

Glucuronic Acid Conjugates of Bilirubin-IX α in Normal Bile Compared with Post-Obstructive Bile

TRANSFORMATION OF THE 1-O-ACYLGLUCURONIDE INTO 2-, 3-, AND 4-O-ACYLGLUCURONIDES

By FRANS COMPERNOLLE,* GUSTAAF P. VAN HEES,† NORBERT BLANCKAERT†
and KAREL P. M. HEIRWEGH†

*Department of Macromolecular and Organic Chemistry, University of Leuven, Heverlee, Belgium,
and †Laboratory of Hepatology, Department of Medical Research, University of Leuven, Leuven, Belgium

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Structures have been determined for bilirubin-IX α conjugates in freshly collected bile of normal rats, dogs and man and in post-obstructive bile of man and rats. The originally secreted conjugate has been characterized as azopigment (I), i.e. a 1-O-acyl- β -D-glucopyranuronic acid glycoside. Conversion of the acetylated methyl ester of azopigment (I) into methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy- β -D-glucopyranuronate (V) indicates the pyranose ring structure for the carbohydrate and a C-1 attachment for the bilirubin-IX α acyl group. Alternative procedures for deconjugation of azopigment (I) and its derivatives are also described. In post-obstructive bile, the 1-O-acylglucuronide is converted into 2-, 3- and 4-O-acylglucuronides via sequential intramolecular migrations of the bilirubin acyl group. The following approach was utilized. (1) The tetrapyrrole conjugates were cleaved to dipyrrolic aniline and ethyl anthranilate azopigments, and the azopigments were separated as the acids or methyl esters. (2) The isomeric methyl esters were characterized by mass spectral analysis of the acetates and silyl ethers. (3) The free glycosidic function was demonstrated by 1-oxime and 1-methoxime derivative formation. (4) The position of the dipyrrolic O-acyl group was determined for the methyl esters by protecting the free hydroxyl groups of the glucuronic acid moieties as the acetals formed with ethyl vinyl ether and by further conversion of the carbohydrates into partially methylated alditol acetates. These were analysed by using g.l.c.-mass spectrometry. The relevance of the present results with regard to previous reports on disaccharidic conjugates is discussed. Details of procedures for the formation of chemical derivatives for g.l.c. and mass spectrometry have been deposited as Supplementary Publication SUP 50081 (15 pages) at the British Library Lending Division, Boston Spa, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978), **169**, 5.

The structure of conjugated bilirubin-IX α , in particular the nature of the carbohydrate bound to the tetrapyrrole, still requires elucidation. Structural studies on conjugated bilirubin mentioned below were mostly performed on the more stable azopigment derivatives obtained by diazonium cleavage at the central methylene bridge of the tetrapyrrole. Talafant (1956), Billing *et al.* (1957) and Schmid (1957) tentatively assigned glucuronide structures to the major bilirubin conjugates of human bile on the basis of hydrolysis studies and paper chromatography of the carbohydrate.

The problem has been re-examined with more precise analytical techniques by several authors. Kuenzle (1970*a,b*) assigned disaccharidic structures, i.e. aldobiuronic acid, hexuronosylhexuronic acid and pseudoaldobiuronic acid, to the carbohydrates

released with aqueous 0.1M-ammonia from azopigments B₄, B₅ and B₆, but failed to detect a glucuronide conjugate.

Heterogeneity of bile pigment conjugates was revealed also by t.l.c. of ethyl anthranilate azopigments prepared from bile of patients with various liver diseases and from post-obstructive rat bile (Fevery *et al.*, 1972). For normal rat bile, however, only azopigment fractions α_0 (unconjugated azodipyrrole) and δ were detected. To the latter δ -azopigment fraction, Compennolle *et al.* (1970) assigned a glucuronide structure based on the mass spectrum of a derivative obtained by methyl esterification of the glucuronic acid carboxyl group and silylation of the hydroxyl groups.

The divergent views held by Kuenzle and our group have been difficult to reconcile. Kuenzle (1973)

attributed our results to an unexplained 'silyl cleavage reaction' giving rise to selective loss of the non-reducing terminal sugar. He also mentioned detection of only glucose and glucuronic acid respectively, when 'disaccharidic' carbohydrates obtained from azopigment B₄ and azopigments B₅ and B₆ were silylated and subjected to g.l.c. analysis. Evidently, the argument can easily be reversed, as the latter unprecedented findings also cast doubt upon the validity of his results.

The study by Gordon *et al.* (1976) was concerned mainly with identification of glucuronic acid released by alkaline methanolysis of a δ -azopigment fraction. The δ -azopigment was the only (or one of two) azodipyrrole product resulting from diazonium cleavage of some major bilirubin-IX α mono- and di-conjugates, separated at the tetrapyrrole stage (Heirwegh *et al.*, 1975; Gordon *et al.*, 1977).

With the present work we intend to determine structures of conjugates of bilirubin-IX α for two categories of bile samples, i.e. 'normal' and post-obstructive biles. The major azopigment derived from bilirubin conjugates in 'normal bile' retains the original C-1 ester linkage with β -glucopyranuronic acid.

For post-obstructive bile, more complex patterns of conjugated azopigments are observed when using either the pH 6.0 aniline diazonium method (Kuenzle, 1970a) or the pH 2.7 ethyl anthranilate diazonium procedure of our group (Van Roy & Heirwegh, 1968). In both cases the observed complexity reflects a sequential migration of the bilirubin 1-*O*-acyl group to positions 2, 3 and 4 of glucuronic acid. The present work deals with the structures of the four positional isomers and is confined to aniline and ethyl anthranilate azopigments obtained by applying the pH 6.0 diazonium method.

Preliminary accounts of this work have appeared (Compernelle *et al.*, 1975, 1977; Heirwegh *et al.*, 1976).

Results and Discussion

Definition of 'normal' and post-obstructive bile

'Normal bile' was freshly collected from healthy rats, dogs and man. Occasionally it was stored at -18°C in the dark for short periods of time (not longer than 1 week). This bile was 'stabilized' with regard to acyl migration by acidifying to pH 4-6.

Initial post-obstructive bile samples from man and rats were obtained as described previously (Fevery *et al.*, 1972). In the course of the investigation it was found, however, that the same t.l.c. patterns of azopigments and their methyl esters (see below) were formed by simple incubation of fresh bile from normal rats and man. For this purpose bile was kept under N₂ at 37°C or 20-25°C for 1-20h (Blanckaert *et al.*, 1978). In the subsequent

work, azopigments were therefore prepared from incubated bile.

Diazonium cleavage and isolation of δ -azopigment fraction

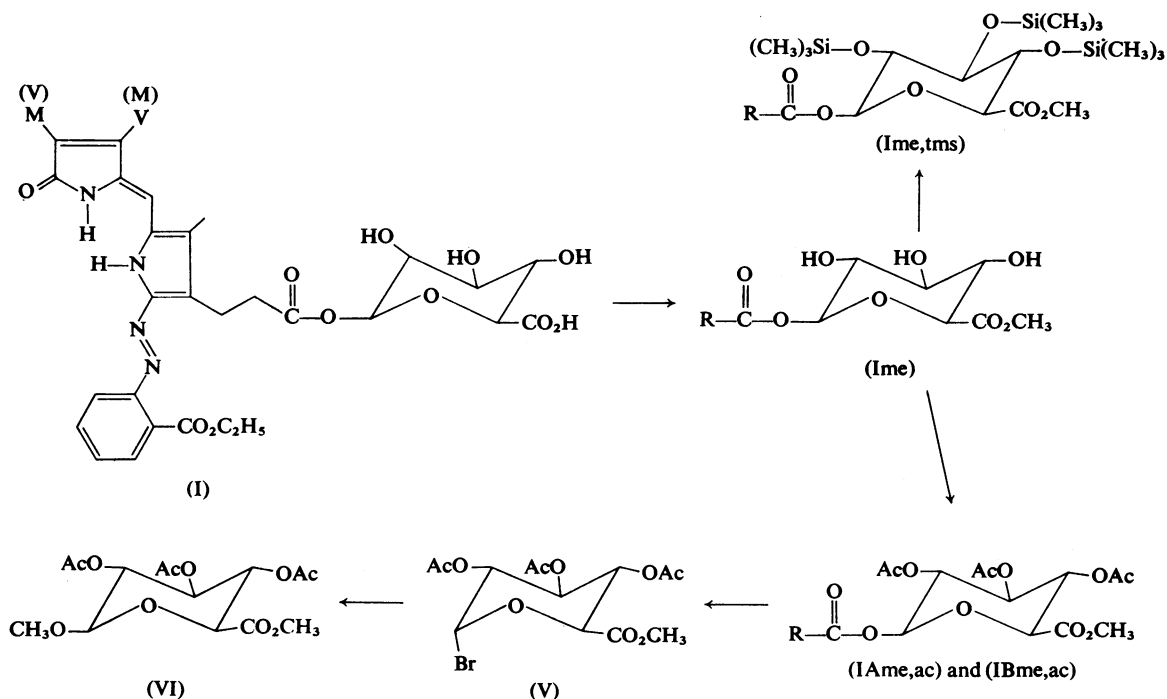
Analysis of bilirubin conjugates in bile was performed by diazonium cleavage of bilirubin conjugates at the central bridge of the tetrapyrrole. Completely analogous results were obtained by using either ethyl anthranilate or aniline diazonium reagents. The pH 6.0 procedure (Kuenzle, 1970a) adopted in the present work utilizes equivalent amounts of aromatic amine and NaNO₂. This prevents acid-catalysed reaction of the free glycosidic function of 2-, 3- and 4-*O*-acyl glucuronides with an excess of aromatic amine, which has been observed (F. Compernelle, unpublished work) when using the pH 2.7 method of Van Roy & Heirwegh (1968). The ethyl anthranilate *N*-glucuronides formed in this reaction are detected as so-called azopigments β and γ (Fevery *et al.*, 1972).

When starting from normal bile, both the pH 2.7 and the pH 6.0 procedures yielded the 1-*O*-acyl glucuronide [azopigment (I)] as the major derivative. A δ -azopigment fraction was isolated by using column chromatography and t.l.c. on ordinary silica-gel plates. Small and larger amounts (for fresh and older biles respectively) of the 2-, 3- and 4-*O*-acylglucuronides were separated on silane-treated plates. The pure azopigment (I) was hydrolysed completely with β -glucuronidase (Blanckaert *et al.*, 1978). It remained homogeneous on t.l.c. of the acid azopigment, the methyl ester (Ime) and the acetylated methyl ester (Ime,ac), except for the separation at the last stage of the two isomers designated (IAme,ac) and (IBme,ac) with interchanged methyl and vinyl groups (Scheme 1).

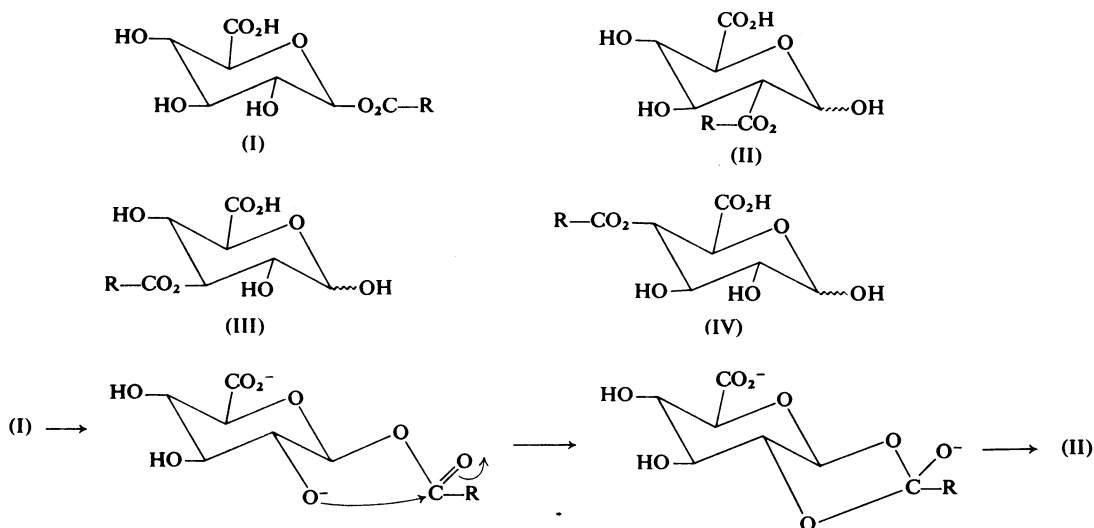
Treatment of post-obstructive or incubated bile with the pH 6.0 ethyl anthranilate and aniline diazonium reagents also yielded a δ -azopigment fraction, isolated by column and thin-layer chromatography. However, t.l.c. analysis of this δ -fraction on ordinary silica-gel plates revealed the presence of a poorly separated mixture of azopigments. Partial separation was obtained by using silane-treated plates. By conversion into the structurally characterized methyl esters (see below), it was shown that the most-mobile acid fraction was a mixture of 2- and 3-*O*-acylglucuronide azopigments (II) and (III), and the intermediate and least-mobile acid fractions corresponded to the 1- and 4-*O*-acylglucuronide azopigments (I) and (IV) respectively (Scheme 2).

Derivatives of azopigment (I): methyl ester formation, silylation and acetylation

Derivatives prepared for structure elucidation of azopigment (I) are shown in Scheme 1. First, the

Scheme 1. Structure of δ -azopigment (I) and derivatives

R = azodipyrrole moiety. Two isomers with interchanged methyl (M) and vinyl (V) groups were separated on t.l.c. as the acetates (IAme,ac) and (IBme,ac) [(IAme,ac), *endo*-vinyl group, less-mobile isomer; (IBme,ac), *exo*-vinyl group, more-mobile isomer].

Scheme 2. Structures of ethyl anthranilate and aniline azopigments present in the δ -fraction of azopigments obtained by diazonium reaction on incubated or post-obstructive bile at pH 6.0

R = ethyl anthranilate or aniline azodipyrrole moiety. The mechanism of conversion of a 1-O-acylglucuronide into a 2-O-acyl compound is shown to involve an ortho acid intermediate.

carboxyl group of glucuronic acid was esterified with diazomethane. The solvent plays a crucial role in this reaction. With chloroform, dry tetrahydrofuran or *NN*-dimethylformamide incomplete methylation was observed. (This reaction was even more difficult with the 2-, 3- and 4-*O*-acylglucuronides.) With methanol, results varied from incomplete and complete methylation to methanolysis of the glucuronic acid ester group. Aqueous pentan-2-one and aqueous tetrahydrofuran gave satisfactory results. Water probably ruptures intramolecular and intermolecular hydrogen bridges, thereby rendering the azopigment glucuronides more soluble and the carboxyl group more accessible for the reagent.

Field-desorption mass spectrometry was applied to the ethyl anthranilate azopigment acid (I) and the methyl ester (Ime). The former yielded an ion at *m/e* 462, corresponding to the aglycone carboxylic acid, produced by thermal elimination of the glucuronic acid moiety. Possibly, the presence of Na⁺ and K⁺ cations was the cause of the failure to obtain a satisfactory spectrum (Holland *et al.*, 1976). The methyl ester (Ime) gave the expected molecular ion at *m/e* 652 together with cationization product ions [M+Na]⁺ and [M+K]⁺ found at *m/e* 675 and 691.

The n.m.r. spectrum (Table 1) of the methyl ester (Ime) exhibits the anomeric proton (δ 5.45 p.p.m) with *J* 7 Hz. The high value for the coupling constant indicates the β -configuration for the glycosidic linkage, as the same value, *J* 7 Hz, was found for the β -anomer (δ 5.75 p.p.m) of model

compounds (VII) (Scheme 3) and a smaller value, *J* 3 Hz, for the α -anomer (δ 6.28 p.p.m.). Further assignments in the n.m.r. spectrum of methyl ester (Ime) were made by comparison with the spectra of the acetates (VII) and with the spectra of the azopigment aglycones (Compernelle *et al.*, 1971).

Silylation or acetylation of methyl ester (Ime) yields derivatives suitable for electron-impact mass spectrometry. The silylation with *NO*-bis(trimethylsilyl)acetamide reported previously (Compernelle *et al.*, 1970) gave a mixture of bis- and tris-(trimethylsilyl) derivatives yielding molecular ions at *m/e* 796 and 868. By using the more powerful reagent *NO*-bis(trimethylsilyl)trifluoroacetamide and longer reaction times, almost complete conversion into a tris(trimethylsilyl) ether (Ime,tms) was observed. In the present paper and previous work it is noteworthy that silylation of the lactam group does not occur for ethyl anthranilate azopigments, whether conjugated or not (see below; Blanckaert *et al.*, 1977). By contrast, the lactam group of aniline azopigments and of bilirubin-IX isomers is silylated readily (Salmon & Fenselau, 1974; Compernelle *et al.*, 1976). Consistent with the latter results is the finding that the phenylazo analogue of methyl ester (Ime) gave a tetrakis(trimethylsilyl) derivative (*M*⁺ at *m/e* 868). However, as observed from the relative intensities of the molecular ions at *m/e* 796 and 868, conversion into the tetrakis-(trimethylsilyl) derivatives was only 70% after 15 h, showing that one of the glucuronic acid hydroxyl groups is even more resistant to silylation than observed for the ethyl anthranilate azopigment methyl ester (Ime).

In the spectrum of the phenylazo analogue, an abundant fragment ion at *m/e* 403 forms by cleavage between the two methylene groups of the propionic acid side chain, thus suggesting an ester linkage for the latter with glucuronic acid. This fragmentation occurs with charge retention on the silylated phenylazodipyrrole fragment (Salmon & Fenselau, 1974). The analogous reaction proceeds poorly with the ethyl anthranilate azopigment (Ime,tms), for which the lactam group is not silylated.

A characteristic mass spectrum (Fig. 1, *M*⁺ at *m/e* 778) was obtained for the acetate (IAme,ac) (isomer with *endo*-vinyl group). Carbohydrate fragment ions (*m/e* 317, 257, 215, 197, 186, 155, 144, 127 and 115) are consistent with a 1-*O*-acylglucuronide structure and were assigned by referring to the spectra of the glucuronic acid derivatives (V) and (VI) (see below). For the isomer (IBme,ac) with *exo*-vinyl group, the molecular ion (*M*⁺ 778) exhibited a weak relative intensity, probably due to thermal breakdown.

Copper chelate formation observed in Fig. 1 (*M*⁺ 839 to 842) is a general feature in mass

Table 1. *N.m.r. spectrum of ethyl anthranilate azopigment methyl ester (Ime) in [2H₆]dimethyl sulphoxide*
m = multiplet, unresolved or not further analysed.

δ (p.p.m.)	Multi-plicity	Assignment and remarks
1.38	t	CH ₃ -CH ₂ <i>J</i> 7 Hz
2.01	s	CH ₃ on pyrrolinone ring (<i>endo</i> -vinyl isomer)
2.06	s	CH ₃ on pyrrole ring (<i>endo</i> -vinyl isomer)
2.06	s	CH ₃ on pyrrole ring (<i>exo</i> -vinyl isomer)
2.20	s	CH ₃ on pyrrolinone ring (<i>exo</i> -vinyl isomer)
2.80	m	CH ₂ -CH ₂ propionic side chain
3.3	m	CHOH C ₍₂₎ , C ₍₃₎ and C ₍₄₎ of sugar
3.66	s	CO ₂ CH ₃
3.90	d	CH(CO ₂ CH ₃) C ₍₅₎ of sugar
4.49	q	CH ₂ -CH ₃ <i>J</i> 7 Hz
5.45	d	Anomeric proton (β -linkage) <i>J</i> 7 Hz
5.6-7.1	m	Vinyl group
6.17	s	-CH= methine bridge
7.3-7.9	m	Aromatic protons

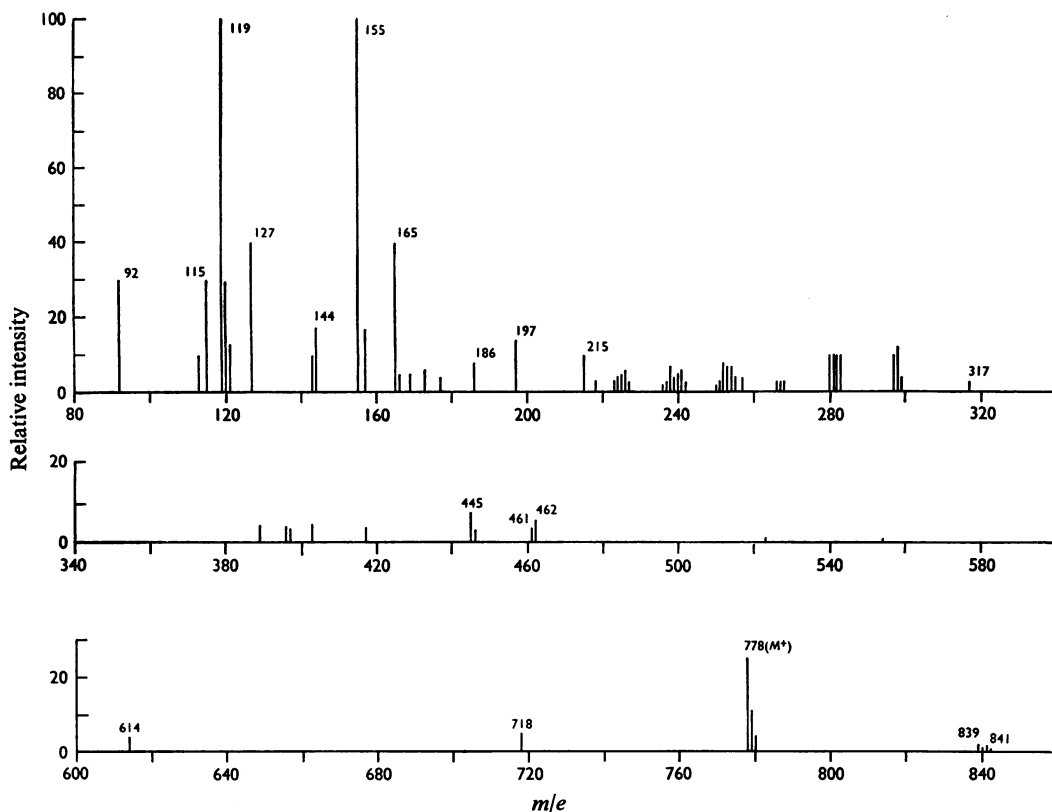


Fig. 1. Mass spectrum of azopigment methyl ester triacetate (IAme,ac)
The isomer (IAme,ac) contains an *endo*-vinyl group on the azodipyrrole moiety.

spectrometry of ethyl anthranilate azopigments. Possibly the ion chamber serves as a source of copper.

Chemical degradation of acetylated azopigment (I)

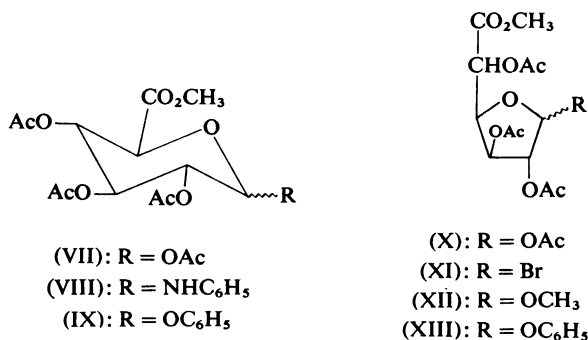
Assignment of structures to the azopigment part of isomers (IAme,ac) and (IBme,ac) was based on their conversion into the known azodipyrrole methyl esters by alkaline methanolysis (Heirwegh *et al.*, 1975).

The chemical degradation of the acetates (IAme,ac) and (IBme,ac) outlined in Scheme 1 proves the pyranose structure and the C-1 linkage of the glucuronic acid moiety. An analogous procedure for structure elucidation of xylopyranose and glucopyranose conjugates of bilirubin has been reported previously (Compennolle *et al.*, 1971). Treatment with HBr and acetic acid yields the glycosyl bromide (V), which was converted into the methyl glycoside (VI) with methanol and Ag_2CO_3 . Both glucuronic acid derivatives (V) and (VI) were identified by comparison with authentic

samples by using combined g.l.c.-mass spectrometry. The known methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy- α -D-glucopyranuronate, identical with compound (V), is the more stable anomer and was derived from methyl 1,2,3,4-tetra-*O*-acetyl- β -D-glucopyranuronate (VII) in the same way as from the acetates (IAme,ac) and (IBme,ac) (Bowering & Timell, 1960).

The methyl glycoside (VI), prepared from unknown compound (V) and reference compound (V), presumably has the β -configuration since the Koenigs-Knorr reaction generally leads to 1,2-*trans*-glycosides when an acetoxy group at C-2 participates in the reaction (Conchie & Levvy, 1963; Hough & Richardson, 1967).

To differentiate between pyranose and furanose isomers and to exclude possible interconversions of ring structures, the same reaction sequence was applied to the furanosyl model compound (X) (Scheme 3). Only methyl furanosides (XII) were demonstrated by g.l.c.-mass spectrometry of the reaction products. The ring isomers (VI) and (XII)



Scheme 3. Structures of acetylated pyranose (VII) and furanose (X) model compounds and derivatives formed by reaction of the 1-*O*-acetyl group

Compounds (VIII) and (IX) were obtained from the azopigment acetates (IAme,ac) and (IBme,ac).

can be differentiated readily by virtue of different retention times and mass spectra.

Compound (X) was synthesized from D-glucofuranosyl-3,6-lactone. Acetylation yields the 1,2,5-tri-*O*-acetate of glucuronolactone. Hydrolysis of the lactone with NaHCO₃ at 0°C liberates the carboxyl group and the C-3 hydroxyl group, which are esterified with diazomethane and acetylated respectively.

Alternative chemical degradation of azopigment (I)

Alternative routes for chemical degradation of azopigment (I) were explored. Reduction of the 1-bromo acetate (V) with LiAlH₄ produced the 1,5-anhydroalditol of glucose, identified by g.l.c.-mass spectrometry as the 2,3,4,6-tetra-acetate. The reduction of 1-bromo derivatives constitutes a more general procedure for determining the ring structure of acetylated azopigment conjugates, which was applied also to methyl 1,2,3,5-tetra-*O*-acetyl- $\alpha\beta$ -glucofuranuronate (X) and to acetylated 2-, 3- and 4-*O*-acylglucuronides (see below).

A particularly gentle deconjugation method consists of treating the acetates (IAme,ac) and (IBme,ac) with aniline and a small amount of acetic acid, yielding the aniline *N*-glucuronide (VIII), identified by mass spectrometry (M^{+} 409). As this procedure yields intense molecular ions (M^{+} 711) for the aniline *N*-glycosides derived from disaccharidic acetates (maltose, lactose and cellobiose were tested), the aniline deconjugation of acetylated C-1 ester conjugates constitutes a powerful method for detection of mono- and di-saccharides.

Alternatively, the anomeric phenyl glycosides (IX) can be prepared from the acetates (IAme,ac) and (IBme,ac) by reaction with a ZnCl₂/phenol

reagent (Montgomery *et al.*, 1942). Reaction of the model compounds (VII) and (X) gives the anomeric phenyl glucopyranosides (IX) and glucofuranosides (XIII) respectively, differentiated by g.l.c.-mass spectrometry.

Direct ammonolysis of azopigment (I) (Compernelle *et al.*, 1970) and hydrolysis with β -glucuronidase inhibited by the addition of glucaro-1,4-lactone (Blanckaert *et al.*, 1978) have been described. Glucuronic acid produced by ammonolysis was identified by using t.l.c. (Blanckaert *et al.*, 1978). A thorough identification of glucuronic acid obtained by alkaline methanolysis was performed by Gordon *et al.* (1976) with g.l.c., n.m.r. and mass spectrometry.

Treatment of azopigment (I) with 2M-HCl in methanol at room temperature (22°C) yields a mixture of α - and β -methyl glycosides of various glucuronic acid derivatives, i.e. lactones and pyranose and furanose methyl esters. An identical mixture was obtained from glucuronic acid treated in the same way, as seen on t.l.c. and confirmed by comparative g.l.c.-mass spectrometry.

A single glucuronic acid derivative was obtained by NaBH₄ reduction of the hydrolysed azopigment (I), conversion of the aldonic acid into the 1,4-lactone and silylation (Perry & Hulyalkar, 1965). The reduction was carried out in the medium (0.5M-NH₃ in water/methanol) used for hydrolysis, either with simultaneous or consecutive addition of hydrolysing and reducing agents.

Thus no carbohydrates other than glucuronic acid could be characterized as constituents of azopigment (I), although gentle methods of deconjugation and conversion of carbohydrates into stable derivatives were devised for detection of the labile mono- and di-saccharides claimed by Kuenzle (1970b).

Isolation of ethyl anthranilate and aniline azopigment methyl esters [(I_{me}), (II_{me}), (III_{me}) and (IV_{me})] and of an ethyl anthranilate azopigment lactone (III_{lact})

Whereas the ethyl anthranilate or aniline δ -azopigment fraction prepared from fresh normal bile consists only of the 1-*O*-acylglucuronide azopigment (I) yielding a single methyl ester (I_{me}) (see above), esterification of the ethyl anthranilate or aniline δ -azopigment fraction derived from post-obstructive or incubated bile gave four methyl esters. It is shown here that the four methyl esters correspond to the 1-, 2-, 3- and 4-*O*-acylglucuronide azopigments shown in Scheme 2 [(I), (II), (III) and (IV)]. T.l.c. mobilities for the methyl esters decreased in the order: (II_{me}), (III_{me}), (I_{me}) and (IV_{me}).

When normal bile was incubated for various periods of time, the azopigment methyl esters were detected in sequential order (Blanckaert *et al.*, 1978). A short incubation gave rise to detection of methyl esters (I_{me}) and (II_{me}), the latter corresponding to the 2-*O*-acylglucuronide as the only transformation product formed from the original 1-*O*-acylglucuronide. Increasing reaction times lead to higher relative amounts of 3-*O*-acylglucuronide and finally of 4-*O*-acylglucuronide, detected as azopigment methyl esters (III_{me}) and (IV_{me}) respectively.

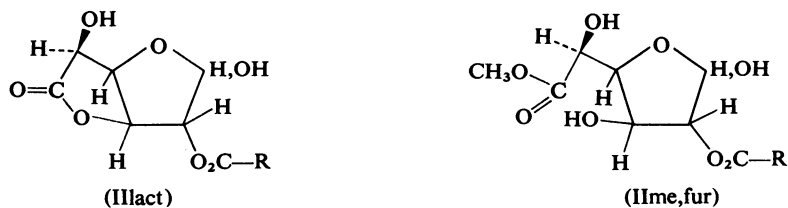
The methyl ester group of azopigments (I_{me})–(IV_{me}) was hydrolysed by storing the esters adsorbed on the silica-gel plates. Hydrolysis of the azopigment (II_{me}) to the acid (II) was especially rapid. The furanose methyl ester (II_{me,fur}) and the 3,6-lactone (III_{lact}) are probable intermediates in the hydrolysis reaction of methyl ester (II_{me}) (Scheme 4). The occurrence of a 3,6-lactone as an intermediate was confirmed by the finding that the ethyl anthranilate azopigment lactone (III_{lact}) was a contaminant of ethyl anthranilate azopigment methyl ester (II_{me}). This was shown (a) by t.l.c. separation of methyl ester and lactone, which revealed continuous conversion of the former into the latter during development and isolation, (b) by mass spectrometry of acetyl and silyl derivatives of the

lactone (M^{+} 704 and 764 respectively) and (c) by demonstrating the furanose ring for the acetylated lactone (see below). Storage of the ethyl anthranilate azopigment lactone (III_{lact}) on t.l.c. plates yielded the final hydrolysis product, ethyl anthranilate azopigment acid (II).

Acetylation and silylation of ethyl anthranilate and aniline azopigment methyl esters

Silylation was performed by reaction of ethyl anthranilate and aniline azopigment methyl esters (I_{me}), (II_{me}), (III_{me}) and (IV_{me}) with *NO*-bis-(trimethylsilyl)trifluoroacetamide and pyridine for various periods of time. Complete conversion (more than 95%) to tris(trimethylsilyl) ethers for ethyl anthranilate compounds and to tetrakis(trimethylsilyl) derivatives for aniline compounds was observed within reaction times of 30 min for the 3-*O*-acylglucuronides (III_{me}) and about 15 h for the 2- and 4-*O*-acylglucuronides (II_{me}) and (IV_{me}). Under the latter conditions, conversion for the 1-*O*-acylglucuronide was almost complete (98%) for the ethyl anthranilate azopigment methyl ester (I_{me}) but was only 70% for the aniline azopigment methyl ester (I_{me}).

The mass spectrum for the trimethylsilyl derivatives showed abundant molecular ions found at m/e 868 for both the ethyl anthranilate and aniline compounds [a carboxyethyl group present for the ethyl anthranilate compounds is compensated for by one more Si(CH₃)₃ group for the aniline compounds]. Incomplete silylation was revealed by molecular ions at m/e 796. Ethyl anthranilate fragment ions were observed at m/e 165, 119 and [M -164] and the corresponding aniline fragment ions were found at m/e 93 and [M -92]. The spectra of aniline compounds further exhibited an abundant fragment ion at m/e 403 due to allylic cleavage of the propionic side chain. A generally abundant carbohydrate fragment ion was found at m/e 217 [(CH₃)₃Si-O-CH=CH-CH=O⁺-Si(CH₃)₃]. Another characteristic fragment ion with structure [(CH₃)₃Si-O-CH=CH-O-Si(CH₃)₃]⁺ (m/e 204)



Scheme 4. Lactone and furanose methyl ester derivatives of ethyl anthranilate azopigment (II)
R = ethyl anthranilate azodipyrrole moiety.

encompasses $C_{(1)}-C_{(2)}$ or $C_{(2)}-C_{(3)}$ of the carbohydrate. It had a very low relative intensity for the 2- and 3-*O*-acylglucuronides, a moderate intensity for the 1-*O*-acylglucuronide and formed the base peak for the 4-*O*-acylglucuronide, thus confirming the structures assigned on the basis of chemical degradation (see below). Very similar results were obtained for the mass spectra of monoacetates of silylated methyl hexopyranosides (Boren *et al.*, 1973). The $C_{(2)}-C_{(3)}$ ion at m/e 204 formed the base peak for 4- and 6-acetates, but showed a small relative abundance for 2- and 3-acetates.

Acetylation of ethyl anthranilate azopigment methyl esters (I_{me}), (II_{me}), (III_{me}) and (IV_{me}) was carried out in the usual way. T.l.c. with the solvent system benzene/ethyl acetate (17:3, v/v) in each case separated the more-mobile *exo*-vinyl and less-mobile *endo*-vinyl isomers. The mass spectra of the ethyl anthranilate azopigment acetates (II_{me,ac}), (III_{me,ac}) and (IV_{me,ac}) resembled that shown above (Fig. 1) for the ethyl anthranilate azopigment (I_{me,ac}), showing molecular ions at m/e 778.

Acid methanolysis of *O*-acyl ester linkages and identification of glucuronic acid

The ethyl anthranilate azopigment methyl esters (I_{me}), (II_{me}), (III_{me}) and (IV_{me}) were treated with 2M-HCl in methanol as described for the ethyl anthranilate azopigment (I) (see above). Only derivatives of glucuronic acid were identified by applying t.l.c. and, after silylation, combined g.l.c.-mass spectrometry.

Ring form of glucuronic acid for the acetylated ethyl anthranilate azopigment methyl esters (II_{me,ac}), (III_{me,ac}) and (IV_{me,ac}) and for acetylated ethyl anthranilate azopigment lactone (III_{lact,ac})

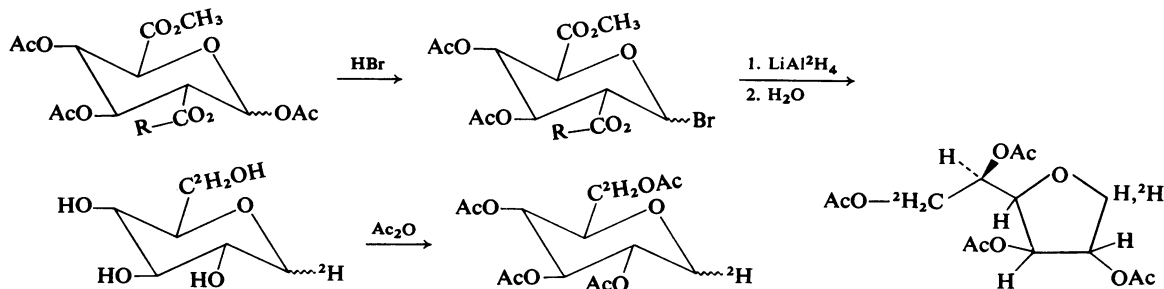
A general procedure for determining the ring

structure of the acetylated glucuronide moiety (exemplified for the 2-*O*-acylglucuronide in Scheme 5) consists of converting the acetates into glycosyl bromides, reducing the 1-bromo, *O*-acyl and methoxycarbonyl groups with $LiAl^2H_4$ or $LiAlH_4$ and acetylation. The method was checked for acetylated pyranose and furanose methyl esters of glucuronic acid and yielded the correctly labelled 1,5- and 1,4-anhydro[1,6,6- 2H_3]alditol acetates of glucose respectively. The 1,4-anhydroalditol acetate (Scheme 5) also was obtained from D-glucurono-3,6-lactone acetate. The 1,4- and 1,5-anhydroalditols were differentiated by their different retention times on g.l.c. (1% QF₁) and mass spectra.

Pyranose ring forms were assigned to the acetylated ethyl anthranilate azopigment methyl esters (II_{me,ac}), (III_{me,ac}) and (IV_{me,ac}) whereas the furanose ring form applies to the acetylated azopigment lactone (III_{lact,ac}). Indeed, carrying these compounds through the procedure respectively yielded the 1,5- and 1,4-anhydro[1,6,6- 2H_3]alditol acetates with exclusion respectively of the 2H_3 -labelled 1,4- and 1,5-isomers.

Reactions of ethyl anthranilate azopigment methyl esters (I_{me}), (II_{me}) and (III_{me}) with hydroxylamine and methoxyamine

The ethyl anthranilate azopigment methyl esters (I_{me}), (II_{me}) and (III_{me}) were treated with hydroxylamine and methoxyamine. For compounds (II_{me}) and (III_{me}) with free glycosidic function these reactions gave rise to 1-oxime and 1-methoxime azopigment derivatives, substituted on the glucuronic acid moiety. Competitive cleavage of the *O*-acyl group by hydroxylamine yielded an azodipyrrole hydroxamate. This cleavage reaction was not observed with methoxyamine. Further, migration of the *O*-acyl



Scheme 5. Pyranose ring structure of acetylated ethyl anthranilate azopigment methyl esters (I_{me,ac}), (II_{me,ac}), (III_{me,ac}) and (IV_{me,ac}), exemplified for 2-*O*-acylglucuronide (II_{me,ac})

R = ethyl anthranilate azodipyrrole moiety (partially degraded by treatment with HBr). A 1,5-anhydro[1,1,6- 2H_3]alditol acetate was identified. The structure of the 1,4-anhydro[1,1,6- 2H_3]alditol acetate derived from furanose ring forms is also shown.

groups seemed to be enhanced for the 1-oxime and 1-methoxime derivatives.

The azopigment reaction products were examined by using t.l.c. and mass spectrometry. When starting from ethyl anthranilate azopigment methyl esters (I_{me}) (1-*O*-acylglucuronide), hydroxamate (for hydroxylamine) and unchanged methyl ester (for hydroxylamine and methoxyamine) were the only azopigments detected.

When ethyl anthranilate azopigment methyl ester (III_{me}) (3-*O*-acylglucuronide) was treated with hydroxylamine, little hydroxamate was produced. Instead, t.l.c. revealed rapid formation of two closely moving and interconverting 1-oxime azopigments. An analogous pair of interconverting 1-methoxime azopigments was formed with methoxyamine. Mass spectrometry was performed for the silylated and acetylated oximes and methoximes. The spectrum of the silyl derivative showed molecular ions at *m/e* 955 for the oxime and 897 for the methoxime. Fragmentations occurring preferentially on carbon atoms bearing charge-stabilizing trimethylsilyloxy groups (Colard *et al.*, 1975), show the location of the *O*-acyl group on a C₍₁₎-C₍₄₎ and C₍₃₎-C₍₆₎ fragment (Scheme 6). This result probably indicates a mixture of 2- and/or 3-*O*-acyl and 4- and/or 5-*O*-acyl compounds, in agreement with the interconversions observed on t.l.c.

The acetylated methoxime gave a molecular ion at *m/e* 807. For the acetylated oxime, thermal loss of acetic acid yielding a nitrile (*M*⁺ 775) occurs very readily (Wolfrom & Thompson, 1931).

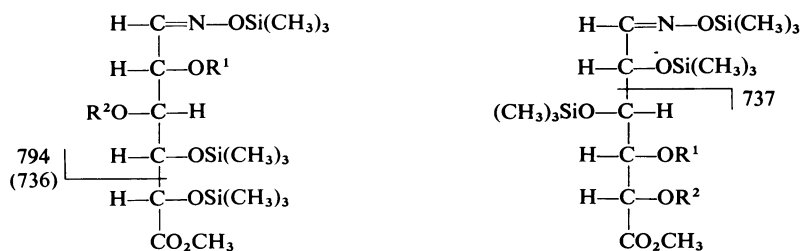
When compared with the 3-*O*-acylglucuronide, reaction of ethyl anthranilate azopigment methyl ester (II_{me}) (2-*O*-acylglucuronide) with hydroxylamine yielded more hydroxamate azopigment. However, t.l.c. and mass spectrometry did not differentiate between oxime and methoxime azopigments formed from either 2- or 3-*O*-acyl compounds, again indicating easy *O*-acyl migration for 1-oxime and 1-methoxime derivatives.

We may conclude that isolation of oximes and

methoximes clearly shows the free glycosidic function for the 2- and 3-*O*-acylglucuronides. However, the position of the *O*-acyl group has to be determined by using alternative methods, which are outlined below. Oximes and methoximes have been used for g.l.c.-mass spectrometry of carbohydrates by Laine & Sweeley (1973).

Determination of position of the azodipyrrole *O*-acyl group

Assignment of positions of *O*-acyl substituents is a difficult task owing to the ready migration and hydrolysis of this labile group (Bouveng, 1961; De Belder & Norrman, 1968). We tried several reaction sequences, aimed at replacing the *O*-acyl group with a stable methyl group and at determining the position of the latter in the alditolreduction product. The key step consisted of protecting the free hydroxyl groups of ethyl anthranilate azopigment methyl esters (II_{me}), (III_{me}) and (IV_{me}) as the acetals formed by reaction with ethyl vinyl ether and trifluoroacetic acid in dichloromethane. The acetal functions introduce new asymmetric carbon atoms, giving rise to a mixture of stereoisomers. The reaction products were analysed by t.l.c. as a function of time either after neutralization or in the presence of trifluoroacetic acid. With the latter procedure, hydrolysis occurs on the t.l.c. plates and the original methyl esters were recovered, showing the positional integrity of the *O*-acyl group under the acid reaction conditions used. For short reaction times (4 h at 0°C), t.l.c. of the neutralized products in each case revealed several groups of azopigments of various polarity, indicating the presence of mainly diacetals and to a smaller extent mono- and tri-acetals. For longer reaction times (3 days at 0°C) and a greater excess of ethyl vinyl ether, triacetals were the main derivatives of compound (III_{me}), whereas for compounds (II_{me}) and (IV_{me}) a mixture of di- and tri-acetals was formed. This interpretation for the t.l.c. behaviour of the acetals agrees with the faster rate of silylation observed for compound (III_{me}) and was



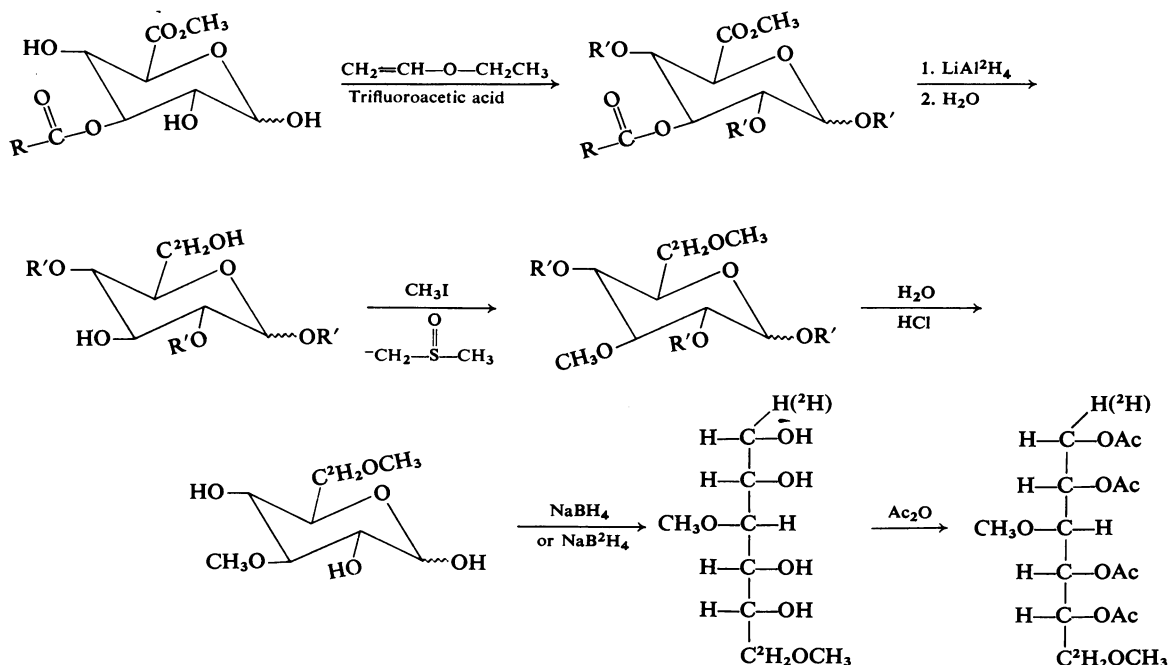
Scheme 6. Fragmentation of silylated oximes and methoximes
R¹ = azodipyrrole acyl; R² = trimethylsilyl; or vice versa.

confirmed by further reaction of the acetals. Two procedures, differing by the reaction time for the acetal formation and by the further treatment of the acetals, gave satisfactory results.

Procedure A. This is exemplified for the 3-*O*-acylglucuronide in Scheme 7. (1) The reaction with ethyl vinyl ether was allowed to proceed for 3 days at 0°C. (2) Reduction with LiAl^2H_4 (or LiAlH_4) removed the azodipyrrole *O*-acyl group, thereby also reducing the methoxycarbonyl function. (3) Free hydroxyl groups produced by the reductive treatment were converted into methyl ethers with methylsulphinyl carbanion and methyl iodide. (4) The acetal functions were removed in aqueous acid medium and (5) the carbohydrates were reduced to the alditols with either NaBH_4 or NaB^2H_4 . (6) Acetylation yielded partially methylated alditol acetates of glucose, which were analysed by combined g.l.c.-mass spectrometry. The results summarized in Table 2 demonstrate the original 2-, 3- and 4-*O*-acyl position for the azodipyrrole groups of ethyl anthranilate compounds (II_{me}), (III_{me}) and (IV_{me}). They gave rise to 2,6-dimethyl-, 3,6-dimethyl- and 4,6-dimethyl-alditol acetates respectively. The mass spectra of the partially methylated alditol acetates show preferential cleavages at carbon atoms bearing

the charge-stabilizing methoxy groups. Fragment ions containing C-1 were shifted with 1 mass unit when NaB^2H_4 was used in reaction (5), and fragments containing C-6 were shifted with 2 mass units when LiAl^2H_4 was used in reaction (2).

Trimethyl alditol acetates were not detected under the conditions of procedure 1. Whether this is due to complete acetal formation (reaction 1) or to preferential loss of trihydroxy precursors during ethyl acetate/water extraction of LiAlH_4 reaction products (reaction 2) has not been fully established. We favour the former explanation for ethyl anthranilate compound (II_{me}) and the latter for ethyl anthranilate compounds (III_{me}) and (IV_{me}) on the basis of the following observations. (a) T.l.c. of the acetals indicates almost complete conversion into triacetals for compound (III_{me}) and incomplete triacetal formation for compounds (II_{me}) and (IV_{me}) (see above). (b) The yield observed on g.l.c. for the 3,6-dimethyl ether derived from compound (III_{me}) was appreciably higher than for 2,6- and 4,6-dimethyl ethers prepared from compounds (II_{me}) and (IV_{me}). (c) For shorter treatments with the acetalizing reagent (3-6 h at 0°C), the yields of dimethyl alditol acetates were lowered. Trimethyl ethers were still not observed, however, unless a more thorough extraction of the



Scheme 7. Site of attachment of acyl group in azopigment methyl esters as determined for 2-, 3- and 4-*O*-acylglucuronides and exemplified for 3-*O*-acylglucuronide

R = azodipyrrole aglycone; R' = $-\text{CH}(\text{CH}_3)-\text{O}-\text{CH}_2-\text{CH}_3$.

Table 2. Determination of *O*-acyl positions by using procedure A. Labelling of positions 1 and 6 was obtained by using NaB³H₄ and LiAl²H₄, respectively, giving rise to corresponding shifts in the mass spectra (numbers in parentheses). Ac represents an acetyl group.

Starting ethyl anthranilate azopigment	Elution temperature of dimethyl alditol acetate*	Dimethyl alditol acetates identified						Characteristic fragment ions	
		1	2	3	4	5	6		
Methyl ester (II _{me}) (2- <i>O</i> -acylgucuronide)	158.5	H-CH(OAc)-CH(OCH ₃)	-CH(OAc)-CH(OAc)	-CH(OAc)-CH(OAc)-CH(OAc)-CH ₂ OCH ₃ (² H ₂)					C ₍₂₎ -C ₍₆₎ , 305 (307); C ₍₁₎ -C ₍₂₎ , 117 (118)
Methyl ester (III _{me}) (3- <i>O</i> -acylgucuronide)	159.5	H-CH(OAc)-CH(OAc)	-CH(OAc)-CH(OCH ₃)	-CH(OAc)-CH(OAc)-CH(OAc)-CH ₂ OCH ₃ (² H ₂)					C ₍₃₎ -C ₍₆₎ , 233 (235); C ₍₁₎ -C ₍₃₎ , 189 (190)
Methyl ester (IV _{me}) (4- <i>O</i> -acylgucuronide)	159	H-CH(OAc)-CH(OAc)	-CH(OAc)-CH(OCH ₃)	-CH(OAc)-CH(OAc)-CH(OAc)-CH ₂ OCH ₃ (² H ₂)					C ₍₄₎ -C ₍₆₎ , 161 (163); C ₍₁₎ -C ₍₃₎ , 261 (262)

* Programmed analysis on 3% OV₁ (130°C, 4°C/min).

LiAlH₄ reduction products was performed. Under the latter conditions the expected trimethyl ethers were obtained in small yield: 2,3,6- and 2,4,6-trimethyl ethers from compound (II_{me}) and 2,3,6- and 3,4,6-trimethyl ethers from compound (III_{me}). (d) Analogous alditols resulting from incomplete acetal formation were the main products when procedure B was applied (see below).

Procedure B. This differs from procedure A in the first two steps. (1) The acetal formation was allowed to proceed for only 4h at 0°C. (2) The *O*-acyl group was removed by treatment with ammonia-saturated methanol. This treatment also transforms the methoxycarbonyl group into an amide. Permethylation of the amide and free hydroxyl groups, hydrolysis of the acetal groups, reduction to the alditol and acetylation were carried out as for procedure A.

G.l.c. and mass spectral results are given in Tables 3 and 4. As a result of incomplete acetal formation, two dimethyl alditol acetates were obtained for each positional isomer (II_{me}), (III_{me}) and (IV_{me}) instead of one monomethyl ether, but assignment of the *O*-acyl position remains possible. Small amounts of trimethyl and monomethyl ethers were also detected.

In the mass spectra (Table 4) of the 2,3-dimethyl- and 3,4-dimethyl-alditol acetates, cleavage between two vicinal methoxy groups gives rise to particularly abundant fragment ions, and fission at the outside of the two methoxy groups is less prominent. For the 2,4-dimethyl ether, intense fragment ions are formed by fission at either side of the isolated methoxy groups.

General comments and comparison with previous work

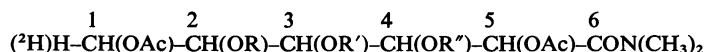
Although evidence accumulated since 1970 (Compernelle *et al.*, 1970, 1975, 1977; Gordon *et al.*, 1976; Heirwegh *et al.*, 1976) confirmed the existence of 1-*O*-acylgucuronides of bilirubin-IX_α, only the present work affords insight into the complexity of conjugated azopigments (Heirwegh *et al.*, 1970; Kuenzle, 1970a; Fevery *et al.*, 1972) and conflicting structural data (Compernelle *et al.*, 1970; Kuenzle, 1970b).

The methods presented characterize azopigment (I) and its phenylazo analogue as 1-*O*-acyl-β-D-glucopyranuronic acid. Esterification of the carboxyl group yielded methyl ester (I_{me}), which was characterized by n.m.r. and field desorption mass spectrometry. Further silylation or acetylation of the hydroxyl groups provided suitable derivatives for electron-impact mass spectrometry.

On the other hand, deconjugation of azopigments and identification of the released carbohydrates demonstrated that glucuronic acid was the only sugar present. To detect labile carbohydrates, possibly lost as a result of derivative formation, i.e.

Table 3. *Determination of O-acyl positions by using procedure B*

The general formula of the alditols is:



where Ac represents an acetyl group and R, R' and R'' represent either an acetyl or a methyl group. Labelling of position 1 was obtained by using NaB^2H_4 instead of NaBH_4 .

Starting ethyl anthranilate azopigment	Structure assigned	Alditol derivatives identified
Methyl ester (II _{me})	2- <i>O</i> -Acylglucuronide	2,3-Dimethyl and 2,4-dimethyl
Methyl ester (III _{me})	3- <i>O</i> -Acylglucuronide	2,3-Dimethyl and 3,4-dimethyl
Methyl ester (IV _{me})	4- <i>O</i> -Acylglucuronide	2,4-Dimethyl and 3,4-dimethyl

methyl ester formation, silylation or acetylation, deconjugation was carried out on the stage of azopigment (I) but this only yielded the expected derivatives of glucuronic acid. Methyl glycosides were formed with 2M-HCl in methanol and they were identified by using t.l.c. and g.l.c.-mass spectrometry. The glucuronic acid released with aqueous ammonia was reduced with NaBH_4 and was characterized as the silylated L-gulono-1,4-lactone,

Selective removal of the 1-*O*-acyl group in the acetylated methyl esters gave rise to the corresponding 1-bromo-, 1-anilino and 1-phenoxy-pyranose derivatives. These results show the pyranose ring structure and the C-1 ester linkage of the glucuronic acid moiety and were verified by applying the same reactions to pyranose and furanose model compounds (VII) and (X).

In bile and buffered solutions of pH 6-9 the original 1-*O*-acylglucuronides of bilirubin-IX α rearrange to form 2-, 3- and 4-*O*-acylglucuronides. The corresponding aniline and ethyl anthranilate azopigments [ethyl anthranilate and aniline azopigments (II), (III) and (IV)] are derived without further affecting the carbohydrate moiety by using a pH 6.0 diazonium reagent devoid of excess of aromatic amine. Complete separation and structural characterization was performed for the four methyl esters of azopigments (I), (II) (III) and (IV). When the pH 2.7 ethyl anthranilate diazonium reagent is utilized, the free glycosidic functions of the rearranged glucuronides react with the excess of ethyl anthranilate (F. Compernelle, unpublished work). This side reaction yields ethyl anthranilate *N*-glucuronides, which constitute the main fraction of β - and γ -azopigments.

How can the present results be related to those of Kuenzle? We mainly consider procedures for collection of bile and for isolation of azopigments (Kuenzle, 1970a), without examining structural assignments made by Kuenzle (1970b). Several lines of evidence suggest that he also worked on rearranged bilirubin-IX α glucuronides that are present in post-obstructive bile. First, relatively large amounts of β - and γ -azopigments, which derive

from 2-, 3- and 4-*O*-acylglucuronides, were detected for T-tube bile obtained from patients operated for cholelithiasis (Feverly *et al.*, 1972). The bile used by Kuenzle (1970a) was obtained under analogous conditions, i.e. after cholecystectomy of patients operated for removal of gallstones. Secondly, large amounts of bile were sampled in bags cooled with ice and the bile was stored in a deep-frozen state for unspecified periods of time. It was shown by Blanckaert *et al.* (1978) that partial transformation of the 1-*O*-acylglucuronides occurs under these collection conditions. Further, complete conversion was observed after storage in a deep-frozen state for 2 years. Thirdly, bilirubin conjugates were pre-fractionated by using reversed-phase chromatography carried out at 20°C for about 24h (Kuenzle, 1970a). A mixture (1:1) of bile and a mobile phase, consisting of a diluted buffer of pH 6.0 (5mm-sodium potassium phosphate), was applied to the chromatographic column. We found a pH value of 7.7 for a mixture (1:1) of fresh rat bile and mobile phase. Storage of this mixture at 20°C under N_2 for 8h yielded 2-, 3- and 4-*O*-acylglucuronides, leaving only a small amount of the starting 1-*O*-acylglucuronide.

The conjugated aniline azopigments B₄, B₅ and B₆ were separated (Kuenzle, 1970a) by using again reversed-phase chromatography with another mobile phase of pH 3.4. When mixtures of the ethyl anthranilate and aniline azopigments (I), (II), (III) and (IV) were subjected to this chromatography system, no clear-cut separation was observed and the azopigments were eluted as a continuous band. However, t.l.c. analysis of subsequent column fractions as their methyl esters revealed partial separation of the azopigment acids. Similar apparent polarities were found for reversed-phase chromatography of the acids as for t.l.c. of the methyl esters. Thus the first, more-polar, fractions contained almost pure ethyl anthranilate or aniline azopigments (IV) mixed with some azopigment (I). Further fractions were enriched in azopigment (I). Finally, mixtures composed of the least-polar azopigments (II) and (III) were detected.

Table 4. *G.l.c. and mass spectral properties of alditol derivatives obtained by using procedure B*
 Programmed analysis was carried out on 3% OV₁ (150°C, 4°C/min). Ac represents an acetyl group. Shifts in the mass spectra raising from H² labelling are shown in parentheses.

Elution temperature of alditol derivative (°C)	Structure of alditol derivative	Characteristic fragment ions
177 (2,4-dimethyl)	$ \begin{array}{ccccccc} & 1 & 2 & 3 & 4 & 5 & 6 \\ & \text{H} & \text{H} & \text{CH}(\text{OAc}) & \text{CH}(\text{OAc}) & \text{CH}(\text{OCH}_3) & \text{CH}(\text{OAc}) & \text{CON}(\text{CH}_3)_2 \end{array} $	C ₍₂₎ -C ₍₆₎ , 304; C ₍₁₎ -C ₍₂₎ , 117 (118); C ₍₄₎ -C ₍₆₎ , 188; C ₍₆₎ , 72; C ₍₁₎ -C ₍₄₎ , 233 (234)
177.5 (3,4-dimethyl)	$ \begin{array}{ccccccc} & 1 & 2 & 3 & 4 & 5 & 6 \\ & \text{H} & \text{H} & \text{CH}(\text{OAc}) & \text{CH}(\text{OAc}) & \text{CH}(\text{OCH}_3) & \text{CH}(\text{OAc}) & \text{CON}(\text{CH}_3)_2 \end{array} $	C ₍₁₎ -C ₍₃₎ , 189 (190); C ₍₃₎ -C ₍₆₎ , 232; C ₍₄₎ -C ₍₆₎ , 188; C ₍₆₎ , 72; C ₍₁₎ -C ₍₄₎ , 233 (234)
178 (2,3-dimethyl)	$ \begin{array}{ccccccc} & 1 & 2 & 3 & 3 & 5 & 6 \\ & \text{H} & \text{H} & \text{CH}(\text{OAc}) & \text{CH}(\text{OCH}_3) & \text{CH}(\text{OAc}) & \text{CH}(\text{OAc}) & \text{CON}(\text{CH}_3)_2 \end{array} $	C ₍₂₎ -C ₍₆₎ , 304; C ₍₁₎ -C ₍₂₎ , 117 (118); C ₍₁₎ -C ₍₃₎ , 161 (162); C ₍₆₎ , 72; C ₍₃₎ -C ₍₆₎ , 260, 158

Taken together, these observations suggest that the B₄, B₅ and B₆ azopigments were 2-, 3- and 4-*O*-acylglucuronides or mixtures of them, in contrast with the disaccharide structures proposed by Kuenzle (1970*b*). It should be noticed that the 'disaccharides', released by dilute ammonia solution from azopigments B₅ and B₆, could not be characterized directly. Their existence was postulated from the production of mixtures of hexuronic acids or hexoses on acid hydrolysis. The preferential loss of the terminal non-reducing carbohydrate moiety, which was claimed to explain detection of only glucuronic acid or glucose upon silylation of the 'disaccharides' (Kuenzle, 1973), casts further doubt on the disaccharide structural proposal. Contamination of the B₄, B₅ and B₆ preparations with compounds containing glucose was suggested by Gordon *et al.* (1976), who found that bile salts especially interfere with chromatography of δ -azopigments. Whereas the latter authors removed most bile salts by precipitation at the stage of the bilirubin conjugates, we used column chromatography on silica gel to isolate a purified δ -azopigment fraction. Remaining bile salts were removed by using t.l.c. Moreover, t.l.c. isolation of the methyl esters provided a further purification step.

The subject of acyl migration in carbohydrates has been reviewed by Bonner (1959) and Haines (1976). The reaction proceeds through ortho acid intermediates (Fischer, 1920) (Scheme 2). The intramolecular nature of the mechanism was substantiated by a radioactive-labelling study (Doerschuk, 1952). For bilirubin-IX α β -D-glucopyranuronic acid, the migration appears to be sequential (but reversible; Blanckaert *et al.*, 1978), each step (1 \rightarrow 2, 2 \rightarrow 3 and 3 \rightarrow 4) involving the ortho acid derivative of *trans*-hydroxyl groups.

Methods for determining the position of labile acyl groups have been developed by Bouveng (1961) for partially acetylated xylans and glucans and by De Belder & Norrman (1968) for partially acetylated dextran. Whereas Bouveng protected free hydroxyl groups by reaction with phenyl isocyanate, De Belder & Norrman (1968) used methyl vinyl ether as protecting reagent. We adapted the latter method to a micro-scale procedure suitable for use with *O*-acyl derivatives of glucuronic acid. After methyl ester formation and protection of free hydroxyl groups by reaction with ethyl vinyl ether, the azodipyrrole *O*-acyl group was removed by treatment with LiAlH₄ or ammonia. The latter reaction steps also transformed the readily lactonizing carboxyl group into C²H₂OH or CONH₂ groups. Finally, the methylated carbohydrates were hydrolysed and converted into the corresponding alditol acetates. The position of the methyl groups was determined by using g.l.c.-mass spectrometry.

The propensity to acyl migration has further

implications with regard to the synthesis of bilirubin-IX α glucuronides. Thompson & Hofmann (1976) reported the synthesis of bilirubin-IX α diglucuronides (esterified at positions 2, 3 or 4). The carboxyl groups of bilirubin were activated by reaction with carbonyldi-imidazole. Bilirubin di-imidazole was isolated and converted into the ester conjugates by treatment with the tetrabutylammonium salt of glucuronic acid. The synthetic glucuronides resisted the action of α - and β -glucuronidase. Diazonium cleavage was performed by using an excess of ethyl anthranilate at pH 2.7. Besides azopigment δ , two prominent azopigments γ were formed, moving on t.l.c. as the compounds derived from bilirubin conjugates in cholestatic bile. As the azopigments γ derive from ethyl anthranilate azopigment (II) (F. Compernelle, unpublished work), this result suggests the presence of mainly 2-*O*-acylglucuronides in the synthetic mixture. In contrast with glycoside formation catalysed by acid, the base-catalysed acylation will not favour reaction at C-1. Furthermore, 1 \rightarrow 2 acyl migration could have occurred during synthesis and extractive work-up. Indeed, the relatively strong base imidazole (pK_a 7.1; Joule & Smith, 1972) is released from bilirubin di-imidazole, and the esterification of alcohols with acylimidazoles even requires base catalysis (Staab, 1962). The supposed 1 \rightarrow 2 acyl migration is equally base-catalysed and could be avoided only by using suitable protecting groups for the hydroxyl functions at positions 2, 3 and 4 of glucuronic acid.

Alternative methods for the synthesis of bilirubin conjugates have been attempted. Using 1-alkyl-3-*p*-tolyltriazenes, Hutchinson *et al.* (1973) prepared alkyl esters of bilirubin, but the triazene procedure has not been adapted for the synthesis of carbohydrate esters. Bilirubin-IX α bis-amides were obtained from 1- and 2-glucosamine and 1-xylosamine (Hancock *et al.*, 1976).

Experimental

The details of chemical derivatization procedures for g.l.c. and mass spectrometry have been deposited with the British Library Lending Division.

Materials

D-Glucuronic acid was purchased from BDH Chemicals (Poole, Dorset, U.K.), and D-glucuronolactone and ethyl vinyl ether from Aldrich-Europe (Beerse, Belgium). ZnCl₂ and phenol were obtained from Union Chimique Belge (Zwijnaarde, Belgium). HBr [40% (w/w) in acetic acid], dichlorodimethylsilane, molecular sieve (pore size 0.4 nm), silica gel H, pre-coated F254 silica-gel plates (layer thickness 0.25 mm), trifluoroacetic acid, hydroxylamine hydrochloride, LiAlH₄, NaB²H₄ and silane-treated Celite (70–100 mesh; art. no. 9751) were purchased from E. Merck A.-G. (Darmstadt,

Germany). NaH dispersion in paraffin oil (4:1, w/w) was obtained from Merck-Schuchardt (Hohenbrunn, Munich, Germany). Methoxyamine hydrochloride and liquid phases 1% JXR, 3% QF₁, 1% OF₁, 3% OV₁ and 3% Dexsil 300 GC on Gas-Chrom Q (100–120 mesh) for g.l.c. were from Applied Science Laboratories (Sint-Stevens Woluwe, Belgium). Pyridine (dried on KOH pellets), dimethyl sulphoxide (Carbo-Erba, Milan, Italy) (dried on molecular sieve, pore diam. 0.4 nm) and aniline were distilled before use. *NO*-bis(trimethylsilyl)trifluoroacetamide and *NO*-bis(trimethylsilyl)acetamide were obtained from Pierce Chemical Co. (Europoort, The Netherlands).

Apparatus and chromatography systems

N.m.r. spectra. Spectra were recorded at 100 MHz with a Varian XL-100 spectrometer. Solvents were [²H₆]dimethyl sulphoxide for azopigment methyl ester (Ime) and [²H]chloroform for the anomers of compound (VII). Chemical shifts are given relative to tetramethylsilane as an internal standard.

Mass spectra. Spectra of azopigments were run by using the direct-insertion inlet of an MS902S mass spectrometer. Conditions of the ion source were: temperature 180–250°C, as required; electron voltage 70 eV; accelerating voltage 8 kV; ionizing current 500 μ A.

Field-desorption mass spectra were recorded for ethyl anthranilate azopigment (I) and methyl ester (Ime) on a Varian CH-5D mass spectrometer with a combined field desorption and electron-impact ion source. Benzonitrile-activated emitters were dipped into solutions of compounds (I) and (Ime) in acetic acid or methanol.

G.l.c.-mass spectrometry. Solutions were injected on the column (glass; length, 1.8 m; internal diam., 2 mm; carrier gas, He, 50 ml/min) of a Hewlett-Packard gas chromatograph model 5750. Liquid phases and operating conditions of the columns are specified in the Methods section. The outlet of the column was divided into two unequal parts. One-third went to a flame ionization detector and two-thirds went to the ion source via a two-stage jet separator. Injector block, separator and ion source were operated at 250°C.

Thin-layer chromatography. Azopigments were purified by t.l.c. on precoated silica-gel plates by the procedures described by Heirwegh *et al.* (1970, 1974). Silane-treated t.l.c. plates were prepared by exposing the above plates for 3 min to an atmosphere of dichlorodimethylsilane; 30 min before treatment, the reagent (about 5 ml) was poured into a chromatography tank provided with glass rods to avoid direct contact of the plates with the liquid. Without delay the plates were developed once with toluene, dried in the air for 30–60 min and developed once with methanol.

They were kept moisture-free in a box. Before chromatography the azopigment solutions were carefully made water-free. These silane-treated plates were used for separation of ethyl anthranilate and aniline azopigments (I), (II)+(III) and (IV).

For elution of the azopigments, the silica gel material was suspended in pentan-2-one/formamide (4:1, v/v) or pentan-2-one (for less-polar compounds) by vigorous mixing by means of a Polytron mixer (Kinematica G.m.b.H., Lucerne, Switzerland). While mixing was continued, an equal volume of glycine/HCl buffer, pH 2.0, was added (prepared by adjusting a 0.4M-HCl solution to pH 2.0 with glycine). For less-polar non-acidic azopigments, this buffer solution was replaced with water. The mixture was centrifuged and the formamide was removed from the supernatant solution by repeated washings with water.

Methods

Collection and stabilization of normal bile. Dog gall-bladder bile, normal rat bile and human duodenum bile of normal adult volunteers were collected as described previously (Fevery *et al.*, 1972). Bile samples were used freshly or were stored in a deep-frozen state for not longer than 1 week.

In the later stages of the work the bile was stabilized by adding 1 vol. of citrate/phosphate buffer, pH 4.0 (prepared from 61.45 ml of 0.1M-citric acid and 38.55 ml of 0.2M-Na₂HPO₄). Under these conditions only slight conversion is observed when bile is kept under N₂ at 25°C for 17h, whereas untreated bile (pH 7.8) shows almost complete conversion into 2-, 3- and 4-*O*-acylglucuronides. The conversions were observed by diazonium cleavage at pH 6 (see below) and t.l.c. analysis with the solvent system chloroform/methanol (9:1 or 17:3, v/v). For the azopigment acids, t.l.c. was performed on silane-treated plates, whereas for the methyl esters untreated t.l.c. plates gave good separations (for methyl ester formation, see below).

Post-obstructive and incubated bile. Post-obstructive human and rat biles were collected by using the procedure of Fevery *et al.* (1972). Incubated bile was prepared by keeping fresh normal biles from man and rat under N₂ at 20°C (6–20h) or 37°C (4h) (Blanckaert *et al.*, 1978).

Preparation of azopigment (I) and its phenylazo analogue. These were prepared from dog, rat and human bile with diazonium reagents derived from ethyl anthranilate and aniline. In the present work we used a diazonium procedure with equivalent amounts of aromatic amine and NaNO₂ at pH 6.0. Whereas the same azopigment (I) or its phenylazo analogue was obtained from fresh normal bile by using an excess of aromatic amine at pH 2.7 (Van Roy & Heirwegh, 1968; Heirwegh *et al.*, 1974), *N*-glycosides (azopigments β and γ) were

formed from rearranged bilirubin-IX α glucuronides having a free glycosidic function (F. Compennolle, unpublished work).

The pH 6.0 reaction was carried out for 30 min at 0°C with careful precooling of bile, buffer and reagent solutions. The diazonium reagent was prepared by reaction of 0.4 g of NaNO₂ in 5 ml of water for 30 min at 0°C with aniline or ethyl anthranilate solution (0.5 ml and 0.88 ml, dissolved in 9 ml of 1.5M-HCl by vigorous shaking; the ethyl anthranilate solution was briefly centrifuged and the clear lower layer was used). The reagent (15 μ l) was added to a mixture of diluted bile (10 ml) (bile diluted with 1 vol. of distilled water) and citrate/phosphate buffer (10 ml) (7.74 g of citric acid and 22.5 g of Na₂HPO₄ adjusted to 1 litre with distilled water). The azopigments were extracted by shaking vigorously with a minimal volume of pentan-2-one and centrifuging briefly. The organic layer was cooled to -77°C and precipitated ice was removed by quick filtration on a glass filter. The pentanone solution was diluted with an equal volume of chloroform and the mixture was applied to a column of silica gel H suspended in chloroform. The column was washed thoroughly with chloroform, and azopigment (I) (or its aniline analogue) was eluted with pentan-2-one/formamide (4:1, v/v). The pigment solution was then washed, first with glycine buffer (0.4M-HCl adjusted to pH 2.5 with solid glycine), then several times with an equal volume of water. (Occasionally, the first wash contained some azopigment. This was set aside and was used as the aqueous phase at a later stage of the washings.) The final pentanone solution was cooled to -77°C and filtered on a glass filter. Purification was carried out by t.l.c. with the solvent system chloroform/methanol/water (65:25:3, by vol.) (Heirwegh *et al.*, 1970, 1974). A better separation from 2-, 3- and 4-*O*-acylglucuronides was obtained with silane-treated plates and the solvent system chloroform/methanol (17:3, v/v). The latter t.l.c. procedure followed the former t.l.c. purification when other than 1-*O*-acylglucuronides were present.

Conversion of ethyl anthranilate and aniline azopigment (I) into methyl ester (Ime) and into phenylazo analogue of methyl ester (Ime). The silica-gel material from t.l.c. bands corresponding to azopigment (I) or its phenylazo analogue, was suspended in pentanone/formamide (4:1, v/v) by vigorous mixing by means of a Polytron mixer (Kinematica G.m.b.H.). While mixing was continued, an equal volume of glycine/HCl buffer, pH 2.0, was added (prepared by adjusting a 0.4M-HCl solution to pH 2.0 with solid glycine). The mixture was centrifuged and the formamide was removed from the supernatant solution by repeated washing with water. For small volumes an excess of water was separated from the organic phase by centrifuging. For larger volumes the

pentanone layer was briefly cooled to -77°C and rapidly filtered on a glass filter. Ethereal diazomethane was added in an excess to the organic layer and the solution was evaporated. The methyl esters were finally purified by t.l.c. in the solvent system chloroform/methanol (17:3, v/v).

Isolation of ethyl anthranilate and aniline azopigments (I), (II)+(III) and (IV)

Post-obstructive or incubated biles from rat or man were treated with the pH 6.0 diazonium reagents derived from aniline and ethyl anthranilate. The azopigments were extracted with pentan-2-one, and the δ -fraction of azopigments was isolated by column chromatography on silica gel H, as described above for azopigment (I). The δ -fraction was further purified by t.l.c. with the solvent system chloroform/methanol/water (65:25:3, by vol.). Separation into mainly three bands was achieved on silane-treated t.l.c. plates (see above) by using the solvent system chloroform/methanol (17:3 or 9:1, v/v). Esterification of the fractions with diazomethane and t.l.c. of the methyl esters (see below) showed that the most-mobile fraction was a mixture of ethyl anthranilate or aniline azopigments (II)+(III). The second and third fractions corresponded to ethyl anthranilate or aniline azopigments (I) and (IV) respectively.

Pure azopigment (II) was obtained from rat bile incubated at 37°C for 20 min (Blanckaert *et al.*, 1978).

Isolation of ethyl anthranilate and aniline azopigment methyl esters (Ime), (IIme), (IIIme) and (IVme)

A δ -fraction of azopigments was purified by using chromatography and t.l.c. as described in the preceding section. The pentan-2-one solution resulting from elution of the azopigments (see under 'Thin-layer chromatography') was briefly cooled to -77°C and rapidly filtered on a glass filter to remove the excess of water. Ethereal diazomethane was added in excess and the solution was evaporated. The methyl esters were separated by t.l.c. with the solvent system chloroform/methanol (17:3 or 9:1, v/v). Storage of the t.l.c. plates in the dark gave rise to extensive loss of the methyl ester group, especially for the azopigment (II). The conversion into acid azopigments was verified by re-chromatography, by using the t.l.c. systems and marker compounds described above [see under 'Isolation of ethyl anthranilate and aniline azopigments (I), (II)+(III) and (IV)']. The acids were re-converted into the methyl esters by treatment with diazomethane.

Isolation of ethyl anthranilate azopigment lactone (IIIact)

The material from the most-mobile methyl ester

band, corresponding to ethyl anthranilate azopigment methyl ester (IIme), was re-chromatographed on commercial t.l.c. plates. Development with ethyl acetate/benzene (3:2, v/v) gave two bands. Separation was more reproducible when the empty plates had been developed first with chloroform/methanol/water (65:25:3, by vol.), or when the $10\text{cm} \times 20\text{cm}$ or the $5\text{cm} \times 20\text{cm}$ Merck plates were used. Isolation and re-chromatography of the two fractions with the same solvent system showed further conversion of the less-mobile into the more-mobile compound, whereas the latter did not change. Lactone structure (V) was assigned to the more-mobile IIIact azopigment. The lactone and the methyl ester exhibited the same R_F value on t.l.c. with the solvent system chloroform/methanol (9:1, v/v).

Treatment of bile under reversed-phase conditions

Fresh rat bile (approx. pH 8) (1 vol.) and 1 vol. of 5 mM-phosphate buffer (prepared by dissolving 0.68 g of KH_2PO_4 in 0.9 litre of water, adjusting to pH 6.0 with 1M-NaOH and diluting to 1 litre), saturated with butan-1-ol, were mixed and left to stand under N_2 at 20°C for 8 h (final pH 7.7). Aniline and ethyl anthranilate azopigments were prepared by using the pH 6.0 diazonium procedure and were analysed as the acids on silane-treated plates or as the methyl esters on ordinary t.l.c. plates (see above). Conversion of the 1-*O*-acyl compound into 2-, 3- and 4-*O*-acyl compounds was almost quantitative.

Reversed-phase column chromatography of aniline and ethyl anthranilate azopigments (I), (II), (III) and (IV)

A two-phase system, pH 3.4, was prepared from octan-1-ol / di-isopropyl ether / ethyl acetate / methanol / 0.2M-acetic acid (1:2:2:3:4, by vol.). Silane-treated Celite (6.0g; Merck A.-G.) was impregnated with 3 ml of the organic phase by mechanical rotation. Aqueous phase (40 ml) was added and mixed by means of a Polytron mixer. Columns (diam. 1 cm, height approx. 30 cm) were prepared from these suspensions.

The aniline or ethyl anthranilate azopigment mixtures (I), (II), (III), and (IV) were applied to the column, either dissolved in mobile phase (about 1 ml) or bound to a small amount of impregnated Celite. The pigments were eluted as a continuous band. The fractions collected were transformed into methyl esters and analysed by t.l.c. The compositions of subsequent fractions were: (a) (IV); (b) (IV)+(I); (c) (I) + (II) + (III); (d) (II) + (III)

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