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Excretion in Dog Bile of Glucose and Xylose Conjugates of Bilirubin

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1. T.l.c. with neutral solvent systems of ethyl anthranilate azopigments derived from bile of man, dog and rat revealed pronounced species variation. The less polar components (α-group) could be separated conveniently by development with chloroform–methanol (17:3, v/v). 2. The azopigment material derived from gall-bladder bile of dog contained about 10% of azobilirubin β-D-monoxyloside (azopigment α_2) and 30% of azobilirubin β-D-monoglucoside (azopigment α_3). The sugar moieties were identified by t.l.c. with acidic, neutral and basic solvent systems and by anion-exchange column chromatography of their boric acid complexes. Treatment of the purified azopigments with ammonia vapour led to the formation of the amide of azobilirubin, indicating that both pigments are ester glycosides. The β-D configuration was demonstrated by enzymic studies with emulsin (an adequate source of β-glucosidase activity) and with Mylase-P (an adequate source of β-glucosidase and β-xylosidase activities). 3. Hydrolysis studies with model substrates and with the α_2- and α_3-azopigments suggested that in Mylase-P the β-glucosidase and β-xylosidase activities reside in separate enzymes. 4. Compared with the accepted conjugation with glucuronic acid as a major route of detoxication in mammals, the detection of large amounts of xylose and glucose conjugates of bilirubin in dog bile suggests that the underlying biosynthetic pathways may be important alternative routes of detoxication.

In animals, birds, amphibia and fish conjugation with glucuronic acid constitutes a major pathway in the end metabolism of endogenous compounds (Dutton, 1966*a*) and drugs (Smith & Williams, 1966), glucuronic acid being transferred enzymically from UDP-glucuronic acid to the aglycones (Dutton, 1966*a*). In plants, bacteria and insects the formation of glucosyl derivatives is a major mechanism for detoxication (Dutton, 1966*b*; Hassid, 1967), although β-D-glucuronides can be detected (Marsh, 1955).

Until recently bilirubin was generally thought to be excreted mainly as glucuronosides (for a review see With, 1968). However, Kuenzle (1970*a*,*b*,*c*) demonstrated that disaccharidic azodipyrryl conjugates can be derived from post-obstructive bile of man. Work with the diazonium salt of ethyl anthranilate (Heirwegh, Van Hees, Leroy, Van Roy & Jansen, 1970*a*) also indicated a pronounced complexity of bile pigments. From human bile was obtained an azopigment that was chromatographically very similar to azobilirubin β-D-monoglucuronide. In addition, considerable amounts of the so-called β- and γ-azopigments, which contain hexuronic acid (not necessarily identical with glucuronic acid) and typical acid-labile structures, were found. Small amounts of alkali-labile azopigments (azopigments α_2 and α_3) that were free of hexuronic acid were also detected.

In the present work it is shown that the α_2- and α_3-azopigments derived from dog bile are major components (30–40% of total azopigment colour) and correspond to azobilirubin β-D-monoxyloside and azobilirubin β-D-monoglucoside respectively. They are both ester conjugates. More complete structural pictures have been established by Compernolle, Van Hees, Fevery & Heirwegh (1971).

The nomenclature used in the present work is based on t.l.c. of dipyrryl azopigments (Heirwegh *et al.* 1970*a*). The use of a single symbol to indicate one spot characterized by a given R_F value does not imply chemical homogeneity. Similarly, denotation by the same symbol of chromatographically identical components derived from different sources does not prove chemical identity.

The present work has been communicated in part at the 507th Meeting of the Biochemical Society (Heirwegh, Van Hees, Compernolle & Fevery, 1970*b*).

MATERIALS AND METHODS

Chemicals. Reference carbohydrates were from the following sources: L-arabinose, D-galactose, D-glucose, D-mannose, L-rhamnose and D-xylose from E. Merck A.-G. (Darmstadt, Germany); D-ribose from British Drug Houses Ltd. (Poole, Dorset, U.K.); D-fructose from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). *p*-Nitrophenyl α-D-xylopyranoside was from Koch–Light Laboratories Ltd. and the β-D-xylopyranoside from British Drug Houses Ltd. Glucono-(1→5)-lactone, *p*-nitrophenol and the α-D- and β-D-glucopyranosides of *p*-nitrophenol were purchased from E. Merck A.-G. Mylase-P (from *Aspergillus oryzae*) was obtained from Mann Research Laboratories (New York, N.Y., U.S.A.) and emulsin (from sweet almonds) was from British Drug Houses Ltd. The other chemicals were from the sources indicated previously (Van Roy & Heirwegh, 1968; Heirwegh *et al.* 1970*a*).

Biological materials. Through the courtesy of Dr R. Suy gall-bladder bile was obtained at operation from 10 healthy dogs, used for experiments in vascular surgery. Hepatic bile samples were obtained from one dog through a cannula inserted in the common bile duct after cholecystectomy. The animals were anaesthetized by intravenous administration of pentothal.

Quantitative determination of azopigments by t.l.c. Diluted dog bile was treated with diazotized ethyl anthranilate, and the azopigments were separated by t.l.c. on pre-coated silica gel plates (DC-Alufolien 5553/0025; from E. Merck A.-G.). They were developed at room temperature in the dark with chloroform–methanol–water (65:25:3, by vol.) for 12cm, dried in an air stream and developed again for 16cm with chloroform–methanol (17:3, v/v). Reference azopigment mixtures were obtained by diazotization of human post-obstructive fistula bile and of normal rat bile (Heirwegh *et al.* 1970*a*).

The separated azopigments were determined quantitatively either by photometric determination of methanol eluates (Heirwegh *et al.* 1970*a*) or by densitometry at 536nm. In the latter case chromatograms were developed on pre-coated glass plates (DC-Kieselgel F254, 5715/0025; from E. Merck A.-G.). The extinction of the light transmitted through the plates was recorded and integrated by using a Flying spot TLD100 densitometer (Vitatron, Dieren, The Netherlands) at a scanning speed of 0.5cm/min. The plates used for densitometry were either freshly developed or were stored overnight in the dark.

Preparation of azopigments α_2 and α_3. Azopigment extract was submitted to preparative t.l.c. with chloroform–methanol (17:3, v/v) on pre-coated silica gel plates. The separated α_2- and α_3-bands were scraped from the plates, transferred to empty tubes (internal diameter 6–8mm) and eluted as rapidly as possible with methanol. Without undue delay the eluates were dried at room temperature in a rotary evaporator connected to an oil vacuum pump.

T.l.c. of the conjugating moieties. Azopigments α_2 and α_3 and aqueous solutions of reference sugars were applied to pre-coated silica-gel plates (DC-Alufolien no. 5553/0025; from E. Merck A.-G.) and kept overnight at room temperature in a tank saturated with the vapour produced by 27% (w/v) ammonia. After complete evaporation of the excess of ammonia, fresh samples of the reference sugars and of the initial azopigments were applied, and the plates were developed for 18cm with chloroform–methanol (17:3, v/v) to remove as far as possible any azopigment material from the vicinity of the origin. Complete release of the conjugating moieties was indicated by complete conversion of the alkali-labile azobilirubin conjugates into mixtures of azobilirubin and its carboxylic acid amide (Heirwegh *et al.* 1970*a*; Compernolle, Jansen & Heirwegh, 1970). None of the sugars examined moved appreciably with the solvent system mentioned above, whereas the azopigment derivatives had R_F values near to 1.0.

The plates were dried in air and developed further for 18cm with one of the following solvents (Lewis & Smith, 1967): (*a*) propan-1-ol–water (17:3, v/v); (*b*) butan-1-ol–acetic acid–water (12:3:5, by vol.); (*c*) butan-1-ol–pyridine–water (10:3:3, by vol.). Plates were dried in air and submitted to a final development with chloroform–methanol–water (65:25:3, by vol.) before colour development. Sugars were localized with *p*-anisidine–phthalate or with naphtharesorcinol–H_2SO_4 mixtures (Krebs, Heusser & Wimmer, 1967) as the spraying reagents.

Isolation of conjugating moieties and analysis by anion-exchange column chromatography. Purified azopigment α_2, applied as a band to a pre-coated silica-gel plate (DC-Alufolien no. 5553/0025), was submitted to treatment with ammonia and further developed with chloroform–methanol (17:3, v/v) as outlined above. After drying in air, the silica gel near the origin of the plate was scraped off, transferred to a centrifuge tube and extracted vigorously by shaking with 10–20ml of water. After centrifuging the suspension for 10min at $1000g_{av.}$ the supernatant solution was applied to a chromatographic column (internal diameter 5mm) containing successive layers (10cm) of Dowex 1 (X8; 200–400 mesh; acetate form) and of Dowex 50W (X8; 200–400 mesh; H^+ form), eluted with water and freeze-dried.

The freeze-dried preparation was redissolved in 0.1M-H_3BO_3 (adjusted to pH7 with 5M-NaOH) and subjected to anion-exchange chromatography by the procedure of Kesler (1967). The analyses were repeated with the initial material fortified with D-xylose.

Exactly the same procedure was followed to purify and analyse the conjugating material of azopigment α_3. In this case D-glucose was used in the final mixed chromatographic run.

Enzymic incubations. Dried purified azopigments were redissolved by vigorous shaking in 0.2M-sodium acetate–acetic acid buffer, pH5, and centrifuged for 10min at $1000g_{av.}$. Solutions of azopigment α_2 (2ml) and of azopigment α_3 (1ml) were incubated with shaking at 37°C with 0.1ml of buffer containing emulsin or Mylase-P. Controls were prepared (*a*) by omission of enzyme and (*b*) by the addition of glucono-(1→5)-lactone as the dry powder to the incubation mixtures. After extraction of the incubation mixtures with 1ml of pentan-2-one the degree of hydrolysis was assessed by densitometry of thin-layer chromatograms (Table 1).

The hydrolysing activities of the enzymes were determined (Beck & Tappel, 1968) with the α-D- and β-D-xylopyranosides (final concentration 2.5mM) and with the α-D- and β-D-glucopyranosides (final concentration 1mM) of *p*-nitrophenol as the substrates (Table 2).

Table 1. *Enzymic hydrolysis of azopigments α_2 and α_3*

Incubations were carried out as indicated in the Materials and Methods section.

Azopigment preparation					% of azopigment hydrolysed		Final concn. of glucono-(1→5)-lactone
Nature	No.	Enzyme	Amount of enzyme (mg/test)	Incubation time (min)	With enzyme	With enzyme and inhibitor	(mM)
Azopigment α_2	1	Emulsin	10	60	1	0	50
	1		10	120	2–5	0	50
	1		10	1320	25	5	50
	1	Mylase-P	10	30	9.6	9.2	50
	1		10	60	15	12	50
	1		10	120	29	22	50
	1		10	1320	62	57	50
	2		10	120	46	30	25
	3		50	120	72	58	100
Azopigment α_3	4	Emulsin	0.2	60	40	1	50
	4		2	60	80	3	50
	5		10	120	95	8	25
	5		10	120	90	5	25
	6	Mylase-P	10	120	97	68	25
	7		20	120	66	42	50
	7		20	120	90	70–80	100

Portions (1.5ml) of 0.2M-sodium acetate–acetic acid buffer, pH5.2, containing substrate and enzyme were incubated with shaking at 37°C. Controls (*a*) and (*b*) were prepared as above. Hydrolysis was stopped by adding 2ml of 2M-NH_3 to the incubation mixtures. Without delay the colours were read at 400nm. After correction for extinction obtained with controls (*a*) the amounts of *p*-nitrophenol produced in the assay mixtures were read from a calibration curve for *p*-nitrophenol.

Diazotization at pH2.8 *and* pH5.85. The following buffers were used: (*a*) 0.4M-HCl adjusted to pH2.80 with 2M-glycine; 0.5M-citric acid adjusted with 1M-Na_2HPO_4 (*b*) to pH2.80 or (*c*) to pH6.00.

In simultaneous reaction series samples of diluted dog bile (1ml) were treated at 25°C with 2ml of buffer (*a*, *b* or *c*) and 1ml of diazotized ethyl anthranilate (prepared as described by Heirwegh *et al.* 1970*a*). After the required reaction times (Fig. 2) the reaction mixtures were treated with 2ml of ascorbic acid solution (100mg/ml) followed by 2ml of buffer (*a*). Azopigment colour was extracted with 2–5ml of pentan-2-one and determined photometrically. The extracted azopigments were separated by t.l.c. and determined quantitatively both by densitometry and by photometry after methanol elution.

RESULTS

Chromatographic analysis of the azopigments. By t.l.c. the azopigment patterns derived from dog bile were shown to be similar in complexity to those found with human post-obstructive bile (Fig. 1). For comparison a typical pattern obtained with normal rat bile is also shown. The quantitative differences are quite marked. By photometry after methanol elution the following percentages were found with gall-bladder bile of dog: ($\alpha_0+\alpha_1$)-azopigments, 10.2±2.7S.D.; α_2-azopigment, 9.6±1.9S.D.; α_3-azopigment, 27.6±4.2S.D.; γ-azopigment, 8.6±1.7S.D.; δ-azopigment, 43.0±3.7S.D. (separate analyses on bile samples of ten dogs). Trace amounts of pigments of the β-group have been neglected. In general, the α_0- and α_1-azopigments were not sufficiently well separated to allow separate determination (e.g. see the tracing obtained with dog bile, Fig. 1*b*). In one case quantitative determination was possible and yielded, for azopigments α_0 and α_1, 7.0% and 2.7% of total azopigment colour respectively. From one dog hepatic bile was obtained over three successive 15min collection periods. Analysis of the samples indicated azopigment compositions comparable with those found with gall-bladder bile: ($\alpha_0+\alpha_1$)-azopigments, 8.6%; α_2-azopigment, 6.0%; α_3-azopigment, 22.0%; γ-azopigments, 6.4%; δ-azopigment, 55.6%. In two cases the α_0- and α_1-azopigments were completely separated and amounted to 6.4 and 2.1% of total azopigment colour respectively (mean values are given in all cases).

Photometry after methanol elution and densitometric analysis yielded very similar values. With the densitometer used the best results were obtained by scanning freshly developed plates (negligible base-line correction) at the lowest possible scanning speed available for the instrument (nearly linear response). If due precautions are taken the technique is of value for the quantitative determination of complex azopigment mixtures, and has the advantage that notable plates are not destroyed (in the dark at room temperature colour patterns lasted for at least 1 year). Simple patterns as obtained, for example with normal rat bile are

Table 2. *Activities and inhibition of reference glycosidases with glucono-(1→5)-lactone*

Enzyme assays were carried out as described in the Materials and Methods section. In general for each assay at least three time-points were obtained in the constant portion of the velocity–time curve. Values of enzyme activities marked with an asterisk (*) are to be considered as rough estimates of upper limits, checked only at one time-point. The following symbols are used to denote the *p*-nitrophenyl derivatives of: PNPαGlc, α-D-glucopyranose; PNPβGlc, β-D-glucopyranose; PNPαXyl, α-D-xylopyranose; PNPβXyl, β-D-xylopyranose.

Enzyme preparation	Amount of enzyme (μg/test)	Substrate	Time-period (min)	*p*-Nitrophenol formed (μmol/min per mg of protein)	Inhibition with glucono-(1→5)-lactone: Concn. of inhibitor (mM)	Inhibition with glucono-(1→5)-lactone: % of uninhibited activity
Emulsin	50	PNPαGlc	30	0.001*	Not tested	
	2.5; 5	PNPβGlc	0–20	1.17	0	100
					25	1.8
					50–100	0.7–0.4
	50	PNPαXyl	20	0.003*	Not tested	
	50; 125	PNPβXyl	0–20	0.026	0	100
					25	8
					50–100	4–3
Mylase-P	1000	PNPαGlc	0–30	0.0018	0	100
					25	11
					50–100	6–3
	250	PNPβGlc	0–30	0.0125	0	100
					25	2.4
					50–100	1
	1000	PNPαXyl	20	0.0002*	Not tested	
	1000	PNPβXyl	0–30	0.0037	0	100
					25	100
					50	87
					75	76
					100	68

analysed most economically and rapidly by photometry after methanol elution.

Successive developments with chloroform–methanol–water (65:25:3, by vol.) followed by chloroform–methanol (17:3, v/v) yielded clearcut separations of the major azopigment groups (Figs. 1*b*, 1*d* and 1*f*). Development of the plates with chloroform–methanol (17:3, v/v) alone separates well the major components of the α-group (Figs. 1*a*, 1*c* and 1*e*). In the latter system purified preparations of azopigments α_2 and α_3 have repeatedly been shown to move with exactly the same R_F values as the corresponding components in the initial complex mixtures resulting from diazotization of dog bile.

Diazotization of dog bile at pH 2.8 *and* pH 5.85. Even with relatively large amounts (95 nmol) of conjugated bile pigments (assayed as diluted dog bile) diazotization at room temperature in glycine–HCl buffer, pH 2.8, was virtually complete after 2.5 min (Fig. 2). The standard reaction time in this system is 30 min (Heirwegh *et al.* 1970*a*). The azopigment composition remained nearly unchanged when the reaction was prolonged to 30 min, with a small increase of the pigments of the γ-group. A similar behaviour was observed when citric acid–Na_2HPO_4 mixtures were used to buffer the reaction systems (final pH values 2.8 and 5.85). At pH 5.85 final colour yields generally were 10–20% low.

Nature of the aglycones. In solution and on thin-layer plates azopigments α_2 and α_3 showed the typical colours of azobilirubin (Heirwegh *et al.* 1970*a*). Ammonia treatment on thin-layer plates of δ-azopigment from rat bile (reference material) and of the azopigments α_2 and α_3 in each case led to the formation of the same mixture of two more hydrophobic derivatives as shown by t.l.c. in chloroform. With the reference material the derivatives have been shown to correspond to azobilirubin and its carboxylic acid amide (Compernolle *et al.* 1970).

Analysis of the conjugating groups. Ammonia treatment of azopigments α_2 and α_3 on thin-layer plates led to the release of D-xylose and D-glucose respectively, as was demonstrated by t.l.c. with acidic, neutral and basic solvent systems for sugars (Table 3). No other material reacting positively with anisidine–phthalate or with naphtha-resorcinol–sulphuric acid mixtures was noticed. The control experiments indicated that the sugars tested, and in particular D-xylose and D-glucose, were not affected chromatographically by the ammonia treatment.

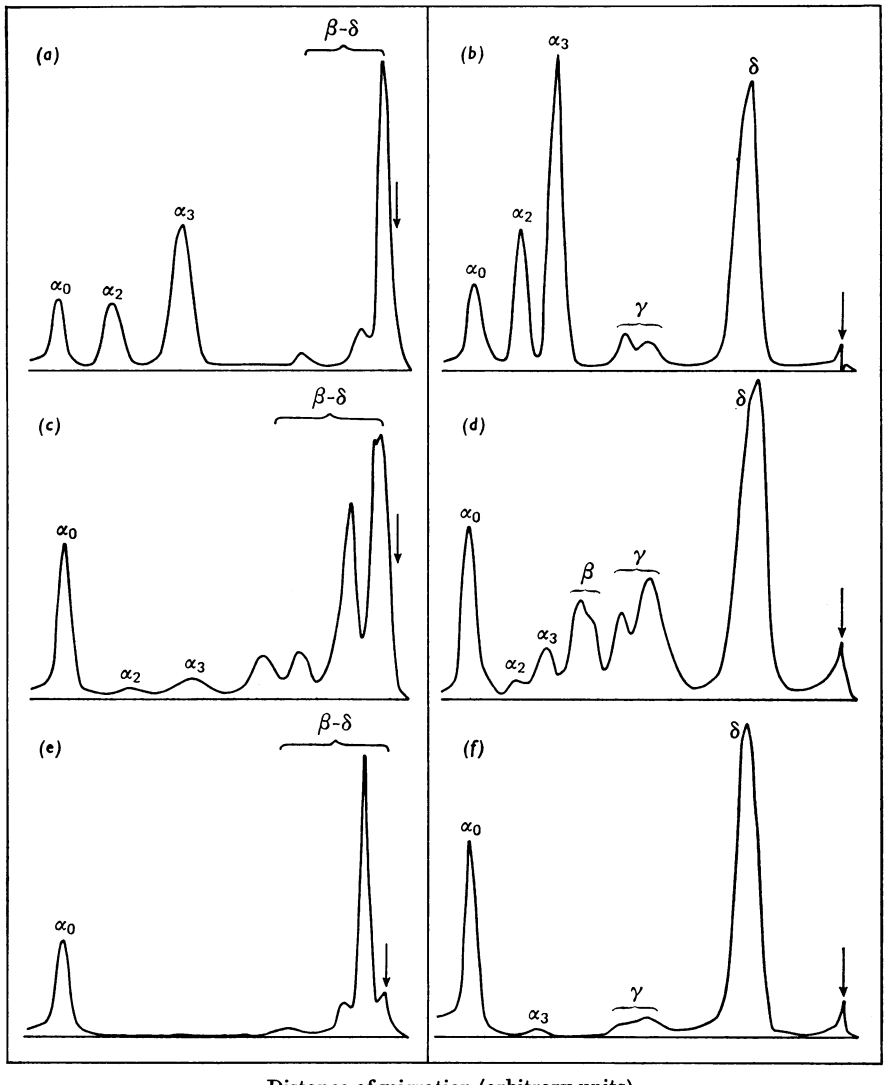

Fig. 1. T.l.c. of azopigments derived from gall-bladder bile of dog, post-obstructive bile of man and normal bile of Wistar (R-strain) rat. Samples of azopigment extracts obtained from bile of dog (*a* and *b*), man (*c* and *d*) and rat (*e* and *f*) were applied to two silica-gel plates. One plate was developed with chloroform–methanol (17:3, v/v) (*a*, *c* and *e*), and the second plate was developed with chloroform–methanol–water (65:25:3, by vol.) followed by chloroform–methanol (17:3, v/v) (*b*, *d* and *f*). Densitometric scans are shown. The starting line is indicated by an arrow. Migration is from right to left. Chromatographically distinguishable azopigments or azopigment groups are denoted by Greek letters (Heirwegh *et al.* 1970*a*). Further details are given in the Materials and Methods section.

The results obtained by t.l.c. were confirmed by anion-exchange chromatography in boric acid, pH 7 (Kesler, 1967). The technique was of value for confirming or disproving the suspected identity of the isolated sugars, as (1) the order of elution of sugars from the column is different from their relative R_F values on thin-layer plates with the solvent systems used in the present work, and (2) D-xylose and D-glucose are well separated from each other, from the other reference sugars tested and from some additional sugars examined by Kesler (1967): gentiobiose, lyxose and 2-deoxy-D-ribose. The material derived from azopigment α_2 moves as a single peak that coincided exactly with D-xylose

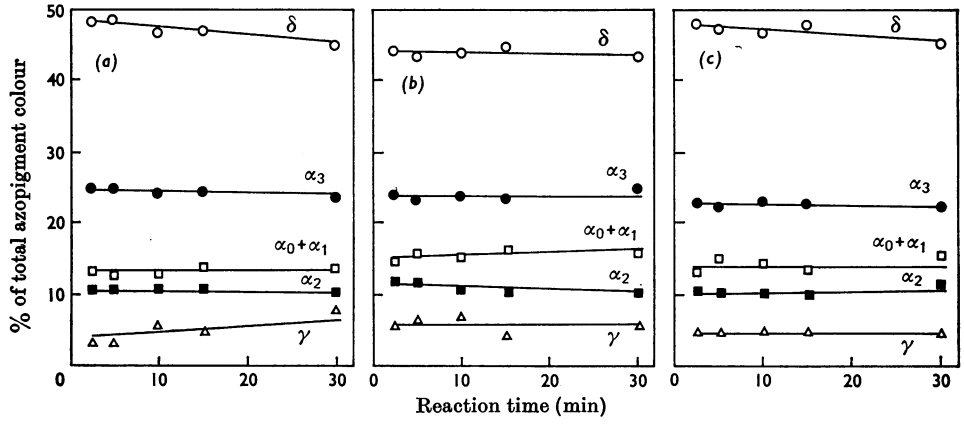

Fig. 2. Percentage composition of the azopigments obtained from gall-bladder bile of dog on treatment with diazotized ethyl anthranilate for various times. Reaction systems were buffered with glycine–HCl buffer (*a*) or with citric acid–phosphate buffer (*b* and *c*) at final pH values 2.80 (*a*), 2.80 (*b*) and 5.85 (*c*). The azopigment mixtures resulting from diazo-coupling were separated by t.l.c. and analysed by densitometry as outlined in the Materials and Methods section. Chromatographically distinguishable azopigments or azopigment groups are indicated by Greek letters (Heirwegh *et al.* 1970*a*).

on mixed chromatographic analysis. D-Glucose was detected as the main component in the hydrolysate obtained from azopigment α_3. However, in the latter case trace amounts of rapidly moving material were also found.

The purified conjugating material obtained from azopigment α_3 showed an immediate and very strong reaction with glucose oxidase test paper (Clinistix; from Ames Co., Stoke Poges, Bucks., U.K.) whereas the reaction was very weak and slow with azopigment α_2.

Azopigment α_3 was hydrolysed nearly completely by Mylase-P and by emulsin. In the latter case reaction was inhibited completely by glucono-(1→5)-lactone (Table 1). The emulsin preparation showed pronounced β-glucosidase activity, which was inhibited by gluconolactone (Table 2). Its α-glucosidase activity was negligible. Azopigment α_2, which is suspected to contain D-xylose (see above), was hydrolysed only slightly by emulsin and to a large extent by Mylase-P (Table 1) in accordance with the relatively large β-xylosidase activity of the latter enzyme preparation (Table 2). Inhibition of the enzymic hydrolysis required rather high gluconolactone concentrations when Mylase-P was tested either with azopigment α_2 (Table 1) or with *p*-nitrophenyl β-D-xylopyranoside (Table 2) as the substrate.

DISCUSSION

The application of the diazonium salts of aniline (Kuenzle, 1970*a*,*b*,*c*) and of ethyl anthranilate (Van Roy & Heirwegh, 1968; Heirwegh *et al.* 1970*a*) for the analysis of bile pigments has revealed much greater heterogeneity than hitherto suspected. In studies with post-obstructive human bile Kuenzle (1970*c*) demonstrated the existence of disaccharidic conjugating groups composed of aldobiuronic acid or pseudoaldobiuronic acid or equimolecular amounts of hexuronic acids. By using the methods developed by Heirwegh *et al.* (1970*a*) relatively large amounts of the azopigments α_2 (about 10% of total azopigment colour) and α_3 (about 30% of total azopigment colour) were derived from dog bile. Much smaller amounts of chromatographically similar compounds were obtained from human post-obstructive bile (Fig. 1). They were virtually absent from normal rat bile.

The results obtained in the present work demonstrate some features of the structures of the azopigments α_2 and α_3. The ammonolysis experiments, in conjunction with previous findings (Compernolle *et al.* 1970), indicate that the azopigments are ester conjugates of azobilirubin. The isolated conjugating groups of the azopigments α_2 and α_3 behaved chromatographically as D-xylose and D-glucose respectively. In the latter case the identity of the conjugating moiety has been further confirmed by two methods. (1) In agreement with the known specificity of glucose oxidase (Keilin & Hartree, 1948, 1952), the purified conjugating material reacted promptly with glucose oxidase test paper. (2) The azopigment was readily hydrolysed by emulsin and Mylase-P (Table 1) and hydrolysis was inhibited by glucono-(1→5)-lactone, as expected from literature data (Conchie, Gelman & Levvy, 1967) and from model studies (Table 2). As emulsin contained negligible α-glucosidase activity the evidence given above strongly supports

Table 3. *R_{Glc} values of reference sugars and of the conjugating residues obtained from the α_2- and α_3-azopigments derived from dog bile*

The conjugating groups were obtained by ammonolysis of the azopigments and analysed by t.l.c. (see the Materials and Methods section). R_{Glc} values were obtained for the following sugar solvents: (1) propan-1-ol–water (17:3, v/v); (2) butan-1-ol–acetic acid–water (12:3:5, by vol.); (3) butan-1-ol–pyridine–water (10:3:3, by vol.).

	R_{Glc}		
Reference compounds	Solvent (1)	Solvent (2)	Solvent (3)
L-Rhamnose	2.10	1.79	1.90
D-Xylose	1.61	1.40	1.48
D-Ribose	1.44	1.40	1.44
L-Arabinose	1.22	1.17	1.23
D-Mannose	1.13	1.09	1.15
D-Fructose	1.10	1.11	1.13
D-Glucose	1.00	1.00	1.00
D-Galactose	0.83	0.92	0.72
Conjugating group from			
α_2-Azopigment	1.60, 1.62, 1.60	1.41, 1.41	1.49, 1.48
α_3-Azopigment	1.00, 1.00, 1.02	1.00, 0.99	1.00, 0.99

the conclusion that the purified α_3-azopigment corresponds to azobilirubin β-D-monoglucoside. With azopigment α_2, which obviously is a xylose conjugate, the hydrolysis experiments with Mylase-P (Table 1) similarly support a β-D configuration. The relatively slow attack by emulsin remains unexplained.

Several features of the structures of the azopigments, and more in particular the C-1 attachment of the sugars to the propionic acid side chains of azobilirubin, have been confirmed by Compernolle *et al.* (1971). In addition, it was shown that the conjugating groups are monoglycopyranosides.

How closely do the structures of the azobilirubin monoglycosides reflect structures pre-existing in the parent bile pigments? No structural change during the purification was indicated by any variation of R_F values of the purified azopigments α_2 and α_3 from those of unpurified azopigments in diazotized dog bile. However, the isolated azopigments might have been formed from more complex precursors during diazotization. The kinetic results in Fig. 2 invalidate such a hypothesis. Over the time-period examined no evidence for a precursor–product relationship was obtained. It is very unlikely that any change occurred during the short initial reaction period (2.5min) at pH2.8 or 5.85. Azopigment α_3 cannot have originated from aldobiuronic acid conjugates (Kuenzle, 1970c) as the isolation of the latter compounds involved prolonged chromatographic analyses and diazotization for 1h at room temperature at pH about 5.8. With azopigment α_2 acid decarboxylation of azobilirubin β-D-monoglucuronoside or of bilirubin β-D-glucuronosides could be postulated. The reaction conditions required to remove CO_2 from glucosiduronic acids (Lefèvre & Tollens, 1907; Marsh, 1966) are much more drastic than those used in the present diazo-coupling procedures. The isolated azopigments are clearly not artifacts formed from dipyrryl moieties of conjugated bile pigments.

In animals the excretion of only one pentose glycoside, the 1-ribosylimidazol-4(5)-ylacetic acid, has been reported (Schayer, 1956). Disaccharidic bilirubin conjugates containing hexuronic acid residues, aldobiuronic acid or pseudoaldobiuronic acid (Kuenzle, 1970c) have been demonstrated by means of their phenylazo derivatives. Mixed conjugates of steroids containing glucuronic acid and either glucopyranose (Williamson, Collins, Layne, Conrow & Bernstein, 1969; Williamson & Layne, 1970) or *N*-acetylglucosamine (Layne, Sheth & Kirdani, 1964; Layne, 1965; Arcos & Lieberman, 1967; Collins, Williams & Layne, 1967; Collins & Layne, 1968) have also been described. The glycosidic linkages of the steroid conjugates were shown to be in the β-D configuration (Collins *et al.* 1967; Williamson *et al.* 1969; Matsui & Fukushima, 1969). The available evidence supports the contention that, as for conjugation with glucuronic acid, UDP-sugars are involved as donors of D-glucose (Collins, Williamson & Layne, 1970) and *N*-acetylglucosamine (Collins, Jirku & Layne, 1968; Collins *et al.* 1970). Similarly bilirubin conjugated either with D-xylose or with D-glucose has been synthesized *in vitro* by incubating the appropriate UDP-sugars and unconjugated bilirubin in the presence of liver homogenates of dog and rat (J. Fevery, P. Leroy & K. P. M. Heirwegh, unpublished work). The synthesis *in vitro* of *p*-nitrophenyl β-D-glucoside in a similar system has also been reported (Gessner & Vollmer, 1969).

It is rather unexpected to find about 10% of total azobilirubin conjugated with D-xylose, as no other instance of the excretion of xyloglycosides in animals has been described. However, xylose is a constituent of bovine lung heparin (Lindahl & Rodén, 1965; Lindahl, 1968) and nasal chondromucoprotein (Gregory, Laurent & Rodén, 1964). Further, transfer of D-xylose from UDP-xylose to serine (as present in some heparins) by a mouse ascitic mast-cell tumour preparation has been demonstrated by Grebner, Hall & Neufeld (1966), and enzymes that decarboxylate UDP-glucuronic acid have been detected in the tumour preparations (Silbert & DeLuca, 1967) and in hen oviduct (Bdolah & Feingold, 1965).

In animals conjugation of endogenous (and possibly also exogenous) compounds with monosaccharides other than glucuronic acid may be of more frequent occurrence than hitherto suspected. In some cases such processes may constitute major routes in detoxication, as illustrated by the work of Layne and his collaborators (see above for references) and of Kuenzle (1970*a*,*b*,*c*) and by the large proportion of azobilirubin β-D-monoglucoside derived from dog bile.

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REFERENCES

Arcos, M. & Lieberman, S. (1967). *Biochemistry, Easton*, **6**, 2032.

Bdolah, A. & Feingold, D. S. (1965). *Biochem. biophys. Res. Commun.* **21**, 543.

Beck, C. & Tappel, A. L. (1968). *Biochim. biophys. Acta*, **151**, 159.

Collins, D. C., Jirku, H. & Layne, D. S. (1968). *J. biol. Chem.* **243**, 2928.

Collins, D. C. & Layne, D. S. (1968). *Can. J. Biochem.* **46**, 1089.

Collins, D. C., Williams, K. I. H. & Layne, D. S. (1967). *Archs Biochem. Biophys.* **121**, 609.

Collins, D. C., Williamson, D. C. & Layne, D. S. (1970). *J. biol. Chem.* **245**, 873.

Compernolle, F., Jansen, F. H. & Heirwegh, K. P. M. (1970). *Biochem. J.* **120**, 891.

Compernolle, F., Van Hees, G. P., Fevery, J. & Heirwegh, K. P. M. (1971). *Biochem. J.* **125**, 811.

Conchie, J., Gelman, A. L. & Levvy, G. A. (1967). *Biochem. J.* **103**, 609.

Dutton, G. J. (1966*a*). In *Glucuronic Acid, Free and Combined*, p. 185. Ed. by Dutton, G. J. New York and London: Academic Press.

Dutton, G. J. (1966*b*). *Archs Biochem. Biophys.* **116**, 399.

Gessner, T. & Vollmer, C. A. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.* **28**, 545.

Grebner, E. E., Hall, C. W. & Neufeld, E. F. (1966). *Archs Biochem. Biophys.* **116**, 391.

Gregory, J. D., Laurent, T. C. & Rodén, L. (1964). *J. biol. Chem.* **239**, 3312.

Hassid, W. Z. (1967). In *Metabolic Pathways*, vol. 1, 3rd ed., p. 354. Ed. by Greenberg, D. M. New York and London: Academic Press.

Heirwegh, K. P. M., Van Hees, G. P., Compernolle, F. & Fevery, J. (1970*b*). *Biochem. J.* **120**, 17P.

Heirwegh, K. P. M., Van Hees, G. P., Leroy, P., Van Roy, F. P. & Jansen, F. H. (1970*a*). *Biochem. J.* **120**, 877.

Keilin, D. & Hartree, E. F. (1948). *Biochem. J.* **42**, 221.

Keilin, D. & Hartree, E. F. (1952). *Biochem. J.* **50**, 331.

Kesler, R. B. (1967). *Analyt. Chem.* **39**, 1416.

Krebs, K. G., Heusser, D. & Wimmer, H. (1967). In *Dunnschicht-Chromatographie, ein Laboratoriumshandbuch*, 2nd ed., p. 813. Ed. by Stahl, E. Berlin, Heidelberg and New York: Springer-Verlag.

Kuenzle, C. C. (1970*a*). *Biochem. J.* **119**, 387.

Kuenzle, C. C. (1970*b*). *Biochem. J.* **119**, 395.

Kuenzle, C. C. (1970*c*). *Biochem. J.* **119**, 411.

Layne, D. S. (1965). *Endocrinology*, **76**, 600.

Layne, D. S., Sheth, N. A. & Kirdani, R. Y. (1964). *J. biol. Chem.* **239**, 3221.

Lefèvre, K. U. & Tollens, B. (1907). *Ber. dt. chem. Ges.* **40**, 4513.

Lewis, B. A. & Smith, F. (1967). In *Dunnschicht-Chromatographie, ein Laboratoriumshandbuch*, 2nd ed., p. 794. Ed. by Stahl, E. Berlin, Heidelberg and New York: Springer-Verlag.

Lindahl, U. (1968). *Biochim. biophys. Acta*, **156**, 203.

Lindahl, U. & Rodén, L. (1965). *J. biol. Chem.* **240**, 2831.

Marsh, C. A. (1955). *Nature, Lond.*, **176**, 176.

Marsh, C. A. (1966). In *Glucuronic Acid, Free and Combined*, p. 119. Ed. by Dutton, G. J. New York and London: Academic Press.

Matsui, M. & Fukushima, D. K. (1969). *Biochemistry, Easton*, **8**, 2997.

Schayer, R. W. (1956). *Br. J. Pharmac.* **11**, 472.

Silbert, J. E. & DeLuca, S. (1967). *Biochim. biophys. Acta*, **141**, 193.

Smith, R. L. & Williams, R. T. (1966). In *Glucuronic Acid, Free and Combined*, p. 457. Ed. by Dutton, G. J. New York and London: Academic Press.

Van Roy, F. P. & Heirwegh, K. P. M. (1968). *Biochem. J.* **107**, 507.

Williamson, D. G., Collins, D. C., Layne, D. S., Conrow, R. B. & Bernstein, S. (1969). *Biochemistry, Easton*, **8**, 4299.

Williamson, D. G. & Layne, D. S. (1970). *Steroids*, **15**, 327.

With, T. K. (1968). *Bile Pigments*, p. 381. New York and London: Academic Press.