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The Fate of Bilirubin-IXα Glucuronide in Cholestasis and during Storage *in vitro*

INTRAMOLECULAR REARRANGEMENT TO POSITIONAL ISOMERS OF GLUCURONIC ACID

By NORBERT BLANCKAERT,* FRANS COMPERNOLLE,† POL LEROY,* ROSANE VAN HOUTTE,* JOHAN FEVERY* and KAREL P. M. HEIRWEGH*‡
**Laboratory of Hepatology, Department of Medical Research, University of Leuven, Leuven, Belgium, and † Department of Macromolecular and Organic Chemistry, University of Leuven, Heverlee, Belgium*

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1. In aqueous solution above pH7 bilirubin-IXα 1-*O*-acylglucuronide rapidly isomerizes to the non-C-1 glucuronides by sequential migration of the bilirubin acyl group from position 1 to positions 2, 3 and 4 of the sugar moiety. The transformations are enhanced by increasing the pH. Compared with the rates at 37°C, the transformations are rather slow at 0°C. Virtually complete inhibition is observed at values below pH6. The isomerization at 25°C and pH7.4 is not affected by the presence in the solutions of a molar excess of human serum albumin. 2. Isomerization in bile kept at 37°C at pH7.7–7.8 is probably non-enzymic, as the rates of change are similar to those observed under comparable conditions for aqueous solutions of glucuronides of bilirubin-IXα and of azodipyrrole. 3. Analysis without delay of normal biles of man and rats collected at 0°C over a maximum period of 10min shows that the bilirubin-IXα mono- and di-glucuronides consist exclusively of the 1-*O*-acyl isomers. 4. The mixtures of the four positional isomers of bilirubin-IXα glucuronide found in freshly collected biles of man and rats with cholestasis probably originate from initially synthesized 1-*O*-acylglucuronide by the same mechanism of sequential migration as has been observed in aqueous solutions of conjugated bilirubin-IXα.

In most mammals metabolic breakdown of haem leads predominantly to the formation of bilirubin-IXα (Gray *et al.*, 1972; Lathe, 1972). For efficient excretion, previous conversion into more-polar derivatives is required (Lester & Klein, 1966; Heirwegh *et al.*, 1976; Blanckaert *et al.*, 1977*a*). It is now well established that this process involves primarily the esterification of one or two propionic acid side chains of bilirubin with sugar, thus resulting in the excretion of mono- and di-conjugates in bile (Kuenzle, 1973; Heirwegh *et al.*, 1976). The nature of the sugar residues has for long been a standing problem. Talafant (1956), Billing *et al.* (1957) and Schmid (1957) reported that in man and rats bilirubin is excreted in the form of glucuronides. This view was contested by Kuenzle (1970), who claimed bilirubin to be excreted predominantly as disaccharidic conjugates. However, recent investigations have confirmed the existence of 1-*O*-acylglucuronides (Compernolle *et al.*, 1970, 1978; Gordon *et al.*, 1976) and revealed two additional aspects. (*a*) Glucosides and xylosides are also formed, their relative importance depending on species (Fevery *et al.*, 1971, 1977; Gordon *et al.*, 1974). (*b*) The bilirubin conjugates in post-obstructive bile of cholestatic patients, and of rats with obstructed bile ducts, differ from those in normal man and rats by the presence of additional conjugates (Fevery *et al.*, 1972). These compounds have now been characterized as the 2-, 3- and 4-*O*-acylglucuronides (Compernolle *et al.*, 1978).

The present paper deals with the mechanism of formation of these non-C-1 isomers in cholestasis. During storage *in vitro*, glycosidic conjugates of bilirubin-IXα may undergo non-enzymic rearrangement by sequential migration of the bilirubin conjugate *O*-acyl group from position 1 of the sugar moiety to positions 2, 3 and 4. By avoiding migration of the acyl group before and during structural analysis it could be shown that bile from normal man and rats contains exclusively 1-*O*-acylglycosides. In cholestasis, bilirubin-IXα is first transformed into 1-*O*-acylglycosides, but subsequent, probably non-enzymic, migration of the *O*-acyl residue from position 1 of the sugar to positions 2, 3 and 4 during stasis

‡ To whom reprint requests should be addressed.

Table 1. *Partial structures of azodipyrrole glucuronides*

In general, azopigment δ could be separated as the free acid into subfractions (I), (II) and (III+IV) or, after methyl ester formation, into the methyl esters (Ime), (IIme), (IIIme) and (IVme). Freshly collected biles from normal man and rats yield exclusively azopigment (I). The aglycone azodipyrrole is a mixture of both the *endo*- and *exo*-vinyl isomers (Compernolle *et al.*, 1970; Jansen & Stoll, 1971).

Symbol	Structure	References
I	Azodipyrrole 1-*O*-acyl-β-D-glucopyranuronoside	Compernolle *et al.* (1970, 1975, 1978); Gordon *et al.* (1976)
II	Azodipyrrole 2-*O*-acylglucuronide	Compernolle *et al.* (1978)
III	Azodipyrrole 3-*O*-acylglucuronide	
IV	Azodipyrrole 4-*O*-acylglucuronide	

leads to the excretion in bile of mixtures of the 1-, 2-, 3- and 4-*O*-acylglycosides (Table 1).

Preliminary accounts of this work have been given (Blanckaert *et al.*, 1976*a*; Compernolle *et al.*, 1977).

Materials and Methods

Chemicals

UDP-[U-^{14}C]glucuronic acid (sp. radioactivity 231mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Bilirubin (A.R. grade) and precoated silane-treated silica-gel plates (60F254, 5746/0025) were obtained from Merck A.-G. (Darmstadt, Germany). Glucaro-(1→4)-lactone (A grade) was from Calbiochem (San Diego, CA, U.S.A.). β-Glucuronidase (from bovine liver; type B-3) was furnished by Sigma Chemical Co. (St. Louis, MO, U.S.A.). The enzyme preparation liberated 5.3μg of phenolphthalein from 0.5mM-phenolphthalein β-glucuronide in 1h at 37°C and pH5.0 (30min assay). Human serum albumin (crystallized, lot no. 33; from Miles Laboratories, Kankakee, IL, U.S.A.) was defatted by the procedure of Chen (1967). The other chemicals were A.R. grade and/or as specified by Heirwegh *et al.* (1972) and Blanckaert *et al.* (1976*b*).

Collection of bile samples and treatment of rats

Normal bile was obtained by duodenal intubation in healthy volunteers as described by Fevery *et al.* (1972). Bile of cholestatic patients was collected during cholecystectomy from patients with a non-functioning gall bladder and by T-tube drainage during the first 3 days after surgery in patients with obstructive jaundice.

Normal rat bile was obtained by biliary drainage in male Wistar R/A rats (300–400g body wt.). The surgical procedure has been described by Blanckaert *et al.* (1977*a*). After surgery the animals were placed in restraining cages and transferred to a humidified air thermostat to keep their body temperature at 38±0.5°C. They had free access to water. A 5% (w/v) glucose solution in 0.15M-NaCl was infused in the jugular vein at a rate of 1–1.5ml/h. After complete recovery from anaesthesia (3–4h after surgery), collection of bile was started. Cholestasis was induced by sealing the free end of the bile-duct catheter. Alternatively, the unsealed catheter was elevated, thus producing a hydrostatic pressure of 23–24cm, which completely counteracted bile flow. In general, the obstruction was released after 20h. Further details about collection and treatment of bile samples are given in the text.

Preparation of bilirubin-IXα 1-O-acylglucuronide

Bilirubin-IXα mono- and di-glucuronides were isolated from bile of normal rats by the procedure of Heirwegh *et al.* (1975). Bilirubin-IXα 1-*O*-acyl-β-D-mono[U-^{14}C]glucuronide was prepared as follows. Microsomal preparations (2g liver wt. equiv./ml of suspension) were prepared from liver of Wistar R/A rats (Heirwegh *et al.*, 1972) and were pre-incubated at 0°C for 30min with an equal volume of digitonin solution (25mg/ml). This solution of activated enzyme (3ml) was incubated at 37°C for 20min at a final volume of 10ml with the following reagents (final concns. are given): bilirubin-IXα (200μM), $MgCl_2$ (7.5mM), ATP (2mM), glucaro-(1→4)-lactone (1mM), UDP-[U-^{14}C]glucuronic acid (200μM; sp. radioactivity 8880d.p.m./nmol) and Tris/HCl, pH7.6 (0.2M-HCl). After incubation the mixtures were placed on ice and acidified with 1vol. of glycine/HCl buffer, pH1.8 (prepared by adjusting a 0.4M-HCl solution to pH1.8 with solid glycine), that was saturated with NaCl and contained ascorbic acid (20mg/ml). Then 1vol. of chloroform/ethanol (1:1, v/v) was added. After mixing and centrifugation, the extract was taken off and the aqueous phase was re-extracted with 1vol. of chloroform/ethanol mixture. The organic phases containing the yellow pigments were combined and submitted to t.l.c. with chloroform/methanol/water (10:5:1, by vol.). The pigment moving as reference bilirubin monoglucuronide was scraped from the plates, eluted immediately with methanol and rechromatographed with chloroform/methanol/water (30:15:2, by vol.). After elution with methanol, the eluate was evaporated to

dryness and stored overnight under N_2 on solid CO_2. After solution in 1ml of serum, taken from normal Wistar R/A rats, the pigment (50–60nmol) was injected intravenously into rats with obstructed bile ducts.

Radioactivity assays

Determination of radioactivity was carried out as described by Blanckaert *et al.* (1977*a*). The efficiency of counting was in the ranges 80–82% for bile samples and 69–83% for azopigment solutions.

Incubation of bile and of aqueous solutions of azodipyrrole glucuronides

The composition of the solutions was as follows. (i) Bile was diluted with an equal volume of either water (final pH7.7–7.8) or one of the buffers mentioned below. (ii) Dried azopigment (about 15nmol) was first dissolved in 30μl of dimethyl sulphoxide and further diluted with 1ml of one of the following buffers: (*a*) glycine/HCl buffer, pH2.7 (0.4M-HCl adjusted to pH2.7 with solid glycine), (*b*) citric acid/phosphate buffer (0.1M-citric acid adjusted with 0.2M-Na_2HPO_4 to pH2.7, 3.5, 4.0, 5.0, 6.0 or 7.0), (*c*) Na_2HPO_4/KH_2PO_4 buffer, pH7.4 (0.15M-Na_2HPO_4 adjusted to pH7.4 with 0.15M-KH_2PO_4) or Tris/HCl buffer (0.2M-Tris adjusted with 0.2M-HCl to pH8.0, 8.5 or 9.0).

The pigment solutions were flushed with N_2 and then incubated in closed tubes under N_2 in a thermostatically controlled water bath. Incubation was stopped (i) by formation of azo derivatives followed by extraction into pentanone or (ii) by the addition of 2ml of glycine/HCl buffer, pH2.7, and extraction of the azopigments into 2ml of pentanone. Further details are indicated below.

Determination of total diazo-positive bilinoids: formation and isolation of azo derivatives

In general, for determining their structure and concentration, the bilinoids were converted into their more stable azo derivatives by diazonium cleavage at pH6 (Blanckaert *et al.*, 1977*b*). The diazo reagent was prepared from an equimolecular mixture of $NaNO_2$ and ethyl anthranilate, thus avoiding reaction of an excess of aromatic amine with the hemiacetal hydroxy group of non-C-1 glucuronides (F. Compernolle, unpublished work). Explicit mention in the text is made when the standard ethyl anthranilate procedure of Van Roy & Heirwegh (1968) was also employed.

Except when stated otherwise, the azopigments and their derivatives were separated and purified by the t.l.c. procedures of Heirwegh *et al.* (1974) and of Blanckaert *et al.* (1976*b*) (Table 1). The chromatographic fractions were denoted by Greek letters and suffixes according to the nomenclature of Heirwegh *et al.* (1970, 1974) and their relative amounts were determined by densitometry (Fevery *et al.*, 1971). Azopigment δ was rechromatographed on glass plates precoated with silane-treated silica gel provided at the upper edge with a pad of filter paper (Whatman no. 3). Development was for 12–15h with chloroform/methanol (9:1, v/v).

Chemical and enzymic tests on azopigments

Isolated azopigments were treated with acetic anhydride/pyridine as described by Heirwegh *et al.* (1975). Alkaline ethanolysis and methyl ester formation were carried out as described by Blanckaert *et al.* (1976*b*, 1977*b*). Ammonolysis of azopigment δ and of subfractions was carried out as described by Heirwegh *et al.* (1974) with the following modifications: (*a*) the aq. solution of NH_3 was replaced by water-free methanol saturated with NH_3 and (*b*) precoated silane-treated silica-gel plates were used. After exposure of the plates overnight to NH_3 vapour, excess of NH_3 was removed by leaving the plates in the air for 1h before drying in an air stream. They were then developed with chloroform/methanol/water (65:25:3, by vol.) and subsequently, after drying, with propan-1-ol/water (9:2, v/v). The separated sugars were localized by spraying with naphtharesorcinol [20mg in 17ml of ethanol/H_2SO_4 (16:1, v/v)]. The hexuronic acid/azodipyrrole ratio was determined by the method of Fishman & Green (1955) as described by Heirwegh *et al.* (1970).

Results and Discussion

Classification of bile samples

An important aspect of the present work is that rapid structural changes of bilirubin conjugates occur during stasis in the body and during storage in aqueous solutions. Even during collection of bile on ice and storage at −15°C for a few days some changes occurred. It is therefore of practical importance to distinguish bile samples according to (*a*) their biological origin and (*b*) any treatment affecting the structures of bile pigments before structural analysis.

(*a*) 'Cholestatic bile' is obtained from patients or animals with diminished hepatic bilirubin secretion. Such samples in the present study have been obtained from rats with induced obstruction of the bile duct and from patients with overt jaundice due to extrahepatic obstruction. In addition, stagnant bile obtained from non-functioning gall bladders was also investigated.

(*b*) Bile is denoted as 'fresh' when it is collected on ice within a period of not more than 10min and then analysed immediately. 'Stored bile' is collected

on ice over longer periods of time (3–5h) and/or stored for 1–5 days at −15°C. Bile is 'stabilized' against intramolecular rearrangement when collected and stored at 0°C at below pH 6, as demonstrated for fresh normal rat bile.

General composition of the azopigments obtained from bile of man and rats

Typical chromatographic patterns of azo derivatives obtained from normal biles are shown in Fig. 1 of the paper by Blanckaert *et al.* (1977*b*). In all cases azopigments α_0 and δ were the major components. The former pigment is identical with the mixture of *exo-* and *endo*-vinyl isomers of azodipyrrole derived from unconjugated bilirubin-IXα (Compernolle *et al.*, 1970; Jansen & Stoll, 1971). In the present discussion this heterogeneity is of little importance, and the mixture of isomers is here called azodipyrrole. The unconjugated azopigments α_F, α'_F and β_x derive from unconjugated bilirubin-IXβ and -IXδ (Blanckaert *et al.*, 1977*a*). Cholestatic bile yielded only slightly more azopigment γ than normal bile, and azopigment β was never observed. In contrast, when the standard ethyl anthranilate procedure is used (Van Roy & Heirwegh, 1968), detection of azopigment β is typical for cholestatic bile, and azopigment γ content is significantly higher than that for normal bile (Fevery *et al.*, 1972). The β-azopigment and the increased amounts of γ-azopigment arise from reaction of the hemiacetal hydroxy group of non-C-1 glucuronides with an excess of aromatic amine (F. Compernolle, unpublished data).

Chemical and enzymic analysis of azopigment δ

The present work is focused on the major conjugated azodipyrrole fraction, azopigment δ, obtained by t.l.c. with chloroform/methanol/water (65:25:3, by vol.) (Fig. 1 in the paper by Blanckaert *et al.*, 1977*b*). Previous structural work on this fraction obtained from normal bile led to the conclusion that it corresponds to azodipyrrole 1-*O*-acyl-β-D-glucopyranuronic acid (Compernolle *et al.*, 1970, 1975; Gordon *et al.*, 1976). However, 5–25% of azopigment δ was not hydrolysed by β-glucuronidase (Heirwegh *et al.*, 1970; Gordon *et al.*, 1976), suggesting heterogeneity. Similarly, we found that about 15% of azopigment from 'stored' normal bile was not affected by the enzyme, and an even larger fraction when the azopigment was obtained from cholestatic bile (Table 2). Therefore the previous identification can only apply to about 80% of azopigment δ from normal bile and to an even smaller part of azopigment δ from cholestatic bile, when the usual procedures for collection and/or storage are adopted.

In the present work, we first tried to separate the various subfractions of azopigment δ (Table 1). Subsequently, structural information on the isolated azopigment was obtained by various chemical and enzymic tests. These tests can conveniently be applied on a routine basis, in contrast with the more thorough chemical study by Compernolle *et al.* (1978).

Table 2. *Treatment of azopigment δ and its subfractions with β-glucuronidase*

Azopigments obtained from the sources indicated below were purified and incubated for 1 h at 37°C and pH 5.2 in the dark with β-glucuronidase by the method of Blanckaert *et al.* (1977*b*) (*a*). Controls were run in parallel (*b*) with enzyme and the specific inhibitor glucaro-(1→4)-lactone and (*c*) without enzyme. The percentage of glucuronide hydrolysed was determined by azopigment analysis.

Compound tested and its source	Glucuronide hydrolysed (%) (*a*)	(*b*)	(*c*)	No. of expts.
Azopigment δ				
Normal rat bile				
Fresh	98–100	4–7	3–7	3
Stored	85–92	3–6	2–6	5
Normal human bile				
Fresh	96, 98	4, 6	5, 4	2
Stored	82–90	2–6	3–6	6
Cholestatic rat bile				
Fresh	39–69	3–7	4–10	4
Stored	41–65	3–9	2–7	8
Cholestatic human bile				
Fresh	10–65	4–8	5–10	4
Stored	6–64	4–9	5–8	6
Normal rat bile				
Fresh (pH 7.8)	96, 98	4, 3	5, 3	2
Stored for 2h at 37°C				
at pH 7.8	6, 8	6, 8	5, 7	2
at pH 6.0	95, 97	6, 3	4, 6	2
Azopigment (I)	100, 100	6, 7	3, 5	2
Azopigment (II)	6, 8	3, 7	5, 11	2
Azopigment (III+IV)	5, 9	5, 5	3, 9	2

Separation of the subfractions of azopigment δ after conversion into methyl ester derivatives. Treatment of azopigment δ with diazomethane resulted in all cases in complete conversion into one or several methyl ester derivatives. T.l.c. of the reaction products revealed the presence of up to four fractions, Ime, IIme, IIIme and IVme (Tables 3 and 4), which have been characterized unequivocally as the isomeric 1-, 2-, 3- and 4-*O*-acylglucuronides of azodipyrrole (Table 1). As no differences were noted between the properties of the corresponding pigments Ime, IIme, IIIme and IVme from fresh, stored incubated or cholestatic bile, no distinction is made here between the subfractions with regard to the biological origin or pretreatment of the bile samples.

Separation of the free acids. On silane-treated silica-gel plates, resolution of azopigment δ in only three components was achieved. The 3- and 4-*O*-acylglucuronides moved as a single band (III+IV).

Table 3. *Composition of azopigment δ from bile of normal and cholestatic patients and rats*
Bile was treated at pH6.0 with diazotized ethyl anthranilate. Azopigment δ, isolated by t.l.c., was treated with diazomethane. The methyl esters were separated by t.l.c. and quantified by densitometry (see the Materials and Methods section). The numbers of bile samples analysed are indicated in parentheses. –, Absent; ±, absent or present in trace amount.

Source of azopigment	Glucuronide isomer	Percentage of total glucuronide			
	...	1-*O*-Acyl	2-*O*-Acyl	3-*O*-Acyl	4-*O*-Acyl
Normal human bile					
Fresh (4)		96–100	0–4	–	–
Stored (10)		80–88	12–20	±	–
Normal rat bile					
Fresh (5)		96–100	0–4	–	–
Stored (9)		83–90	10–17	±	–
Cholestatic human bile					
Fresh (2)		44, 70	30, 20	19, 9	7, 1
Stored (3)		42, 66, 40	29, 20, 35	21, 12, 18	8, 2, 7
Gall bladder* (2)		2, 5	42, 44	38, 36	18, 15
Cholestatic rat bile					
Fresh (2)		46, 40	30, 36	21, 22	3, 2
Stored (5)		49, 37, 67, 45, 54	28, 35, 22, 42, 30	21, 24, 10, 10, 15	2, 4, 1, 2, 1

* Gall-bladder bile from patients with non-functioning gall bladder.

Table 4. *R_F values of methyl esters of azodipyrrole glucuronide and of their fully acetylated derivatives*
The structures of the positional isomers (I), (II), (III) and (IV) are indicated in Table 1. The solvent systems were (*a*) chloroform/methanol (9:1, v/v), (*b*) benzene/ethyl acetate (17:3, v/v) and (*c*) benzene/ethyl acetate (3:2, v/v). In the last-mentioned system, the derivative obtained from azopigment (II) by treatment with diazomethane separated into the methyl ester (IIme) and the (3→6)-lactone (IIlact) of azopigment (II) (Compernolle *et al.*, 1978).

Compound	Solvent system	R_F values		
	...	(*a*)	(*b*)	(*c*)
Ime		0.43		
IIlact		0.59		0.37
IIme		0.59		0.35
IIIme		0.55		
IVme		0.39		
Ime acetate				
exo-Vinyl isomer			0.28	
endo-Vinyl isomer			0.24	
IIlact acetate				
exo-Vinyl isomer			0.30	
endo-Vinyl isomer			0.27	
IIme acetate				
exo-Vinyl isomer			0.28	
endo-Vinyl isomer			0.25	
IIIme acetate				
exo-Vinyl isomer			0.29	
endo-Vinyl isomer			0.26	
IVme acetate				
exo-Vinyl isomer			0.28	
endo-Vinyl isomer			0.26	

Component (I) moved most rapidly followed by components (II) and (III+IV) in that order. Azopigments (I) and (II) yielded methyl esters (Ime) and (IIme) respectively, whereas azopigment (III+IV) gave both methyl esters (IIIme) and (IVme). The method was used to prepare small amounts of the free acids for subsequent chemical and enzymic tests. Different relative t.l.c. mobilities were observed for the subfractions of azopigment δ with the t.l.c. procedure developed by Compernolle *et al.* (1978).

Acetylation of the methyl ester derivatives. Treatment of the separated methyl esters with acetic anhydride/pyridine in each case yielded the expected pair of *endo*- and *exo*-vinyl isomers of fully acetylated methyl esters (Table 4). Their isomeric status was verified by alkaline ethanolysis of the acetates and t.l.c. of the resulting ethyl esters of azodipyrrole (Blanckaert *et al.*, 1977*b*).

Ammonolysis and t.l.c. of the liberated sugar moieties. Treatment of subfractions (I), (II) and (III+IV) with NH_3 vapour resulted in complete cleavage of the ester bond. In each case the pair of carboxylic acid amides of *endo*- and *exo*-vinyl isomers of azodipyrrole and trace amounts of the corresponding methyl esters of azodipyrrole were obtained. After subsequent t.l.c. of the sugar moieties, only glucuronic acid was detected. In the system used, galacturonic acid moves with an R_{GlcUA} (R_F value relative to that of glucuronic acid) value of 0.51. Disaccharides containing hexuronic acid are expected to move more slowly.

Determination of the hexuronic acid/azodipyrrole ratio. The ratio was close to 1 for azopigment (I) obtained from normal bile of man (0.99±0.02 S.D.,

$n = 3$) and rat (0.96 ± 0.04 S.D., $n = 5$). For cholestatic bile the ratio determined with (II+III+IV) was 0.90 ± 0.06 S.D. ($n = 4$) for man and 1.05 ± 0.05 S.D. ($n = 6$) for the rat. These observations are compatible with the presence of 1 mol of glucuronic acid/mol of azopigment. The occurrence of other carbohydrates has been excluded on the basis of t.l.c. analysis and more thorough g.l.c.–mass-spectrometric analysis in the preceding paper (Compernolle *et al.*, 1978).

Hydrolysis with β-glucuronidase. Depending on the origin and pretreatment of the bile samples the extent of hydrolysis of azopigment δ varied between 6 and 100% (Table 2). In the presence of the specific inhibitor glucaro-(1→4)-lactone (Levvy & Conchie, 1966), the rates of hydrolysis were small and comparable with the rates of spontaneous hydrolysis. Subfraction (I) was hydrolysed completely, demonstrating unequivocally that it has a β-D-glycosidic linkage at C-1. The same conclusion was reached by n.m.r. studies (Compernolle *et al.*, 1978). The enzyme assays do not allow determination of ring structure: pure β-glucuronidase from liver attacks not only β-D-glucopyranuronosides but also β-D-glucofuranuronosides (Levvy & Conchie, 1966). The present observations are thus compatible with the 1-*O*-acyl-β-D-glucopyranuronoside structure proposed for azopigment (I) (Compernolle *et al.*, 1978). As expected, the non-glycosidic glucuronides (II) and (III+IV) were not attacked by the enzyme (Table 2).

Composition of azopigment δ from various bile samples

Two points should be clarified before a meaningful comparison of the present results with previous studies on the nature of bilirubin conjugates is possible. (1) In all cases the tetrapyrrolic pigments were converted into dipyrrolic azo derivatives before structural analysis. In the laboratories of ourselves and Gordon, the ethyl anthranilate procedure of Van Roy & Heirwegh (1968) has been used in the past. With this procedure the excess of aromatic amine was found to react partly with the 2-, 3- and 4-*O*-acylglucuronides, thus leading to partial transformation of azopigment δ into azopigments β and γ (F. Compernolle, unpublished work). In contrast, there is no excess of aromatic amine in the diazonium reagent used by Kuenzle (1970) and in the present work, and the C-1 glycosidic function remains unaffected. (2) During the present work it became apparent that structural changes of bilirubin conjugates may occur during collection and storage of bile. Therefore it is essential to distinguish between fresh and stored bile samples (see above under 'Classification of bile samples'). In general, bile samples used in previous studies were collected and occasionally stored over at least several hours and thus should be classified as 'stored' bile.

Fresh bile samples. To identify the metabolites of bilirubin-IXα that are excreted into the bile, we have attempted to avoid structural changes of the

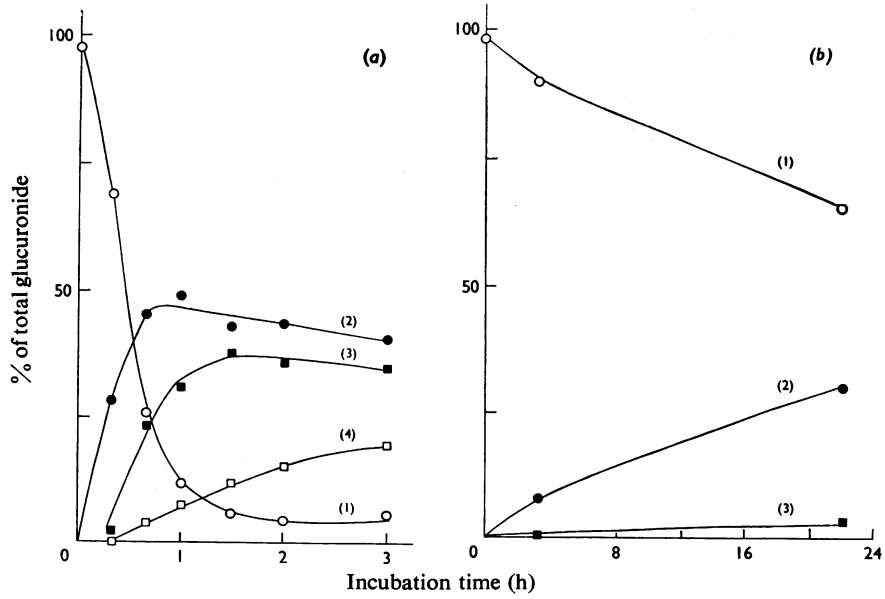

Fig. 1. *Isomerization of bilirubin-IXα glucuronide in bile at pH* 7.8
Fresh normal rat bile, diluted with an equal volume of distilled water, was incubated at (*a*) 37°C or (*b*) 0°C. Curves (1), (2), (3) and (4) refer respectively to 1-, 2-, 3- and 4-*O*-acylglucuronides.

pigments that may occur *in vitro* during collection of bile. Therefore the biliary bilinoids were characterized as quickly as possible after their secretion. Bile was collected over periods of 10min in tubes placed on ice and protected from light. The samples were treated immediately with diazo reagent and the azo derivatives were separated by t.l.c. If prepared in this way, azopigment δ from bile of normal man and rats was homogeneous. It behaved as azopigment (I) when chromatographed on silane-treated silica-gel plates, yielded only methyl ester (Ime) on treatment with diazomethane (Table 3) and was hydrolysed completely by β-glucuronidase (Table 2). In contrast, fresh bile from patients and rats with cholestasis contained mixtures of the four glucuronide isomers (Table 3). In general, about 50% or more of total glucuronide corresponded to non-glycosidic conjugates (Table 3) and resisted attack by β-glucuronidase (Table 2). Gall-bladder bile from patients with non-functioning gall bladder contained only trace amounts of 1-*O*-acylglucuronide (Table 3), as would be expected because of the prolonged stasis.

Stored bile. About 15% of azodipyrrole glucuronide from normal biles that had been stored for 1–7 days in a deep-freeze corresponded to the 2-*O*-acyl isomer

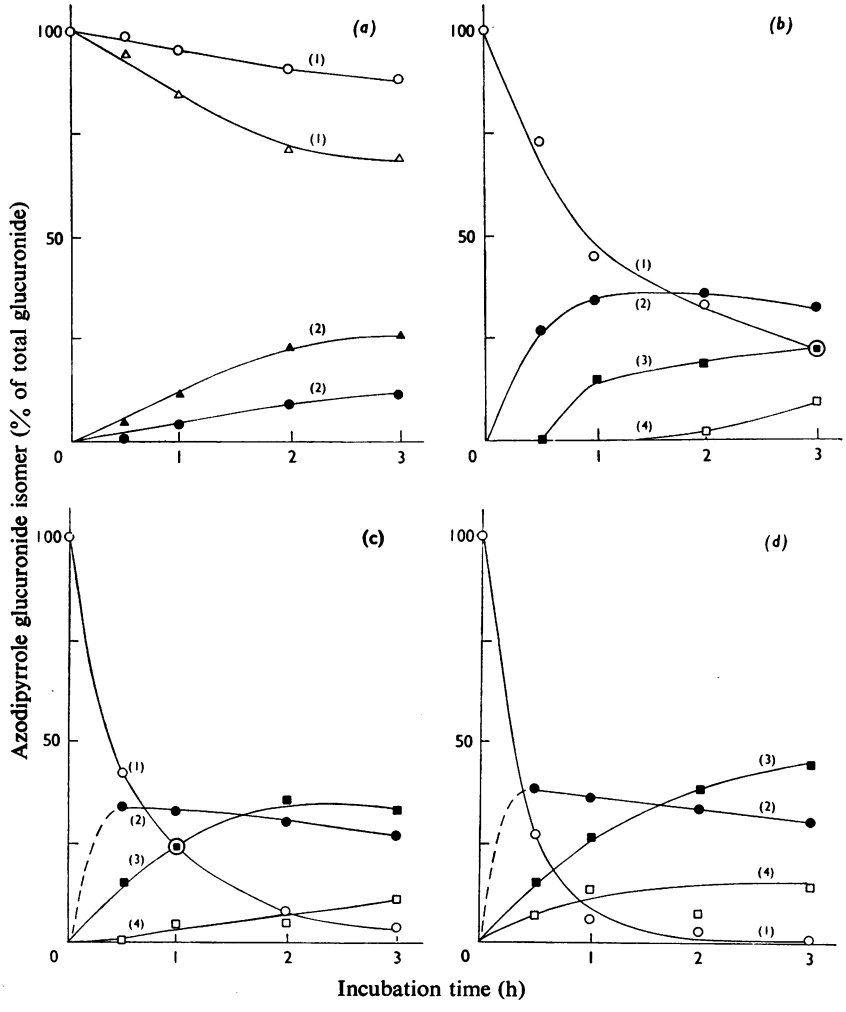

Fig. 2. *Isomerization of azodipyrrole* 1-*O*-*acylglucuronide at* 37°C
Azodipyrrole 1-*O*-acylglucuronide was incubated at pH (*a*) 7.0 (○, ●) and 7.4 (△, ▲), (*b*) 8.0, (*c*) 8.5 and (*d*) 9.0. Curves (1), (2), (3) and (4) refer respectively to 1-, 2-, 3- and 4-*O*-acylglucuronides.

(Table 3) and resisted attack by β-glucuronidase (Table 2). Stored cholestatic biles contained appreciable amounts of all four isomers (Table 3) and considerably more glucuronide resisted enzymic hydrolysis (Table 2).

Structural changes of the bilirubin-IXα glucuronides in vitro

Azopigment δ prepared from stored normal bile contained some 2-*O*-acylglucuronide and trace amounts of the 3-*O*-acyl isomer, which were absent from fresh samples (Table 3). This indicated that isomerization of the 1-*O*-acylglucuronide into non-C-1 glucuronides may occur during collection and/or storage. As it is important to define conditions for preparation and handling of conjugated bilirubin, we decided to study these transformations further.

When freshly collected bile (pH 7.7–7.8) was incubated at 37°C the 1-*O*-acylglucuronide disappeared rapidly, with concomitant formation of the 2-*O*-acyl isomer and, after a lag period of about 20min, of the 3- and 4-*O*-acyl isomers (Fig. 1*a*). After incubation for 2h, the 1-*O*-acyl isomer almost completely disappeared. The 2- and 3-*O*-acyl isomers also tended to decrease after about 60 and 90min respectively, whereas the 4-*O*-acyl isomer gradually increased. Similar changes occurred at 0°C (Fig. 1*b*), thus explaining the difference between 'fresh' and 'stored' normal bile.

To study the extent of the changes under various conditions, further experiments were carried out with normal rat bile, and with buffered solutions of (*a*) bilirubin-IXα mono- and di-1-*O*-acylglucuronides and (*b*) the azodipyrrole 1-, 2- and 3-*O*-acylglucuronides.

Isomerization depends critically on pH. The 1-*O*-acylglucuronide in normal bile remained unchanged after incubation at 22°C for 5h at pH 4–6. No isomerization took place during similar incubation for 3h of buffered aqueous solutions of (*a*) isolated bilirubin-IXα 1-*O*-acylglucuronides at 26°C and pH 3 and (*b*) azodipyrrole 1-*O*-acylglucuronide at 37°C and pH 2.7–6.0. At neutral to slightly alkaline pH, non-glycosidic isomers were formed. Incubation at 37°C of azodipyrrole 1-*O*-acylglucuronide for 3h at pH 7 and 7.4 resulted in conversion of 10 and 18% respectively of initial glucuronide into the 2-*O*-acyl isomer (Fig. 2*a*). At higher pH the 3- and 4-*O*-acyl isomers were also formed. At pH 8, formation of the 3-*O*-acyl isomer started after a lag period of about 30min whereas the formation of the 4-*O*-acyl isomer was more delayed (Fig. 2*b*). The rates of conversion were further enhanced by increasing the pH to 8.5 and 9.0 (Figs. 2*c* and 2*d*). Fig. 3 shows the proportions of the isomeric azodipyrrole glucuronides obtained after incubation for 3h as a function of pH.

The rates of formation of 2-*O*-acylglucuronide in buffered solutions of bilirubin-IXα mono- and di-1-*O*-acylglucuronides at pH 7.4 were comparable with those obtained for solutions of azodipyrrole 1-*O*-acylglucuronide (Table 5). The rates of conversion of the bilirubin conjugates were not affected by the presence of a molar excess of serum albumin (Table 5). This observation suggests that binding of the conjugates to albumin does not directly involve the sugar moieties.

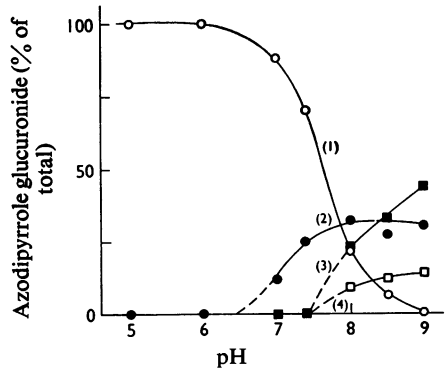

Fig. 3. *Composition of azodipyrrole glucuronide isomers after incubation of the 1-O-acyl isomer for 3h at 37°C as a function of pH*

The curves (1), (2), (3) and (4) refer respectively to the 1-, 2-, 3- and 4-*O*-acyl isomers.

Considerable protection is obtained by working at 0°C. When normal rat bile (pH 7.7) was incubated for 3h at 37, 22 and 0°C, conversion of bilirubin glucuronide into non-glycosidic conjugates was 95, 27 and 7% respectively. However, after 22h at 0°C the percentage of non-glycosidic conjugates had increased to 33%. As already noted, apparently complete inhibition of isomerization is achieved by bringing the pH below 6.

The rates of conversion at 37°C and pH 7.4 were comparable for rat bile and for protein-free solutions of both bilirubin-IXα 1-*O*-acylglucuronide and azodipyrrole 1-*O*-acylglucuronide. It is thus reasonable to assume that in bile the process is non-enzymic. Sequential migration of the acyl group from position 1 to positions 2, 3 and 4 of the sugar residue is strongly supported by the progress curves (Figs. 1 and 2). Typical features are (*a*) accumulation of the 2-*O*-acyl isomer at pH 7 and 7.4 and (*b*) lag periods for the formation of the 3- and 4-*O*-acyl isomers at higher pH values. In accordance with this hypothesis, azodipyrrole 2-*O*-acylglucuronide at pH 8 generated rapidly the 3- and 4-*O*-acyl isomers (Fig. 4*a*) whereas the 3-*O*-acylglucuronide yielded the 2- and 4-*O*-acyl isomers (Fig. 4*b*).

Table 5. *Isomerization of bilirubin-IXα glucuronides in the presence and absence of serum albumin*
The following mixtures were flushed with N_2 and then incubated at 25°C in closed tubes under an N_2 atmosphere in a thermostatically controlled water bath (final concns. given in parentheses): bilirubin-IXα mono- or di-glucuronide (25μM), defatted human serum albumin (0–125μM), NaCl (0.1M) and Tris/HCl buffer, pH7.4 (20μM-Tris). For conjugated bilirubin-IXα, the same molar extinction coefficient, 46×10^3 litre·mol^{-1}·cm^{-1}, was assumed as for the bilirubin-IXα–human serum albumin complex at pH5 (Blauer *et al.*, 1972). For each time point, a separate mixture (1ml) was prepared. After the indicated periods of incubation, the mixtures were placed on ice. After diazonium cleavage and extraction by the method of Blanckaert *et al.* (1977*b*), azopigment δ was isolated by t.l.c., treated with diazomethane and further separated into the methyl ester derivatives. –, Absent.

			Percentage of total glucuronide											
	Molar ratio of albumin/	Glucuronide isomer ... Incubation period	1-*O*-Acyl				2-*O*-Acyl				3-*O*-Acyl			
Bile pigment	bilirubin	(min) ...	0	90	150	210	0	90	150	210	0	90	150	210
Bilirubin-IXα	0		96	85	79	77	4	15	21	22	–	–	–	1
monoglucuronide	0.5		97	87	76	70	3	13	22	26	–	–	–	4
	1		96	81	80	71	4	17	16	25	–	–	–	4
	5		99	85	76	70	1	15	22	25	–	–	–	5
Bilirubin-IXα	0		99	85	79	76	1	15	21	24	–	–	–	–
diglucuronide	0.5		96	83	76	70	4	15	20	24	–	1	3	6
	1		91	78	72	69	9	19	23	27	–	3	5	9
	5		99	80	72	65	1	17	23	26	–	3	5	9

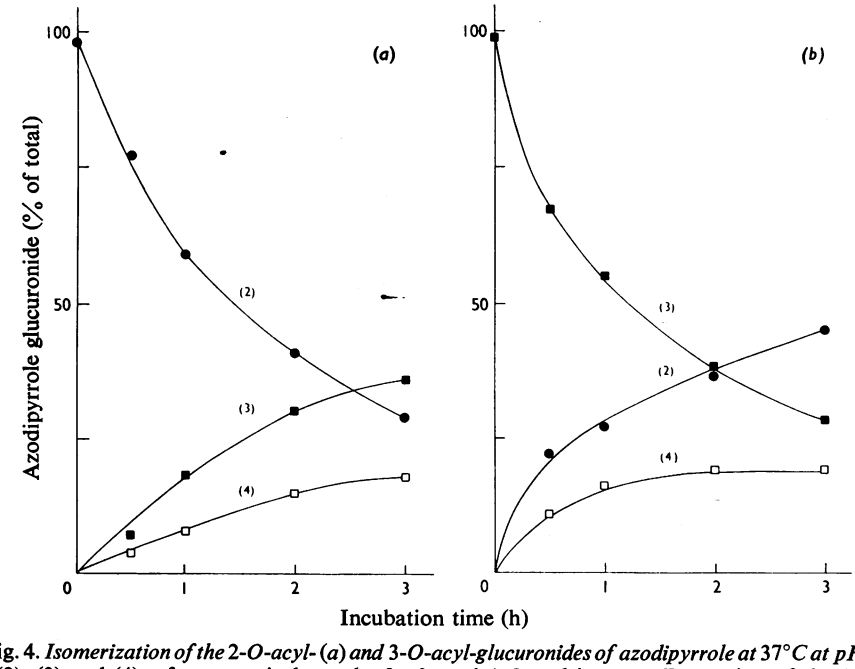

Fig. 4. *Isomerization of the 2-O-acyl-(a) and 3-O-acyl-glucuronides of azodipyrrole at 37°C at pH 8.0*
The curves (2), (3) and (4) refer respectively to the 2-, 3- and 4-*O*-acyl isomers. Formation of the 1-*O*-acyl isomer was not observed.

Formation of the 2-, 3- and 4-O-acylglucuronides in cholestasis

Two possible mechanisms for the formation of non-glycosidic conjugates in cholestatic patients and rats have been considered. (1) All four isomers are formed directly by conjugation of bilirubin-IXα, or (2) conjugation yields exclusively the 1-*O*-acyl isomer followed by isomerization to the 2-, 3- and 4-*O*-acyl isomers.

Enzymic formation of 1-O-acylglucuronide in normal and cholestatic liver. Bilirubin-IXα UDP-glucuronosyltransferase activities in untreated and in digitonin-activated homogenates and microsomal preparations from liver of cholestatic rats were comparable with those for normal rats (Table 6), confirming previous work with human liver (Black & Billing, 1969). With digitonin-activated preparations from liver of both normal and cholestatic rats, only the 1-*O*-acylglucuronide of bilirubin-IXα was formed *in vitro*. The results are compatible with rapid enzymic formation of bilirubin-IXα 1-*O*-acyl-glucuronide in cholestatic liver. They do not allow one to conclude whether or not conjugation occurs *in vivo* with glucuronic acid at positions other than C-1. Indeed, in transferase assays with UDP-glucuronic acid one would expect exclusive formation of glycosidic conjugates, as the sugar moiety to be transferred is activated at C-1. However, conjugation at non-C-1 positions seems unlikely, as to the best of our knowledge glucuronic acid donors activated at positions other than C-1 have not been detected in tissues.

Fate of bilirubin-IXα glucuronoside in cholestasis. To test whether changes by migration of the acyl group similar to those observed *in vitro* also occur during cholestasis, we injected bilirubin-IXα 1-*O*-acylmono[U-^{14}C]glucuronide into Wistar rats with bile ducts that had been obstructed for 20h (Table 7). Obstruction was maintained for another 150min

Table 6. *Activity of bilirubin-IXα UDP-glucuronosyltransferase in preparations from livers of cholestatic rats*

Cholestasis was induced in three adult male Wistar R/A rats by ligation of the common bile duct. After 20h the rats were submitted to light ether anaesthesia and their livers were perfused with ice-cold 0.25M-sucrose solution to remove blood containing conjugated bilirubin. Subsequent preparation of homogenates and microsomal fractions, activation of part of these by pre-treatment with digitonin, enzymic incubation and assay of synthetic conjugated bilirubin were performed by the procedures of Heirwegh *et al.* (1972). Enzymic incubation was for 15min at 37°C. Results are expressed as means±S.D. for the numbers of preparations given in parentheses.

Source of enzyme	Bilirubin conjugated (nmol/10min per g wet wt. equiv. of liver)	
	Cholestatic rat	Normal rat*
Homogenate		
Untreated	30±6 (3)	55±10 (6)
Activated	327±7 (3)	731±116 (6)
Microsomal preparation		
Untreated	13±3 (3)	13±3 (6)
Activated	96±15 (3)	138±25 (5)

* Normal values taken from Heirwegh *et al.* (1972).

Table 7. *Recovery of glucuronide isomers after injection of bilirubin-IXα monoglucuronide in obstructed rats*

Synthetic bilirubin-IXα 1-*O*-acylmono[U-^{14}C]glucuronide (sp. radioactivity 8880d.p.m./nmol), freshly dissolved in 1ml of serum obtained from normal Wistar R/A rats, was injected into a Wistar R/A rat that had been obstructed for 20h. After further maintenance of the obstruction for 150min, bile was collected at 0°C over three successive 20min periods, then over four 30min periods. After determination of volume, samples (50μl) were removed for subsequent counting of radioactivity and the remaining volumes were stored at −15°C. As soon as possible, all samples (Expt. 1) or samples corresponding to successive 1h collection periods (Expt. 2) were pooled. About four-fifths of the samples were submitted to diazonium cleavage at pH6 (Blanckaert *et al.*, 1977*b*), the remainder being treated with the standard procedure at pH2.7 (Van Roy & Heirwegh, 1968). Azopigment obtained with the pH6 procedure was isolated by t.l.c. and treated with diazomethane. The resulting derivatives were separated and quantified (see the Materials and Methods section). n.d., Not determined.

No. of expts.	Body wt. of rat (g)	Amount of conjugate injected (nmol)	Recovery of radioactivity: Collection period (no.)	In bile (% of injected dose)	In azopigment (% of radioactivity in bile)	Glucuronide isomer ...	Azopigment glucuronide (% of total): Azo colour 1	2	3	4	Radioactivity 1	2	3	4	Radioactivity (d.p.m.)/azopigment spot: 1	2	3	4
1	345	48.4	1	12.8	n.d.													
			2	6.7	n.d.		57	22	16	5	43	36	18	3	7879	6596	3298	550
			3	4.9	n.d.													
2	370	64.5	1	5.8	96		55	27	13	5	47	32	17	4	2133	1490	764	175
			2	12.3	86		55	27	13	5	41	38	17	4	4389	4122	1884	446
			3	5.8	106		56	27	12	5	42	35	17	6	5724	4787	2392	827

followed by fractional collection of bile over 180min.

At the end of the collection period, 24% of injected label had been recovered in bile (two rats tested). After diazo treatment of the samples, 86–106% of this label was accounted for in the azopigment extracts, predominantly by glucuronic acid-containing azo derivatives. When the diazonium cleavage reaction was carried out at pH2.7 with the standard ethyl anthranilate procedure of Van Roy & Heirwegh (1968), the percentages of label in the β- (10; 12), γ- (33; 29) and δ-fractions (51; 55) and in an unidentified fraction found near the application line (5; 6) were as indicated between parentheses. The β- and γ-azopigment fractions are derived from non-C-1 glucuronides of bilirubin-IXα by reaction of an excess of aromatic amine at the free C-1 position (F. Compernolle, unpublished work). When the same bile samples were submitted to diazonium cleavage at pH6 in the absence of an excess of aromatic amine, only a small percentage of the label was found in the γ-azopigments (1; 1.1), larger portions being present in the δ-azopigment (88; 91) and near the origin of the chromatograms (9; 7). After treatment of the latter δ-fractions with diazomethane, t.l.c. of the methyl esters showed unequivocally that labelled injected material had been transformed to a considerable extent into the 2-, 3- and 4-*O*-acylglucuronides. Consequently, injected 1-*O*-acylglucuronide serves as a precursor in the generation of the positional isomers. The latter assays carried out *in vivo* do not exclude a partial or complete enzymic process for the isomerization. However, as the extent of conversion was similar to those observed after 150min at 37°C *in vitro*, operation of the same intramolecular and non-enzymic mechanism of acyl migration is strongly suggested.

The relatively poor recovery of injected labelled bilirubin-IXα monoglucuronide obtained over an excretion period of 3h deserves some comment. In non-obstructed rats, 79–91% of injected mono- and di-glucuronides of bilirubin-IXα are excreted into the bile and 2–7% in urine after 2h (Ostrow & Murphy, 1970). During obstruction for 20h preceding injection of labelled glucuronide, considerable amounts of conjugates must have been stored in various parts of the body, such as the distended bile ducts and the blood. Indeed, in experiments of similar duration, Van Damme *et al.* (1971) showed that complete removal of altered conjugates from rats required at least 6h. As in the present experiments, the additional obstruction for 150min probably allowed extensive mixing of labelled material with bilirubin glucuronides already present in the body and further synthesized from endogenous bilirubin-IXα, and relatively slow excretion of label is expected after release of the obstruction. That isomerized conjugates are being evacuated from one or several rather large pools is also suggested by the nearly constant composition of positional isomers over the entire 3h excretion period (Table 7).

General comments

Several types of structural changes initiated by light and/or oxidizing agents such as molecular O_2 (McDonagh, 1975; Pedersen *et al.*, 1977) render preparation and handling of bilirubin conjugates difficult. Easy isomerization of the glucuronic acid moiety at neutral to slightly alkaline pH, observed in the present work, further indicates that great care is required to obtain meaningful results in work with conjugated bilirubin. Isomerization even affects the otherwise more stable dipyrrolic azo derivatives (Figs. 2 and 3) that are frequently used for analytical purposes. Several observations indicate that cholestatic bile contains small amounts of positional isomers of bilirubin-IXα glucoside, i.e. isolation from such bile of an azodipyrrole glucoside that resists hydrolysis by β-glucosidase, yields glucose on ammonolysis and shows the expected molecular ion on mass spectrometric analysis of the fully acetylated derivative (N. Blanckaert, F. Compernolle & K. P. M. Heirwegh, unpublished work). It is likely that alkali-catalysed isomerization may also affect other glycosidic ester conjugates such as benzoic acid glucuronide.

In chemical and biochemical work with glycosidic ester conjugates, one may profitably buffer any aqueous systems to a pH just below 6 (Fig. 3) when this is permissible. If it is not, isomerization can be limited by working at low temperature and by making any contacts with aqueous solutions of neutral to alkaline pH as short as possible.

As outlined by Fevery *et al.* (1972), treatment of biological samples at pH2.7 with the standard ethyl anthranilate diazo reagent of Van Roy & Heirwegh (1968) and determination of the relative amounts of azopigments β and γ offer a valuable and convenient way to characterize the degree of cholestasis. The present work (Table 7) and demonstration that azopigments β and γ derive from non-C-1 glucuronides generated during stasis of bile pigments in the body (F. Compernolle, unpublished work) amply support this conclusion. Analysis of methyl esters of azodipyrrole glucuronides, as explored in the present study, offers an alternative approach that may be more suitable for fundamental studies.

Lack of hydrolysis of non-C-1 glucuronides by β-glucuronidase suggests that formation of positional isomers during stasis in the body and probably also during intestinal transit may protect the organism against undesirable effects of unconjugated bilirubin-IXα. Indeed, it may inhibit formation of bile-pigment stones and re-absorption of the potentially toxic bilirubin-IXα.

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