The Isolation of an Azobilirubin β -D-Monoglucoside from Dog Gall-Bladder Bile

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An ethyl anthranilate azopigment of bilirubin conjugated to β -D-monoglucoside was isolated from dog gall-bladder bile. Glucose was cleaved from the azopigment by treatment with β -glucosidase and β -glucuronidase. Mild alkaline hydrolysis of the compound by sodium methoxide yielded two kinds of compounds, water-soluble and organic-soluble. The former were shown, by enzymic analysis, t.l.c., nuclear magnetic resonance, and combined g.l.c. and mass spectrometry, to contain glucose. No evidence was obtained from these data that a disaccharide was present in this fraction. The organic-soluble compounds formed during this methanolysis were shown, by t.l.c. and mass spectrometry, to be the isomeric dipyrrole azopigments of bilirubin. These findings contribute further evidence to the controversy surrounding the nature of conjugated bilirubin.

During the past decade it has been generally accepted that bilirubin is secreted in bile as a bilirubin glucuronide, although it has never been isolated and characterized as a chemically pure compound (Talafant, 1956; Billing *et al.*, 1957; Schmid, 1957). In the current decade, this concept was challenged by two groups of investigators (Heirwegh *et al.*, 1970; Kuenzle, 1970*a,b,c*). Their studies indicated that bilirubin was not secreted in the bile as a simple glucuronide. From dog gall-bladder bile, Heirwegh *et al.* (1970) isolated and characterized a series of dipyrrolic azoderivatives, an azobilirubin β -D-monoxylsoid, an azobilirubin β -D-monoglucoside, and an azobilirubin β -D-monoglucuronide (Heirwegh *et al.*, 1970; Fevery *et al.*, 1971; Compennolle *et al.*, 1971; Heirwegh *et al.*, 1973). However, from human bile, Kuenzle (1970*a,b,c*) was unable to isolate simple monosaccharide conjugates of bilirubin, but instead identified a group of complex biouronic acids conjugated to bilirubin. The experiments outlined in the present paper are directed at this problem. We present data which document the presence in dog gall-bladder bile of a simple β -D-glucoside conjugate of bilirubin.

Materials and Methods

Bile obtained from young mongrel dogs under light pentobarbital anaesthesia was stored in the dark at -10°C until processed, generally for a period of less than a week. As the pigment moiety of bilirubin is easily oxidized, stable azoderivatives

were made from these pigments and used in subsequent procedures.

Preparation of the azopigment

Ethyl anthranilate derivatives of the bile pigments found in dog gall-bladder bile were prepared essentially as described by Heirwegh *et al.* (1970). One volume of dog gall-bladder bile (diluted 1 part in 10 with water) was treated in the dark at room temperature (25°C) with 1 vol. of 0.4M-glycine-HCl buffer (pH 2.85) and 1 vol. of the diazonium reagent. The reaction was stopped after 30 min by the addition of butan-1-ol (approx. 3 vol.). The azopigments were extracted from the water phase by butan-1-ol, and the extraction was repeated until the water phase became colourless. The butan-1-ol phases were combined, washed twice with an equal volume of water and dried at room temperature in a rotary flash evaporator. The residue was dissolved in chloroform-methanol (1:1, v/v), and the concentrations of the azopigments (expressed in bilirubin equivalents/litre) were calculated from the E_{530} values, by using an extinction coefficient of $44.4 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Van Roy & Heirwegh, 1968). Spectral-grade chloroform-methanol (1:1, v/v) was used in the reference cuvette. The diazonium reagent, freshly prepared, consisted of ethyl anthranilate (0.1 ml dispersed in 10 ml of 0.15M-HCl) to which 0.3 ml of NaNO_2 (5 mg/ml) was added just before use. The mixture after 5 min at room temperature was treated with 0.1 ml of ammonium sulphamate (10 mg/ml) and used within 3 min.

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Separation of the azopigments

Initial separation of the azopigments was performed by preparative t.l.c. in a manner similar to that of Heirwegh *et al.* (1970). The azopigments, dissolved in a small volume of chloroform-methanol (1:1, v/v), were spotted on silica gel H [Brinkman Co. (Fine Chemicals), Montreal, Que., Canada] t.l.c. plates (0.750mm $\times$ 20cm $\times$ 20cm). The chromatograms were first developed at room temperature in the dark in the solvent system chloroform-methanol-water (65:25:3, by vol.). The separation obtained was similar to that described by Heirwegh *et al.* (1970) and the nomenclature of the azopigments used by this group of investigators was adopted. The separation obtained in this first purification step is illustrated in Plate 1.

Preliminary investigations demonstrated that the azopigment α_3 was one of the simplest of the conjugates, and so our initial efforts were devoted to the elucidation of its chemical structure. For this, azopigment α_3 was subjected to further purification by scraping from the preparative plate and eluting from the gel with methanol and then with butanol-water (1:2, v/v). The combined extracts were concentrated at room temperature in a rotary flash evaporator. The residue was redissolved in chloroform-methanol (1:1, v/v) and re-spotted on silica gel H t.l.c. plates (0.250mm $\times$ 20cm $\times$ 20cm). The chromatograms, developed first in diethyl ether, were dried and then developed in a solvent system of chloroform-methanol (17:3, v/v). The last purification step was then repeated until the azopigment α_3 appeared as a single band. It was then extracted from the gel, evaporated to dryness and stored in the dark under N_2 at $-10^\circ C$.

Acid hydrolysis of azopigment in 0.1M-HCl or in formic acid

The azopigment α_3 (equivalent to 700–800 μg) was dissolved in 0.1 ml of methanol; 2 ml of 0.1 M-HCl or 0.1 M-formic acid was added and the sample heated in a sealed vessel for 1 h at temperatures ranging from 60° to $100^\circ C$. The vessels were cooled and the azopigment extracted into butan-1-ol, until the water phase was colourless. The organic phases were combined, washed twice with water and evaporated to dryness; the aqueous phases were pooled and dried. The residue of azopigments from the organic phase was dissolved in a small amount of methanol, spotted on silica gel H t.l.c. plates (0.250mm $\times$ 20cm $\times$ 20cm) and developed in the solvent system chloroform-methanol (17:3, v/v). The azopigments formed during the hydrolysis of the azopigment α_3 were separated in this system, extracted from the gel and the amount of azopigment in each band was then determined as outlined under

'Preparation of the azopigment'. The combined aqueous phases, after acid hydrolysis, were subjected to chemical and enzymic analyses for hexoses (glucose, galactose, fructose), pentoses, uronic acids and hexosamines.

In some experiments, the water phase was freeze-dried, the residue redissolved in a small volume of 50% (v/v) ethanol and spotted together with a set of sugar standards on pre-coated silica gel sheets (E. Merck, Montreal, Que., Canada, no. 5538; 0.250mm $\times$ 20cm $\times$ 20cm). The chromatograms were developed twice in propan-1-ol-water (17:3, v/v), air-dried, and then sprayed with aniline diphenylamine, air-dried, and heated at $110^\circ C$ for 15 min. Blue areas indicated a reducing carbohydrate.

Alkaline hydrolysis of azopigment α_3

The hydrolysis of the azopigments with 0.1 M-NaOH or 0.1 M-NH₃, and the procedures for handling the products formed, were essentially the same as those outlined for acid hydrolysis, with one exception. The water phases of the samples were mixed with freshly washed resin (Resyn 101 H⁺, Fisher Scientific Co., Montreal, Que., Canada) to remove the Na⁺ ions, filtered, dried and then chromatographed or subjected to various chemical analyses.

Hydrolysis of azopigment α_3 on chromatograms by NH₃ vapour

The procedure followed was essentially the same as that outlined by Fevery *et al.* (1971). The azopigment α_3 and standards (glucose, ribose, xylose and glucuronic acid) were spotted on pre-coated silica gel plates (Merck; D.C. Alufolien no. 5533), and kept overnight at room temperature in a tank saturated with NH₃ vapour (27%, w/v). After complete removal of the NH₃ vapour at room temperature, 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 air-dried and developed in propan-1-ol-water (17:3, v/v). The chromatogram was again air-dried, sprayed with aniline diphenylamine, and heated at $110^\circ C$ for 15 min to produce blue spots indicative of reducing carbohydrates.

Hydrolysis of azopigment α_3 by enzymes

The effects of various concentrations of β -glucosidase (EC 3.2.1.21), β -glucuronidase (EC 3.2.1.31) (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and mylase P (*Aspergillus oryzae*) (EC 3.4.24.4) (Nutritional Biochemical Co., Cleveland, Ohio, U.S.A.) on the azopigment α_3 were determined. Throughout the procedure sterile techniques

were used which prevented bacterial degradation of the azopigment during the 2h incubation at 37°C. As controls in each assay, azopigment α_3 was incubated with buffer alone. These assay tubes were treated in the same manner as the test samples and were used as an index of the spontaneous breakdown of the azopigment α_3 . The activities of β -glucosidase and β -glucuronidase were tested by ascertaining the effects of these enzymes on various concentrations of salicin and phenolphthalein β -D-glucuronide respectively. The assay procedure was the same for all three enzymes and was based on previously described methods (Fishman, 1963; Hofman, 1963; Fevery *et al.*, 1971). The azopigment α_3 (300–500 μ g) was dissolved in 0.1ml of methanol, and 2.0ml of sodium acetate buffer (0.05M, pH 5.0) was added. At zero time 0.5mg of β -glucosidase (emulsin from almonds) or β -glucuronidase (bovine liver, 0.2mg/ml), or mylase P (1.0mg/ml) was added to each assay tube. After incubation at 37°C for 120min the reaction was stopped by addition of butan-1-ol and resin (Resyn 101 H⁺). Azopigments were removed from the water phase by repeated extractions with butan-1-ol. The butanol phases were combined and washed twice with an equal volume of water. The resin was removed from the water phase by centrifugation. The water and organic phases were evaporated to dryness. The organic phase was spotted on silica gel H t.l.c. plates (0.250mm $\times$ 20cm $\times$ 20cm) and the chromatogram was developed in chloroform-methanol (17:3, v/v). The azopigments eluted from the t.l.c. plates as described above were made up to a known volume, and the concentration determined at 530nm on a Perkin-Elmer Coleman

124 DU spectrophotometer, by applying the calculation described under 'Preparation of the azopigment'. Samples of the water phase were then tested for the presence of glucose, pentose and uronic acids.

Methanolysis of azopigment α_3

Sodium methoxide was prepared in the following manner: small amounts of sodium were dissolved in super-dry methanol until it existed in excess. This methoxide was added dropwise to anhydrous methanol until the solution was slightly basic (pH 8–9). Methanolysis in a sealed vessel was continued at room temperature for 2h, and was stopped by addition of approx. 500mg of resin (Resyn 101 H⁺). The samples were well mixed on a Vortex mixer, centrifuged to remove the resin, and the supernatant fluid was removed. The resin was washed repeatedly with small volumes of methanol, and the supernatants were combined and dried in a rotary flash evaporator. Water-butan-1-ol (1:1, v/v) was then added and the sample was well mixed. After centrifugation, the butanol phase was removed, and the water phase was again re-extracted with butanol until all the azopigments had been removed. The organic phases were pooled and washed twice with water. The water and organic phases were then evaporated to dryness. The analytical procedures applied to these two phases are summarized in Scheme 1.

Fraction A: organic-soluble compounds. The azopigments from the organic phases were dissolved in a small volume of methanol and spotted on t.l.c. plates (silica gel H; 0.250mm $\times$ 20cm $\times$ 20cm),

Scheme 1. *Treatment of azopigment α_3 with sodium methoxide*

which were then developed in chloroform-methanol (17:3, v/v). The azopigments, identified by comparison with those of bilirubin, were isolated and determined as outlined above. The degree of hydrolysis of the azopigment α_3 was ascertained from these data. Azopigments appearing in the above plates were re-chromatographed on silica gel H t.l.c. plates (E. Merck pre-coated plates no. 5763) in chloroform-benzene (1:1, v/v), which separates the methylated azopigments of bilirubin (Heirwegh *et al.*, 1973).

Fraction B: Water-soluble compounds. The following analyses were conducted on the components in this fraction.

(1) T.l.c. A portion of fraction B (approx. 4–10 μ g) was spotted on t.l.c. plates (E. Merck; D.C. Alufolien no. 5533), which were developed twice in propan-1-ol-water (17:3, v/v). Reducing carbohydrate was detected with aniline diphenylamine and amino acids by spraying with ninhydrin.

(2) Chemical analysis. Fraction B was tested for the presence of hexoses (glucose, galactose, and fructose) and pentose and uronic acids.

(3) Formation of derivatives of unknowns in fraction B. (a) Methylation procedure 1 and acetylation. A portion of fraction B was methylated with diazomethane (1 ml/mg of dry sample) for 5 min at room temperature. The diazomethane solution was freshly prepared by distilling a solution of 1 g of *N*-methyl-*N*-nitrosoguanidine in 10 ml of KOH (10 g/10 ml) with 10 ml of ether. The reaction mixture was dried under N_2 . To the residue a mixture of dry pyridine and acetic anhydride (2:1, v/v) was added and acetylation was continued at room temperature for 4 h. The acetylated compound was then extracted into chloroform and excess of acetic anhydride was removed by washing with 1M-NaHCO₃. The chloroform phase was washed with water three times and finally dried. Remaining traces of pyridine were removed by dissolving the residue in chloroform, evaporating the solution to dryness and repeating this procedure several times with benzene as solvent. The methylated-acetylated sample was then stored in a desiccator and analysed later by g.l.c.

(b) Methylation procedure 2 and acetylation. Another sample of fraction B was evaporated to complete dryness and dissolved in a solution of super-dry methanol-acetyl chloride (125:3, v/v). This solution was boiled under reflux for 60 min at 60°C with a drying tube at the top of the condenser to exclude water. The reaction mixture was cooled, neutralized by addition of freshly prepared diazomethane, and then dried. The unknown sample was then methylated and in most instances was acetylated in dry pyridine with acetic anhydride as outlined above.

(c) Reduction and acetylation. A portion of fraction B was treated with 2% (w/v) NaBH₄ in 1M-NH₃

for 60 min at room temperature. The reaction mixture was neutralized by a dropwise addition of acetic acid and then evaporated under N_2 . Methanol was added and evaporated five times. The unknown sample, thus reduced, was then acetylated as described above.

Gas-liquid chromatography

These analyses were conducted on a Hewlett-Packard Research Chromatography model 5750, under the following operating conditions: two matched stainless-steel columns 6 ft $\times$ $\frac{1}{8}$ in (1.8 m $\times$ 3 mm) were packed with 3% OV-1 on Chromosorb W MP, 80–100 mesh. The injection-port temperature was maintained at 280°C, the column temperature was programmed from 120 to 275°C at a rate of 4°C/min, with an initial hold of 2 min at 120°C; detector 290°C; helium flow was approx. 60 ml/min.

Mass spectra

The mass spectra of methyl esters of the azopigment of bilirubin were recorded with an AEI MS 902 mass spectrometer, electron energy 70 eV, ionizing current 500 μ A and accelerating voltage 8 kV. The mass spectra of components in fraction B were analysed as the trimethylsilyl derivatives [made from a mixture of trimethylsilylimidazole in dry pyridine (1.5 meq/ml)] by g.l.c.-mass spectrometry on an LKB-9000 instrument. Operating conditions were as follows: glass column, 6 ft $\times$ $\frac{1}{8}$ in (1.8 m $\times$ 6 mm), 3% OV-1 on Chromosorb W HP, 80–120 mesh, isothermal at 210°C; injection port 290°C; helium flow rate approx. 40 ml/min; separator 280°C, 70 eV at 60 μ A; scan range m/e 10–700 in 5 s. The mass-spectral data were handled, on-line, by a Varian SS-100 MS data system.

Nuclear magnetic resonance

The nuclear-magnetic-resonance spectrum of fraction B of the azopigment α_3 was recorded on a 220 MHz Varian spectrometer.

Biochemical analyses

The concentration of glucose in the samples was measured by the glucose oxidase method (Kaplan, 1957), that of pentose by the orcinol method (Ashwell, 1957b), that of uronic acids by utilizing the naphthoresorcinol and the carbazole reaction as outlined by Ashwell (1957c), that of hexosamine by the Elson-Morgan reaction (Ashwell, 1957d), that of fructose by the method of Roe as outlined by Ashwell (1957a) and that of galactose by using the Boehringer Mannheim Biochemica kit (15921 TGAN).

Results and Discussion

The separation of the ethyl anthranilate azo-pigments derived from dog gall-bladder bile by t.l.c. is illustrated in Plate 1. Four major groups of azo-pigments were separated in this neutral solvent system and, as the separation pattern and number of azo-pigments obtained were similar to those described by Heirwegh *et al.* (1970), the nomenclature utilized by these investigators was adopted. The azopigment α_3 was purified further (as outlined in the Materials and Method section) and subjected to various analytical procedures to elucidate its chemical nature.

Alkali- and acid-stability of the azopigment α_3

The azopigment α_3 was both acid- and alkali-labile, and a summary of the effects of such treatment on the pigment is given in Table 1. In all instances the organic-soluble compounds found after either alkali or acid hydrolysis were the azopigments of bilirubin and the azopigment α_3 . The concentration of these pigments indicates the degree of hydrolysis of the azopigment α_3 . The data are summarized in Table 1 and indicate that complete hydrolysis was noted only after alkali (0.1M-NaOH) treatment at 100°C for 1h. Glucose was detected by enzymic analysis in the water phase after acid hydrolysis (0.15M-HCl or 0.1M-formic acid) but was not detected in this phase after treatment with alkali (0.1M-NaOH or 0.1M-NH₃). This observation was expected, since it is a well-known fact that in an alkaline solution monosaccharides are extremely unstable (White *et al.*, 1964). No other hexoses, pentoses, uronic acids or hexosamines were detected in the water phases after acid or alkali hydrolysis. Paper or thin-layer chromatograms of a sample of the water fractions developed in a number of solvent systems utilized to separate carbohydrates confirmed these findings. There was no evidence obtained from

these data that a disaccharide was present in these fractions.

Exposure of the azopigment α_3 to NH₃ vapour overnight cleaved azopigment α_3 to the carboxylic acid amide of the deconjugated azopigment and glucose (Jansen & Billing, 1971). Glucose was detected on the thin-layer chromatograms as the only water-soluble product of the reaction. These data confirm the original observations of Fevery *et al.* (1971) that glucose appears as a product after mild alkaline hydrolysis of azopigment α_3 (the source material for his azopigment was also dog gall-bladder bile). These data, however, do not agree with the findings of Kuenzle (1970c). He noted that of the six azobilirubin conjugates he isolated from human bile, five were alkali-labile, and identified biouronic acids as the water-soluble products formed during alkaline hydrolysis. Glucose was never detected as the only compound formed but was identified as a component of the biouronic acids isolated. The apparent differences in the results of these two investigators may derive in part from the different sources of bile used in their studies.

Effect of hydrolytic enzymes on azopigment α_3

The treatment of azopigment α_3 with β -glucuronidase (bovine liver) and β -glucosidase (emulsin) produced partial hydrolysis of the pigment under our experimental conditions (Table 2). The butanol-extractable azopigments formed during the hydrolysis were shown by t.l.c. to be the azopigments of bilirubin. In the water-soluble phase glucose was detected by enzymic analysis and t.l.c. The fraction of the azopigment α_3 converted into the azopigments of bilirubin (α_0) was determined as outlined under 'Preparation of azopigment' and the data obtained are presented in Table 2. Control assays in buffer but without enzyme caused only 20% of degradation of the azopigment α_3 . The azopigments α_0' (methyl ester of the azopigment of bilirubin) and α_0 (the

Table 1. *Products formed after acid and alkali hydrolysis of azopigment α_3*

α_0 is the azopigment of bilirubin. See the text for details of treatments.

Treatment	Chemical analysis			Detection by t.l.c.		Distribution of azopigments (%)	
	Glucose	Pentose	Uronic acid	Sugars	Amino acids	α_0	α_3
0.15M-HCl, 100°C, 1h	+	—	—	+	—	85	15
0.10M-formic acid, 100°C, 1h	+	—	—	—	—	66	35
0.10M-NH ₃ , 100°C, 1h	—	—	—	—	—	71	29
0.10M-NaOH, 100°C, 1h	—	—	—	—	—	100	—
Exposure to NH ₃ vapours on thin-layer chromatograms				+	—		

Table 2. *Effect of enzymes on azopigments α_3*

The amount of each azopigment detected is presented as a percentage of the total $\pm$ S.E.M. The only water-soluble product formed by the reaction of β -glucosidase and β -glucuronidase on azopigment α_3 was glucose.

Treatment	No. of experiments	Distribution of Azopigments					
		With enzyme			Without enzyme		
		α_0'	α_0	α_3	α_0'	α_0	α_3
β -Glucosidase	7	13 $\pm$ 3	49 $\pm$ 7	37 $\pm$ 5	11 $\pm$ 2	11 $\pm$ 1	79 $\pm$ 3
β -Glucuronidase	5	7 $\pm$ 2	43 $\pm$ 6	49 $\pm$ 6	11 $\pm$ 2	11 $\pm$ 1	79 $\pm$ 2
Mylase P	3	6 $\pm$ 2	12 $\pm$ 6	81 $\pm$ 2	7 $\pm$ 2	11 $\pm$ 1	82 $\pm$ 2

Table 3. *Products formed during hydrolysis of azopigment α_3 with β -glucosidase and sodium methoxide*

Details of analysis are outlined in the text. Results are expressed as μ mol of azopigment α_3 converted into azopigment α_0 after treatment with β -glucosidase, or into azopigment α_0' after treatment with sodium methoxide, and as μ mol of glucose cleaved from azopigment α_3 .

Expt. no.	Treatment	Azopigment	Glucose	Ratio (glucose/azopigment)
1	β -Glucosidase	2.38	2.97	1.2
2	β -Glucosidase	1.06	0.99	0.9
3	β -Glucosidase	0.45	0.53	1.2
4	Sodium methoxide	0.20	0.19	1.0
5	Sodium methoxide	0.41	0.45	1.1
6	Sodium methoxide	0.28	0.31	1.1

azopigment of bilirubin) were found in approximately equal amounts (Table 2). The methyl ester of the azopigment of bilirubin was formed during the extraction of the azopigment from the silica-gel plates with methanol. This transesterification reaction takes place because of the large amounts of methanol required to extract minute amounts of the azopigment α_3 from the gel. If the assay was conducted under non-sterile conditions the degree of degradation was 60–80%. Similar proportions of the pigment were degraded if the reaction was carried out in a sodium acetate buffer of higher concentration (0.2–0.5M). Although the azopigment α_3 was hydrolyzed by the action of both β -glucosidase and β -glucuronidase, the reaction with β -glucosidase was always more effective (Table 2, Plate 2a). Mylase P had no effect on the azopigment α_3 (Table 2).

In three experiments the products formed during the hydrolysis of azopigment α_3 with β -glucosidase were measured (Table 3). The proportions of the azopigments α_0 and glucose formed approximate to a 1:1 molar ratio. The slight deviation accrues from the mild instability of the ethyl anthranilate azopigments.

Our data on the effects of β -glucosidase on the azopigment α_3 confirm the findings of Fevery *et al.* (1971), but the effect of mylase P on the pigment is not in agreement with their data. This apparent discrepancy may result from differences in the

enzyme concerned. Our preparations of mylase P, unlike those of Fevery *et al.* (1971), exhibited no measurable β -glucosidase activity under our experimental conditions.

Methanolysis of azopigment α_3 with sodium methoxide

The azopigment α_3 was completely cleaved by treatment with sodium methoxide at room temperature for 2 h. The azopigment products formed during this reaction were chiefly α_0' , with traces of α_0 . When azopigment α_0' was chromatographed in a solvent system which separates the methyl esters of this azopigment, it separated into six fractions (Plate 2b). Two bands remained at the origin. The other, more polar, bands were extracted and analysed by mass spectrometry. The mass spectra obtained for two of the azopigments (bands 1 and 3) agree with data published by Jansen & Stoll (1971) for the methyl esters of the isovinyl and vinyl isomers of azobilirubin. Their structures were therefore identified by t.l.c. and spectroscopic comparison with authentic samples. The identity of the two other components (bands 2 and 4) has not been unequivocally established. Their mass spectra showed molecular ions at 14 mass units less than bands 1 and 3. It is assumed from these mass-spectral data and also from their mobilities on t.l.c. that bands 2 and 4 have structures analogous to bands 1 and 3 but with the ethyl ester group of the anthranilate

EXPLANATION OF PLATE I

Separation of the ethyl anthranilate azopigments of bile-pigment conjugates isolated from dog gall-bladder bile

The azopigments are denoted by Greek letters. Chromatograms were developed in the solvent system chloroform-methanol-water (65:25:3, by vol). This chromatogram and the others used for illustration were overloaded, in order to provide a reasonable photograph.

EXPLANATION OF PLATE 2

Azopigments of bilirubin formed after treatment of azopigment α_3 with β -glucosidase, β -glucuronidase and mylase P (a) and after methanolysis (b)

For details see the text.

moiety replaced by a methyl group during the reaction.

The water-soluble fraction of this hydrolysis (fraction B) contained the unknown compound conjugated to the azopigment α_3 . To clarify its identity, azopigment α_3 was subjected to a variety of analytical procedures, which are outlined in Scheme 1.

Glucose was detected in the water phase by enzymic analysis (fraction B, Scheme 1). When both the amounts of azopigment hydrolysed and the glucose detected were determined, it was found that these compounds existed in a 1:1 molar ratio (Table 3). Therefore it appeared that glucose was one constituent of the unknown compound conjugated to bilirubin. Further analyses were carried out on fraction B to determine whether glucose was either the only component of the conjugate, or existed as part of a disaccharide.

A portion of fraction B was treated with 0.1 M-HCl at 100°C for 1 h. The amount of glucose in the sample did not change and no other reducing carbohydrates were detected by t.l.c. in this sample. These data substantiated the contention that glucose was the only carbohydrate constituent produced by hydrolysis of the azopigment α_3 with sodium methoxide.

Further proof of the chemical nature of the conjugated portion of the azopigment α_3 was sought by analysing samples of fraction B by g.l.c. For this analysis, samples of fraction B were subjected to a variety of chemical reactions which resulted in a series of different volatile derivatives.

One sample was treated with NaBH_4 to reduce the unknown compound to its respective alditol. The hydroxyl groups were then acetylated with pyridine-acetic anhydride at room temperature. This stable, although more volatile, fraction gave on g.l.c. a single chromatographic peak whose retention time was the same as that of β -D-glucose treated in the same manner (Fig. 1a). Alternatively, methylation of any acidic or enolic hydroxyl groups that might exist in the unknown compound was accomplished by treating a portion of the fraction B for 5 min with diazomethane. The reaction mixture was then acetylated. The reaction products yielded two major chromatographic peaks, whose retention times were the same as those for the two peaks arising from an equivalent mixture of α - and β -D-glucose treated in the same manner (Fig. 1b). Thirdly, a vacuum-dried portion of fraction B was treated with TRI-SIL-'Z' (Chromatographic Specialties Ltd., Brockville, Ont., Canada) and was then analysed by g.l.c. Again two chromatographic peaks were obtained (Fig. 1c) with retention times identical with those of α - and β -D-glucose treated in a similar manner. As further proof that fraction B contained only glucose, a dried portion was treated with *N*-trimethylsilylimidazole and analysed by combined

Fig. 1. Gas-liquid chromatograms of products formed when a sample of fraction B was treated in various ways

See the text for details. (a) Reduction of aldehyde groups with NaBH_4 followed by acetylation; (b) methylation of acidic or enolic groups with diazomethane and acetylation of secondary hydroxyl groups; (c) formation of the trimethylsilylated derivative of fraction B.

Fig. 2. 70 eV mass spectrum of a sample of fraction B of azopigment α_3 treated with *N*-trimethylsilylimidazole (a) and the n.m.r. spectrum of a sample of fraction B (b)

For details see the text.

g.l.c. and mass spectrometry. Two chromatographic peaks were observed, which were identical with peaks obtained from α - and β -D-glucose treated in the same manner. The fragmentation pattern obtained by mass spectrometry of the two chromatographic peaks were identical with those obtained for α - and β -D-glucose (Fig. 2a,b), and are in agreement with the reported mass spectra of these compounds (DeJongh *et al.*, 1969). Further, the nuclear-magnetic-resonance spectrum of fraction B was identical with that obtained for an authentic sample of α - and β -D-glucose (Fig. 2b) and agrees with published data (Koch & Perlin, 1970). There was no indication that other components were present in this fraction. It should be noted that the chemical reactions used in making these different derivatives of the compounds in fraction B are those commonly used to identify disaccharides. No evidence was obtained

from the many analyses of fraction B that there were any other components present apart from α - and β -D-glucose.

Although the treatment of the azopigment α_3 with sodium methoxide is considered a very mild procedure, there is no proof that this reaction has not destroyed a portion of the moiety conjugated to the azopigment of bilirubin. Therefore the following additional procedure was carried out. The azopigment α_3 was refluxed with super-dry methanol and acetyl chloride. This reaction should split the ester linkage between the azopigment and the moiety conjugated to it, and simultaneously methylate the unknown conjugating group. The azopigment and methylated unknown was acetylated. Analysis of this sample by g.l.c. indicated that the major component (95%) was identical with β -D-glucose treated in similar manner. No evidence for

the presence of a disaccharide was found in this preparation. These findings are consistent with the other evidence (above) that the conjugated sugar of the azopigment α_3 is β -D-glucopyranose.

In summary, an azopigment of bilirubin designated as azopigment α_3 has been isolated and purified from dog gall-bladder bile and the identification procedures have shown it to be an azobilirubin β -D-glucoside. These findings confirm the original hypothesis of Fevery *et al.* (1971) and provide additional unequivocal evidence for the presence of a single β -D-glucopyranose conjugated to bilirubin. These results also strongly suggest that at least in this fraction there is no evidence of conjugation to a biouronic acid.

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