed in the solvent system propan-1-ol/water (17:3, v/v).

amounts of the organic-soluble azopigments of bilirubin formed during the reaction (azopigments α_0 and α_0'), and the amount of azopigment δ which remained. During the methanolysis, the methyl esters of the dipyrrolic azopigments of bilirubin (that is, of the unconjugated molecule) were formed. These were separated into their different isomeric forms by t.l.c., and were identified by their mass spectra, as described previously (Gordon *et al.*, 1974).

Fraction B: water-soluble compounds. The following analyses were conducted on the material in fraction B.

(1) T.l.c. A portion of this fraction (10–20μg) was spotted on silica-gel H plates (E.M. 5538; 0.250mm × 20cm × 20cm), and chromatograms were developed twice in propan-1-ol/water (17:3, v/v). Reducing carbohydrates were detected with aniline diphenylamine, amino acids with ninhydrin spray, and biliary lipids by exposure to iodine vapour.

(2) Chemical and enzymic analyses. Samples were tested for the presence of reducing sugars (glucose, fructose or galactose), heptulose, hexosamines, pentose, uronic acids and biliary lipids. The concentration of glucose in the sample was measured by the glucose oxidase method (Kaplan, 1957), that of uronic acids by utilizing the naphtharesorcinol and carbazole reactions (Ashwell, 1957*c*), that of fructose by the method of Roe as outlined by Ashwell (1957*a*), that of hexosamine by the Elson–Morgan reaction (Ashwell, 1957*d*), that of pentose by the orcinol method (Ashwell, 1957*b*) and that of galactose by using the Boehringer Mannheim Biochemica kit (15921 TGAN). Bile acids were analysed by the procedure of Murphy *et al.* (1970).

(3) Combined g.l.c. and mass spectrometry. The following derivatives were made of the compounds in fraction B.

(*a*) Methylated and acetylated derivatives. The compounds were methylated with diazomethane and acetylated with acetic anhydride as previously described (Gordon *et al.*, 1974).

(*b*) Trimethylsilylated derivatives. The silylated derivatives were made by two different methods: (i) 100μl of TRI-SIL-'Z' (Pierce Chemical Co., Rockville, IL, U.S.A.) was added to 100μg of freeze-dried fraction B, and the reaction was allowed to proceed at room temperature for 1h; (ii) super-dry pyridine (0.2ml) was added to 100μg of dried fraction B, then 50μl of trimethylsilylacetamide was added, followed by 5μl of trimethylchlorosilane, and the mixture was heated for 10min at 60°C. The silylated derivatives formed in these reactions, together with known amounts of glucuronic acid and glucuronolactone treated in the same manner, were analysed by g.l.c. and mass spectrometry. Both methods were found to yield acceptable products.

The g.l.c.–mass spectrometry was carried out on an LKB 9000 instrument. Operating conditions were as follows: glass column, 6ft × $\frac{1}{4}$in (1.8m × 66mm); 3% OV-1 on Chromosorb W HP, 80–120mesh, isothermal at 210°C; injection port 290°C; helium flow rate approx. 40ml/min; separation 280°C, 70eV at 60μA; scan range *m/e* 10–700 in 5s. The mass-spectra data were handled, on-line, by a Varian SS-100 MS data system.

Gas–liquid chromatograms were also obtained on a Hewlett–Packard Research Chromatograph, model 5830A. Operating conditions were as follows: two glass columns 2ft × $\frac{1}{8}$in (0.6m × 2mm); 3% OV-101 on Chromosorb W HP, 80–120 mesh; column temperature programmed from 130 to 250°C at 2°C/min with an initial hold of 2min at 130°C; injection port at 265°C; detector at 265°C; nitrogen flow rate 24ml/min.

(4) N.m.r. spectroscopy. Fraction B was chromatographed as described above, and an area on the chromatogram corresponding in position to the carbohydrate moiety detected in this system was extracted with water. The n.m.r. spectrum of this was then recorded in a 220MHz Varian spectrometer.

Results and Discussion

We have isolated from dog gall-bladder bile and human duodenal aspirates the major conjugate of bilirubin, which has been named tetrapyrrole A. Since this compound would not be expected to be very stable, we pursued the classical approach to identification of the bilirubin conjugates. We coupled this conjugated tetrapyrrole with ethyl anthranilate to form the more stable dipyrrolic azopigment products. The two half-molecules were found to yield, in this instance, the single azopigment δ, indicating that the conjugating moiety for each half of the molecule is probably the same. We therefore then directed our efforts to identifying the chemical nature of this more stable azopigment.

In our initial experiments azopigment δ was separated and purified by t.l.c. The water-soluble compounds detected after methanolysis of this preparation were glucuronic acid and glucose, identified both by t.l.c. and by combined g.l.c. and mass spectrometry. However, these constituents were not detected in equimolar amounts, and further analyses of this preparation indicated that it was contaminated with small amounts of taurocholate and of azopigment α_3, adsorbed on to phosphatidylcholine. The removal of phospholipids from the bile before the extraction of the tetrapyrrole, or the formation of the azo derivative, eliminated these contaminants.

Acid and alkaline hydrolysis of purified azopigment δ

After mild acid treatment of purified azopigment δ,

Table 1. *Distribution of azopigments at the end of the 2h period of enzymic hydrolysis*

For details see the text.

Treatment	No. of experiments	Distribution of azopigments: Without enzyme α_0'	α_0	δ	With enzyme α_0'	α_0	δ
β-Glucuronidase	9	5.0	5.0	90.0	6.0	72.0	22.0
β-Glucosidase	6	2.0	2.0	96.0	2.0	49.0	49.0
Mylase P	7	1.0	1.0	98.0	2.0		98.0

bands corresponding to glucuronic acid and minute amounts of glucuronolactone were detected by t.l.c. After exposure of the compound to NH_3 vapour, or mild alkaline treatment, only glucuronic acid was detected. Under the conditions utilized here, the hydrolysis of azopigment δ was not complete. Treatments with stronger acid or alkali resulted in degradation of both the whole compound and the products of the hydrolysis.

Enzymic hydrolysis of the azopigment

Azopigment δ was partially hydrolysed by the action of β-glucuronidase and β-glucosidase (see Table 1 and Plate 3). A band corresponding to glucuronic acid and the azopigment of bilirubin were detected by t.l.c. as the only products formed in these reactions. Azopigment δ was not converted into other azo derivatives by the action of Mylase P, indicating that acyl sulphate groups were not present in the compound. The hydrolysis by β-glucuronidase of azopigment δ supports the suggestion of Compernolle *et al.* (1971) that glucuronic acid is joined to bilirubin by an ester linkage. The β-glucosidase utilized here, derived from almond emulsin, is contaminated by β-glucuronidase activity (Baumann & Pigman, 1957). The hydrolytic effect therefore cannot be construed as being specific.

Methanolysis of azopigment δ

Azopigment δ was completely cleaved by treatment with sodium methoxide at room temperature for 2h. The organic-soluble products formed during this reaction were α_0 (the azopigment of unconjugated bilirubin) in trace amounts and α_0' (the methyl esters of the azopigment of unconjugated bilirubin). These separate clearly in the system described by Gordon *et al.* (1974). When azopigment α_0' formed in this reaction was developed in a solvent system that resolves the methyl esters of the azopigment of bilirubin, five fractions were found. A very small proportion remained at the origin, and the remainder was resolved into four well-defined separate bands (see Plate 4). In this instance, as in our previous analysis of the α_0' azopigments arising from the methanolysis of azopigment α_3 (Gordon *et al.*, 1974), we found, by mass spectrometry, that bands 1 and 3 were the methyl esters of the vinyl and isovinyl isomers of bilirubin, and that bands 2 and 4 had structures analogous to bands 1 and 3, but with the ethyl ester group of the anthranilate moiety replaced by a methyl group during the reaction.

Fraction B (the fraction containing the water-soluble products of the methanolysis) contained the unknown conjugate moiety of azopigment δ. Identification was accomplished by means of the variety of analytical procedures outlined in Scheme 2. On the thin-layer chromatogram a compound with the R_F of glucuronic acid was detected (see Plate 5). No glucose was detected in the products of the methanolysis when azopigment δ had been freed from the contaminating phosphatidylcholine and taurocholate, and the small amounts of apparently absorbed azopigment α_3. When the amount of azopigment δ hydrolysed during the methanolysis was measured and the amount of glucuronic acid formed was quantified by the carbazole reaction and by g.l.c., it was found that there was a 1:1 molar ratio between the two (see Table 2).

Further analyses were then carried out on fraction B to determine whether or not glucuronic acid was the only component of the conjugate. Samples of fraction B were analysed by g.l.c. or combined g.l.c. and mass spectrometry. Silylated derivatives of the compounds present in this fraction gave three chromatographic peaks. Their retention times and fragmentation patterns were compared with those of glucuronic acid and of the lactone form of glucuronic acid, treated in the same manner. The retention time and fragmentation pattern of the first chromatographic peak of the unknown were identical with those of the silylated derivative of the lactone form of glucuronic acid. This peak comprised less than 10% of the total. Silylation of glucuronic acid yielded two chromatographic peaks, corresponding to the α

Table 2. *Products formed during hydrolysis of azopigment δ with sodium methoxide*

Details of the analysis are outlined in the text. Results are expressed as μmol of azopigment δ converted into azopigment α_0' after treatment with sodium methoxide, and as μmol of glucuronic acid cleaved from azopigment δ.

Expt. no.	Azopigment α_0' (μmol)	Glucuronic acid (μmol)	Ratio
1	4.48	4.48	1.00
2	0.39	0.36	1.08
3	1.31	1.32	0.99

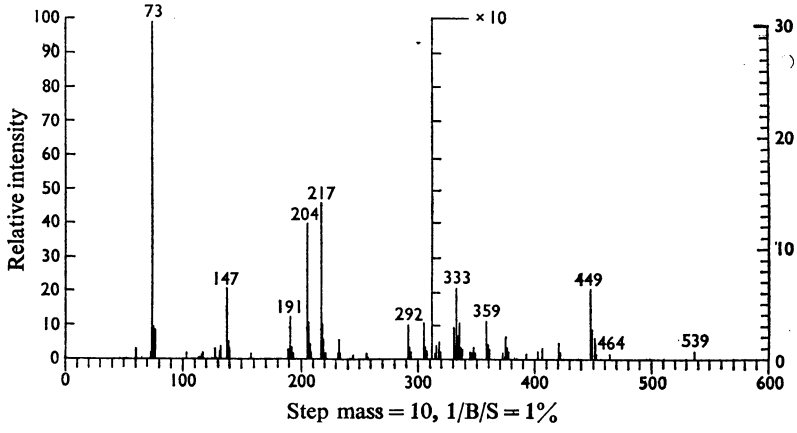
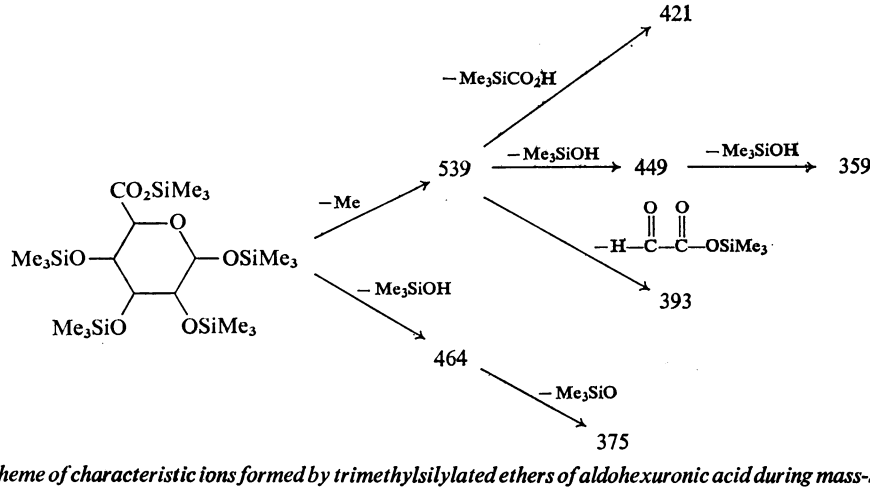


Fig. 2. *Mass spectrum (70eV) of the compounds in the water-soluble fraction B*

The derivative analysed was that formed with *N*-trimethylsilylimidazole. Step mass = 10.



Scheme 3. *Scheme of characteristic ions formed by trimethylsilylated ethers of aldohexuronic acid during mass-spectral analysis*

Abbreviation: Me_3Si, trimethylsilyl.

and β anomers, and the fragmentation pattern of each was the same. The retention times and fragmentation patterns found for the second and third peaks of the unknown were identical with those arising from the two anomeric forms of the glucuronic acid standard.

The fragmentation pattern of the unknown is displayed in Fig. 2. The pattern is identical with that obtained with a known sample of glucuronic acid treated in the same manner. Not unexpectedly the molecular ion ($m/e = 554$) was not detected in this spectrum, but the ion corresponding to the molecular weight (M) minus Me ($m/e = 539$) was present, indicating that the unknown was completely trimethylsilylated. Also present were the characteristic ions $M-(Me_3SiOH)$ ($m/e = 464$) and $M-(Me+Me_3SiOH)$ ($m/e = 449$), which have been proposed as the molecular ion set for trimethylsilyl ethers of glucuronides, when the molecular ion is not detected (Billets *et al.*, 1973). The observed fragmentation pattern for m/e greater than 300 was weak but characteristic of the uronic acid structure, and for m/e less than 300 the characteristic fragmentation pattern common to all trimethylsilylated carbohydrates was found (DeJong *et al.*, 1969). Scheme 3 indicates the steps by which aldohexuronic acids fragment to give their characteristic pattern. As further confirmation, known samples of other

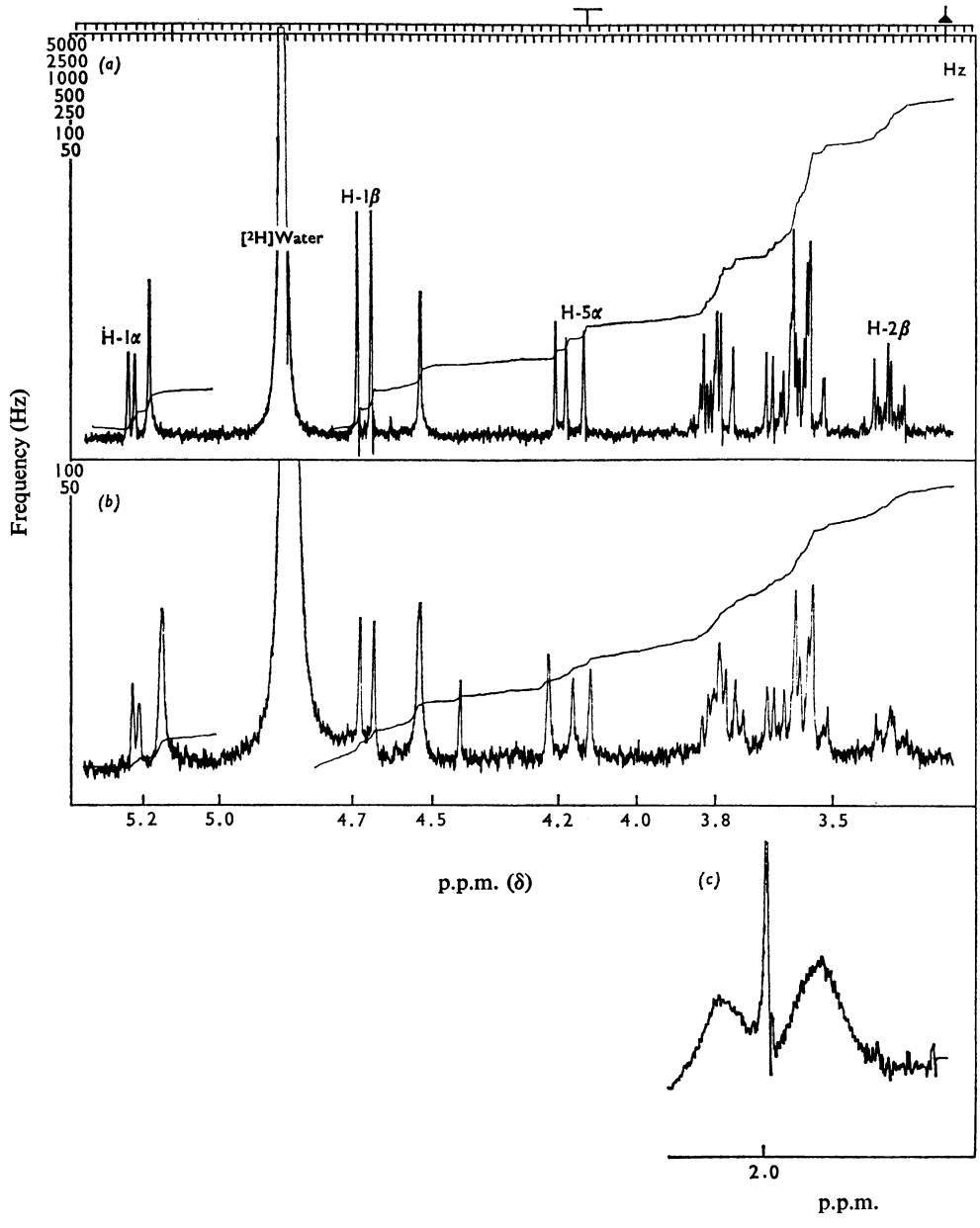


Fig. 3. *Proton-magnetic-resonance spectrum (220 MHz) of (a) sodium α,β-D-glucuronate standard (the signals designated refer to the S–S spinning side bands of the α and β anomers), (b) the water-soluble compound in fraction B and (c) material from the t.l.c. plate*

For further details see the text.

possible uronic acids, in particular iduronic acid and galacturonic acid, were tested. Neither of these gave retention times or fragmentation patterns similar enough to those of the unknown to cause any confusion.

The p.m.r. (proton-magnetic-resonance) spectrum of the moiety in fraction B was found to be virtually indistinguishable from that of the D-glucuronic acid sodium salt (see Fig. 3). A relatively high noise level and strong [²H]water spinning side bands of

the spectrum of the isolated sugar (Fig. 3*b*) reflect the fact that the solution was (of necessity) highly dilute. Despite this, however, all of the chemical shift and splitting parameters are clearly in accord with the data from the standard (Fig. 3*a*). Most readily identifiable in both spectra are the signals for H-1 α (5.2 p.p.m.), H-1 β (4.6 p.p.m.), H-5 α (4.1 p.p.m.) and H-2β (3.3 p.p.m.), although the complex patterns in the region 3.5–3.0 p.p.m. are also essentially superimposable.

It is noteworthy that the spectrum of fraction B is not that of D-glucuronic acid itself. Although similar in many respects, the p.m.r. spectra of the free acid and salt differ, especially in the large downfield shifts of the H-5 signals of the acid form (Perlin *et al.*, 1970). Undoubtedly, during purification, the uronic acid came into contact with an equivalence of cations present in the t.l.c. support and nominally pure solvents used in the various steps, and the salt was formed. Mention should be made also of the fact the upfield portion of the spectrum (Fig. 3*c*) exhibited two strong broad bands, centred at 1.7 and 2.3 p.p.m. respectively. These were due to material extractable from the t.l.c. support itself, and serve as a reminder that a substantial quantity of contaminant may be introduced by t.l.c. (Dietrich *et al.*, 1975). Such an impurity caused little difficulty in the current study, but, since substances of this kind might interfere with certain analyses, their possible presence in chromatographic eluates should be a matter of concern.

We have interpreted these data to signify the identity of the unknown in fraction B with glucuronic acid.

Identity of azopigment δ and of the major tetrapyrrole

The above indicates that the conjugating moiety in the dipyrrolic azopigment δ is glucuronic acid, and that this exists in a 1:1 molar ratio with the dipyrrole. Since, with this purified azopigment δ, no evidence was obtained by t.l.c. or any other form of analysis that other components were present in the conjugating moiety, the conclusion appears well founded.

Azopigment δ, in turn, is the only azo derivative formed when the major tetrapyrrole in dog gallbladder bile or human duodenal aspirates is coupled with ethyl anthranilate. Since each half of this tetrapyrrole yields azopigment δ, the tetrapyrrole must be a diglucuronide.

It is appropriate to review the relations between the present definitive identification of bilirubin diglucuronide and past attempts to define the chemical form of the major conjugate of bilirubin in bile. In the studies by Billing *et al.* (1957) and by Schmid (1957), a conjugating moiety reacting as uronic acid was identified. They suggested that bilirubin was excreted in bile as bilirubin diglucuronide but did not search for other components. The more structurally oriented studies by Compernolle *et al.* (1971) indicated that the major azopigment resulting from coupling of the bilirubin in rat and human bile with ethyl anthranilate was probably azobilirubin β-D-monoglucuronide. In the present studies this azopigment has been isolated cleanly, and analysis shows that, in terms of its structure, its molar ratios and the absence of other conjugating moieties, it is indeed a dipyrrolic azobilirubin β-D-monoglucuronide. Further, the major parent tetrapyrrole of this derivative (it is the most abundant of those present in bile) has been isolated, and cleavage of this molecule during coupling has been shown to result in two halves, both of which migrate chromatographically with the azobilirubin β-D-monoglucuronide. The present studies show unequivocally that the major form in which bilirubin is excreted in dog and human bile is bilirubin diglucuronide and indicate the degree of challenge which the native conjugates themselves present.

There remains a disparity between the present results and those by Kuenzle (1970). Our initial studies of azopigment δ were carried out on samples that had not yet been completely purified, and the results suggested that both glucose and glucuronic acid might be found in the conjugating moiety. The chromatograms of azopigment δ were found to be contaminated by phosphatidylcholine and taurocholate, and small amounts of azopigment α_3, and the initial n.m.r. spectra from fraction B showed the presence of materials in addition to glucuronic acid. Further purification has resulted in the characterization of a pure glucuronide. The work of Kuenzle (1970) may have been plagued by the same kind of contamination. Alternatively, in his samples of bile from patients who have had biliary-tract operations, infection and obstruction may have resulted in the creation of bilirubin conjugates of a variety not ordinarily seen in bile. It is not possible to resolve which alternative applies, at the present time.

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