Biochem. J. (1972) **129**, 635–644
Printed in Great Britain

Structures of Bilirubin Conjugates Synthesized *in vitro* from Bilirubin and Uridine Diphosphate Glucuronic Acid, Uridine Diphosphate Glucose or Uridine Diphosphate Xylose by Preparations from Rat Liver

By J. FEVERY, P. LEROY, M. VAN DE VIJVER and K. P. M. HEIRWEGH
Department of Liver Physiopathology, Rega Instituut, Universiteit te Leuven, B-3000 Leuven, Belgium

(*Received* 23 *February* 1972)

1. In incubation mixtures containing digitonin-activated or untreated preparations from rat liver, albumin-solubilized bilirubin as the acceptor substrate and (*a*) UDP-glucuronic acid, (*b*) UDP-glucose or (*c*) UDP-xylose as the sugar donor, formation of the following ester glycosides was demonstrated: with (*a*), bilirubin β-D-monoglucuronoside, with (*b*), bilirubin β-D-monoglucoside and with (*c*), bilirubin monoxyloside or mixtures of the mono- and di-xyloside. 2. With UDP-glucuronic acid prolonged incubation and variation of the composition of the incubation mixtures yielded equimolar amounts of azodipyrrole (I) and azodipyrrole β-D-monoglucuronoside (II) after treatment of the incubation mixtures with the diazonium salt of ethyl anthranilate. The azo-derivatives were identified by t.l.c. by reference to known compounds and by the following chemical tests. After ammonolysis the conjugated azo-derivative (II) yielded D-glucuronic acid and the carboxylic acid amide of azodipyrrole, indicating transfer of a glucuronic acid residue to the carboxylic acid groups of bilirubin. The β-D-configuration of the sugar moiety and binding at C-1 were demonstrated by enzymic hydrolysis tests. 3. Analogous evidence established the structure of the reaction product obtained with UDP-glucose as the sugar donor, as bilirubin β-D-monoglucoside. 4. With UDP-xylose as the sugar donor xylosyl transfer to the carboxylic acid groups of bilirubin with attachment at C-1 was demonstrated in an analogous way. A β-D-configuration is considered very likely, but requires confirmation. 5. Monoxyloside formation was predominant at pH7.4, whereas at decreasing pH values increasing fractions of the substrate were converted into the dixyloside. Prolonged incubation, low concentrations of bilirubin and high concentrations of UDP-xylose favoured diconjugate formation. The available evidence supports the synthesis sequence:

bilirubin → bilirubin monoxyloside → bilirubin dixyloside.

In bile of normal rats the mono- and di-glucuronoside of bilirubin (Schoenfield & Bollman, 1963; Ostrow & Murphy, 1970) and small amounts of the corresponding glucosidic conjugates occur (Fevery *et al.*, 1971). Larger amounts of bilirubin glucoside and conjugates of xylose are excreted in dog bile. Transfer of glucuronic acid (Grodsky & Carbone, 1957; Schmid *et al.*, 1957; Lathe & Walker, 1958), glucose (Wong, 1971*b*; Fevery *et al.*, 1972) and xylose residues (Fevery *et al.*, 1972) from the respective UDP-sugars to bilirubin occurs *in vitro* in the presence of preparations from rat liver.

The present paper establishes tentative structures of several bilirubin glycosides synthesized *in vitro* from albumin-solubilized bilirubin and UDP-sugar in the presence of homogenates or microsomal material from rat liver. After incubation with UDP-glucuronic acid or UDP-glucose under a variety of reaction conditions, only bilirubin β-D-monoglycosides could be detected. With UDP-xylose as the sugar donor monoxyloside or mixtures of mono- and di-xyloside were formed, depending on the conditions of incubation. A preliminary account of the work has been given (Heirwegh *et al.*, 1971).

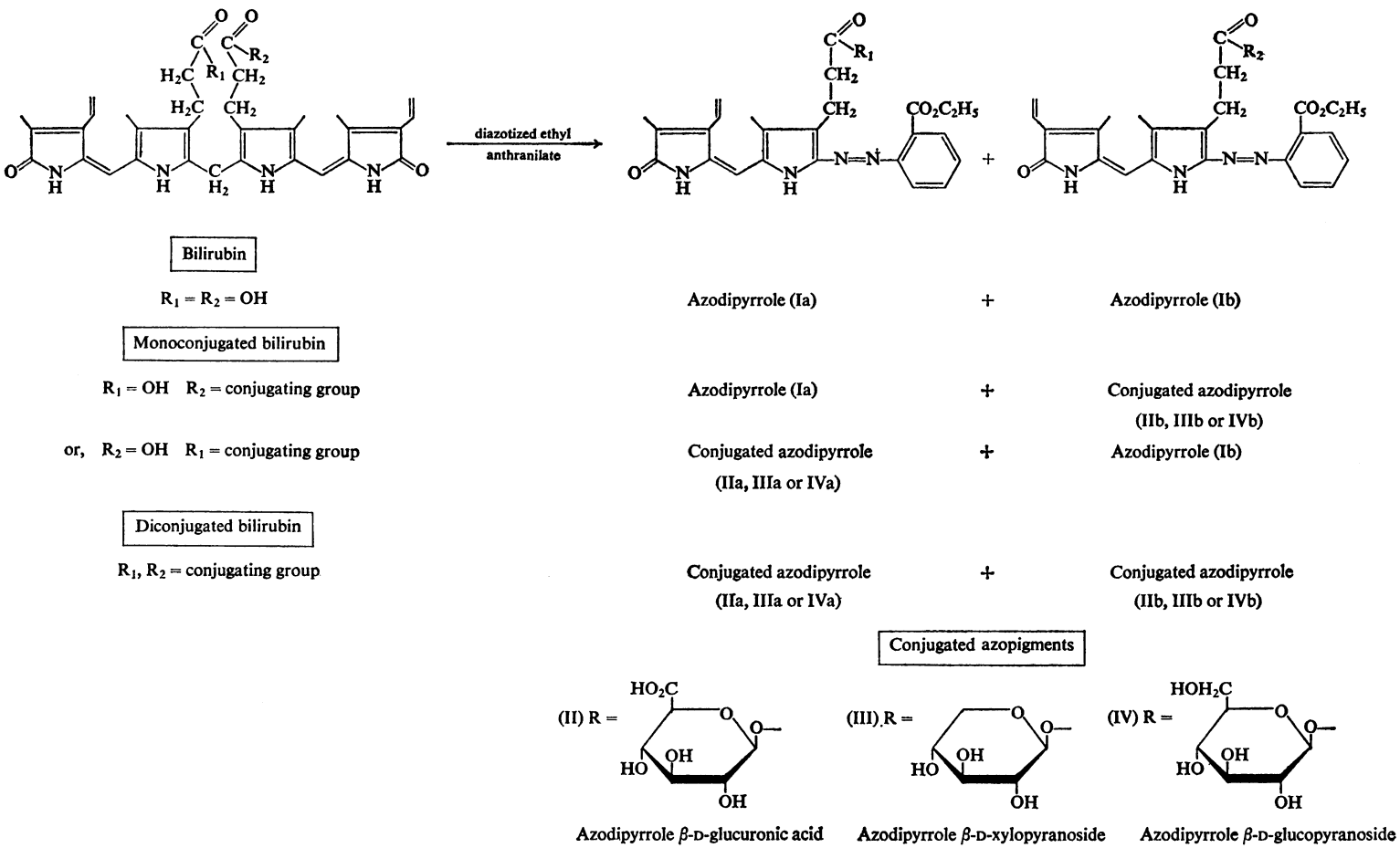
Materials and Methods

Chemicals

Chemicals were as specified by Van Roy & Heirwegh (1968), Heirwegh *et al.* (1972) and Fevery *et al.* (1972).

Synthesis of bilirubin glycosides in vitro and formation of azo-derivatives

Adult Wistar R male albino rats were used. Digitonin-activated homogenates and treated and untreated microsomal preparations from rat liver were incubated with albumin-solubilized bilirubin and



Scheme 1. *Formation and structures of ethyl anthranilate azo-derivatives*

In the present work the vinyl (*a*) and isovinyl isomers (*b*) of unconjugated (I) and conjugated azodipyrrole (II, III and IV) have not been separated. They are discussed as if they were homogeneous compounds.

UDP-sugar (UDP-glucuronic acid, UDP-glucose or UDP-xylose). The bilirubin glycosides thus synthesized were treated with diazotized ethyl anthranilate and the azopigments extracted into pentan-2-one–*n*-butyl acetate (17:3, v/v). Full details about the procedures are given by Heirwegh *et al.* (1972) and by Fevery *et al.* (1972).

Qualitative and quantitative azopigment analysis

Equal volumes of azopigment extracts obtained from the incubation mixtures and from incubation controls (UDP-sugar omitted; Mg^{2+} replaced by disodium EDTA) were applied to a pre-coated silicagel plate (DC-Kieselgel F254, 5717/0025; E. Merck A.-G., Darmstadt, Germany) and separated by successive developments with chloroform (containing 0.6% ethanol) (18cm), chloroform–methanol–water (65:25:3, by vol.) (5cm) and chloroform–methanol (17:3, v/v) (12cm). The first development favours removal of extracted bilirubin (excess of substrate) from areas containing azopigment. Azopigment extracts prepared from dog bile (Fevery *et al.*, 1971) and rat bile (Heirwegh *et al.*, 1970) served as reference standards. Structurally characterized preparations of azodipyrrole (I), azodipyrrole β-D-glucuronoside (II), azodipyrrole β-D-xylopyranoside (III) and azodipyrrole β-D-glucopyranoside (IV) (Compernolle *et al.*, 1970, 1971) were also used (Scheme 1).

Azopigments, after separation, were eluted with methanol and determined photometrically (Heirwegh *et al.*, 1970). The reddish spots obtained in the test run were eluted with methanol. Eluates from the control track, corresponding in shape and R_F value to the azopigment spots, served as blanks. Test and blank eluates were measured separately at 546nm with methanol as a reference.

Calculations

Assuming that the molar extinction coefficients of azodipyrrole (I) and of the conjugated azopigments (II, III and IV) are equal, the ratio R (azodipyrrole/conjugated azodipyrrole) is given by $E/E_{conj.}$ (the corrected extinctions of the azodipyrrole and of the conjugated azodipyrrole spots respectively). This assumption probably does not introduce a significant error, as preparations of both conjugated and unconjugated bilirubin at equal molar concentration showed the same azo-colour extinction (Van Roy & Heirwegh, 1968). A ratio of 1 would then be obtained from monoconjugated bilirubin, a ratio of 0 for diconjugated bilirubin and values between 0 and 1 for mixtures of mono- and di-conjugate (Scheme 1).

The percentage of total conjugated bile pigments present as monoconjugated bilirubin is equal to $200R/(1+R)$. The ranges of experimental error were established by assuming that the total error on the calculated results is mainly due to photometric error (estimated to be ±0.001 on each extinction measurement).

Adequate correction for colour derived from control incubation mixtures (especially in the azopigment-α_0 area) is important. At standard bilirubin concentration, colour formation from excess of bilirubin is approximately constant (diazotization conditions being standard for all systems). Decreased conjugation, owing to decreases in substrate concentration or incubation time, or to the use of enzyme preparations of low activity, will increase the relative magnitude of the blank corrections. This may explain the rather large range of results obtained with bilirubin UDP-glucosyltransferase. Fig. 1 illustrates the relative importance of test and blank values.

Purification of azopigments derived from incubation mixtures

Pooled azopigment extracts were separated by preparative t.l.c. (Heirwegh *et al.*, 1970) by using the sequence of solvents outlined above.

Results and Discussion

Reaction of bilirubin and of conjugated bilirubin with diazonium salts leads to cleavage at the central methylene bridge with the formation of two dipyrrolic azopigments (Scheme 1) (for references, see Jansen & Stoll, 1971). Therefore, as the azo-derivatives are relatively stable compared with bile pigments, the elucidation of structure for the latter compounds can conveniently be done in two stages: structure assignments (1) of separated azopigments and (2) of the parent tetrapyrroles.

Assignment of structure to azopigments derived from bile pigments synthesized in vitro

Studies with slices (Lathe & Walker, 1958; Schoenfield & Bollman, 1963) and broken-cell systems from rat liver (Grodsky & Carbone, 1957; Schmid *et al.*, 1957; Brown *et al.*, 1958; Menken *et al.*, 1966; Wong, 1971*a*), and experiments with isolated rat liver preparations (Schoenfield & Bollman, 1963), support the concept of an enzyme-catalysed glucuronyl transfer from UDP-glucuronic acid to bilirubin. In attempts to establish the configuration of the synthetic product with β-glucuronidase no controls were made with enzyme inhibitor (Schmid *et al.*, 1957; Brown *et al.*, 1958; Menken *et al.*, 1966; Wong, 1971*a*). The acceptor site of the aglycone was not established. Evidence supporting the enzyme-catalysed glucosyl transfer from UDP-glucose to bilirubin has also been obtained (Wong, 1971*b*).

Glucuronoside conjugate. Treatment of incubation

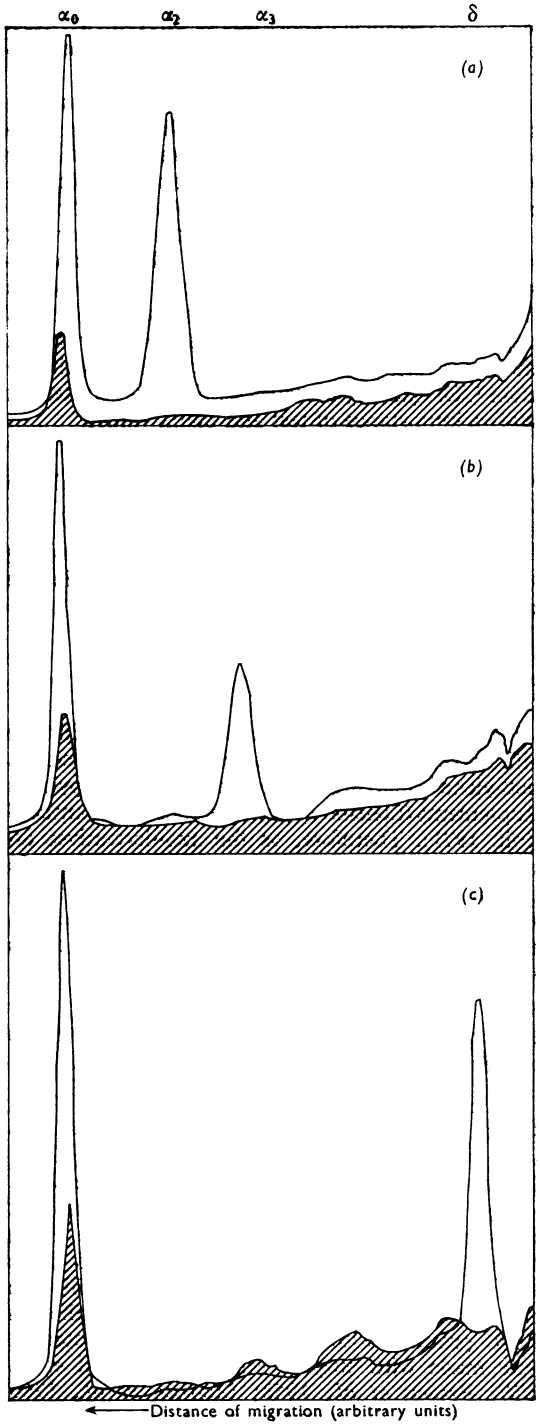


Fig. 1. *T.l.c. of azopigments derived from test and control incubation mixtures*

Equal volumes of azopigment extracts obtained from test (full lines) and control systems (cross-hatched areas) were separated by t.l.c. Densitometric records (E_{536}) obtained with a flying-spot TLD100 densitometer (Vitatron, Dieren, The Netherlands) are shown. Digitonin-activated microsomal preparations were incubated under standard conditions with albumin-solubilized bilirubin and UDP-sugar; the incubation times were 10min with UDP-xylose (*a*) and UDP-glucuronic acid (*c*), and 20min with UDP-glucose (*b*). Azopigment spots are denoted by Greek letters (Heirwegh *et al.*, 1970). Migration is from right to left.

mixtures containing UDP-glucuronic acid with diazotized ethyl anthranilate gave rise to azopigments α_0 and δ (Fig. 1, Table 1). The azopigments had the same chromatographic mobility as azodipyrrole (I) and azodipyrrole β-D-monoglucuronoside (II) respectively. Ammonolysis of the more hydrophilic azo-derivative (azopigment δ) obtained from the incubation mixtures liberated only one sugar that moved chromatographically as D-glucuronic acid (Table 2). Formation of the carboxylic acid amide of azodipyrrole (Table 3) indicates binding of the sugar moiety to the propionic acid side chain of azodipyrrole (Compernolle *et al.*, 1970). Hydrolysis of azopigment δ with β-glucuronidase and inhibition of the process by saccharo-(1→4)-lactone further support the presence of a β-D-glucosiduronic acid residue bound at C-1 (Table 4).

Xylose and glucose conjugates. Analogous observations allow azodipyrrole monoxyloside (III) and azodipyrrole β-D-monoglucoside (IV) structures to be assigned to the conjugated azopigments α_2 and α_3 derived from incubation mixtures containing UDP-xylose and UDP-glucose respectively (Tables 1–4). The emulsin preparation used to establish the configuration of the glucoside (IV) contained negligible α-glucosidase activity (Fevery *et al.*, 1971). Parallel incubations of purified preparations of azopigment α_3 and of azodipyrrole β-D-glucopyranoside (IV) indicated that both compounds were hydrolysed at the same rate. The Mylase-P preparation, used as a source of xylosidase activity, was impure (the ratio of β-xylosidase activity/α-xylosidase activity was 37:2). Although a β-D-configuration of azopigment α_2 is likely, further confirmation with a purer β-xylosidase is essential.

In general the observations demonstrate that glucuronic acid, glucose and xylose residues can be transferred from the respective UDP-sugars to the propionic acid side chains of bilirubin by digitonin-activated and untreated microsomal material from rat liver. Inversion at C-1 accompanies the glucuronyl- and glucosyl-transfer reactions and is likely for xylose.

The ring structures of the sugars were not established. The chromatographic reference compounds

Table 1. *$R_{azodipyrrole}$ values of azopigments α_0, α_2, α_3 and δ derived from synthetic bile pigments*

Incubation mixtures containing digitonin-activated microsomal preparations were used. The derived azopigments were separated by t.l.c. Azopigment mixtures obtained from human cholestatic bile (H) and from dog bile (D), and purified samples of azodipyrrole β-D-xylopyranoside (III) and of azodipyrrole β-D-glucopyranoside (IV), served as references. Azopigment extracts from individual incubation mixtures (total number 19) were analysed on plates nos. 1–5. At the bottom of the table paired values indicate ranges of $R_{azodipyrrole}$ values of the unconjugated and conjugated azopigments; the number of samples analysed is given in parentheses; purified preparations of azopigments α_2 and α_3 obtained from pooled extracts were used in Expt. no. 6.

	$R_{azodipyrrole}$						
Sugar donor used in the incubation mixture ...	UDP-xylose	UDP-xylose	UDP-glucuronic acid	UDP-glucuronic acid	UDP-glucuronic acid	UDP-xylose	UDP-glucose
No. of t.l.c. plate ...	1	2	3	4	5	6	6
Reference compounds							
Azopigment-α_0 (H)	1.00	1.00	1.00				
Azopigment-α_2 (H)	0.82	0.80					
Azopigment-α_3 (H)	0.70	0.68					
Azopigment-δ (H)			0.20				
Azopigment-α_0 (D)						1.00	1.00
Azopigment-α_2 (D)						0.80	
Azopigment-α_3 (D)							0.69
Compound (III)						0.80	
Compound (IV)							0.71
Azopigments derived from enzymic incubation mixtures							
Azopigment-α_0	1.00–1.01 (3)	1.04–1.06 (3)	0.99–1.00 (4)	1.00	1.00		
Azopigment-α_2	0.80–0.82 (3)	0.78–0.81 (3)				0.82	
Azopigment-α_3							0.69
Azopigment-δ			0.22–0.23 (4)	0.22–0.24 (4)	0.21–0.23 (5)		

(III) and (IV) (Compernolle *et al.*, 1971) and (II) (F. Compernolle, G. P. Van Hees & K. P. M. Heirwegh, unpublished work) derived from bile are glycopyranosides. It is likely that the glycopyranose structures present in UDP-sugars are conserved during enzyme-catalysed glycosyl transfer.

Table 2. $R_{glucose}$ *values of conjugating groups*

A modification of the procedure of Fevery *et al.* (1971) was used. Combined azopigment extracts derived from incubation mixtures containing digitonin-activated microsomal preparations and UDP-xylose, UDP-glucose or UDP-glucuronic acid were used to prepare azopigments α_2, α_3 and δ respectively. Samples of the purified azopigments were applied to a t.l.c. plate (DC-Kieselgel F254, 5715/0025) leaving free spaces for subsequent application of reference sugars. The plate was kept overnight at room temperature in an atmosphere equilibrated with 27% (w/v) ammonia. After complete evaporation of excess of ammonia in an air stream reference sugars were applied and the plate was developed sequentially with: (1) chloroform – methanol (19:1, v/v) (18cm); (2) chloroform – methanol – water (65:25:3, by vol.) (15cm); (3) propan-1-ol – water (17:3, v/v) (12cm); (4) chloroform – methanol – water (65:25:3, by vol.) (15cm). Sugars were localized with a *p*-anisidine – phthalate spray (Krebs *et al.*, 1967).

Reference compounds		
D-Xylose	1.42, 1.41	1.39, 1.36
D-Glucose	1.00, 1.00	1.00, 1.00
D-Glucuronic acid	0.23, 0.23	
Conjugating groups from		
Azopigment-α_2	1.42	1.36
Azopigment-α_3	1.00	1.00
Azopigment-δ	0.23	

Assignment of structure to bile pigments synthesized in vitro

Available literature relates only to the synthesis of glucuronic acid conjugates of bilirubin. Synthesis of diconjugated bilirubin by liver slices and by isolated liver preparations from rat (Schoenfield & Bollman, 1963), and of mono- and di-conjugated bilirubin by a clonal strain of rat hepatoma cells (Rugstad *et al.*, 1970), has been demonstrated. In contrast, with homogenates from human liver (Black *et al.*, 1970), and with microsomal material from rat liver (Van Roy & Heirwegh, 1968), treatment of incubation mixtures containing bilirubin and UDP-glucuronic acid with diazotized ethyl anthranilate yielded equimolar amounts of unconjugated and conjugated azodipyrrole. The results suggested synthesis *in vitro* of bilirubin monoglucuronide.

In the present work broken-cell preparations from rat liver were tested under a variety of incubation conditions, in an attempt to obtain values of the ratio R (azodipyrrole/azodipyrrole monoconjugate) significantly smaller than 1.

With UDP-glucuronic acid as the sugar donor R was 0.97 ± 0.08 (S.D.; $n = 32$). No systematic trends were apparent when either the incubation time was lengthened from 15 to 120min or when the concentration of the sugar donor was increased threefold (Table 5). Addition of UDP-*N*-acetylglucosamine, a known inhibitor of UDP-glucuronic acid pyro-

Table 3. $R_{azodipyrrole}$ *values of amide derivatives*

Pooled azopigment extracts derived from incubation mixtures containing UDP-xylose, UDP-glucose or UDP-glucuronic acid were used to prepare azopigment α_2, α_3 and δ respectively. Samples of the purified azopigments and of azodipyrrole β-D-monoglucuronoside (II) were applied to a t.l.c. plate, leaving free spaces between the spots for subsequent application of azodipyrrole (I). The plate was kept overnight at room temperature in an atmosphere equilibrated with 27% (w/v) ammonia. After removal of excess of ammonia in an air stream reference samples of azodipyrrole (I) were applied. The plate was developed with chloroform – methanol – water (65:25:3, by vol.) (5cm), dried in an air stream and developed again with redistilled chloroform (18cm).

	$R_{azodipyrrole}$ of amide derivative			
Expt. no. ...	1	2	3	4
Reference compound				
Azodipyrrole (I)	1.00	1.00	1.00	1.00
Amide from conjugated azopigment				
α_2	1.12	1.14	1.24	
α_3	1.12	1.14	1.23	
δ	1.10	1.14	1.23	1.46
Compound II	1.09		1.20	

Table 4. *Enzyme hydrolysis of azopigments α_2, α_3 and δ derived from synthetic bile pigments*

Pooled azopigment extracts derived from incubation mixtures containing digitonin-activated microsomal preparations and UDP-xylose, UDP-glucose or UDP-glucuronic acid were used to prepare azopigments α_2, α_3 and δ respectively. The purified azopigment preparations were incubated with Mylase-P (azopigment α_2), emulsin (azopigment α_3) or β-glucuronidase from *Escherichia coli* (azopigment δ). Tests were run in the absence and in the presence of 50mM-glucono-(1→5)-lactone (Mylase-P; emulsin) or of 3mM-saccharo-(1→4)-lactone (β-glucuronidase). The final incubation mixtures were extracted into pentan-2-one and analysed by t.l.c. The percentages of azopigments hydrolysed were obtained by visual inspection of the plates (mean values estimated by three independent observers). Details about the origin and activities of the enzyme preparations and about the methods used are given by Heirwegh *et al.* (1970) and by Fevery *et al.* (1971).

	Preparation no.	Enzyme	Amount of enzyme (mg/test)	Incubation time (h)	Azopigment hydrolysed (%) With enzyme	Azopigment hydrolysed (%) With enzyme and inhibitor
Azopigment α_2	1	Mylase-P	10	3	90	90
	2		10	1	90	90
	2		10	2	95	90
Azopigment α_3	3	Emulsin	2	4	90	0
	4		2	3	85	0
	5		2	1	90	0
	5		2	2	95	0
Azopigment δ	6	β-Glucuronidase	2	4.5	95	0

Table 5. *Synthesis in vitro of bilirubin β-D-monoglucuronoside*

Homogenate or microsomal material, either digitonin-activated (*D*) or not activated (*U*), from rat liver was incubated at 37°C with albumin-solubilized bilirubin and UDP-glucuronic acid for the times indicated in the table. Conjugated bilirubin was then treated with diazotized ethyl anthranilate and the derived azopigments (α_0 and δ) were analysed by quantitative t.l.c. The following variants of the standard procedures were also used. (*a*) Identical incubation mixtures each received 1mg of UDP-glucuronic acid at zero time; when the first tube was withdrawn for analysis the tubes remaining at 37°C received a further addition of 1mg of UDP-glucuronic acid; the procedure was repeated until the last incubation period was started. (*b*) Incubation mixtures were supplemented at zero time with UDP-*N*-acetylglucosamine (final concn. 3.2mM). (*c*) The pH of the incubation mixtures was varied. The results are expressed as the ratio (*R*) of azopigment α_0/azopigment δ.

Enzyme preparation	Incubation procedure	*R* 15 min	30 min	60 min	90 min	120 min
Homogenate (*D*)	Standard system		1.04	1.11	1.03	0.82
	UDP-glucuronic acid added (*a*)		0.97	0.94	0.97	1.01
Microsomal preparation no. 1 (*U*)	Standard system		0.97	0.98	0.99	
	UDP-*N*-acetylglucosamine added (*b*)		1.04	0.99	0.95	
(*D*)	Standard system		0.97	0.98	1.17	
	UDP-*N*-acetylglucosamine added (*b*)		0.90	0.94	0.99	
Microsomal preparation no. 2 (*D*)	Standard system	0.86	0.89	0.91	0.93	
	UDP-glucuronic acid added (*a*)		0.89	0.90	0.80	
Microsomal preparation no. 3 (*D*)	Variation of pH (*c*)					
	pH6.6	0.93		0.91		
	pH7.0	0.97		0.94		
	pH7.6	1.03		1.06		
	pH8.0	1.11		1.05		

phosphatase (Pogell & Leloir, 1961; Adlard & Lathe, 1970), had little effect on the ratio *R*. It may be noted that addition of the inhibitor to broken-cell preparations not treated with digitonin considerably increased glucuronyl-transfer rates (Heirwegh *et al.*, 1972).

Table 6. *Enzyme synthesis in vitro of bilirubin β-D-monoglucoside*

Microsomal material (either digitonin-activated or not activated) was incubated at 37°C with albumin-solubilized bilirubin and UDP-glucose for the times indicated in the table. Deviations from the standard procedures are indicated under the heading 'Variable parameter'. Conjugated bilirubin was treated with diazotized ethyl anthranilate and analysed by quantitative t.l.c. The results are expressed as the ratio R of azopigment α_0/azopigment α_3. With $R = 1$ (only monoconjugate formed) as the value to be found, then experimental values marked with an asterisk were within the experimental limits of error (see under 'Calculations' in the Materials and Methods section).

Preparation no.	Variable parameter	Incubation time (min)	Microsomal material: Activated	Microsomal material: Non-activated
1	pH6.8	8	0.89*	
		16	1.13*	
	pH7.3	8	1.53	
		16	1.06*	
2	pH6.42	30	0.92*	
	pH6.72	30	1.02*	
	pH6.78	30	1.00*	
	pH7.08	30	1.06*	
	pH7.40	30	1.33	
	pH7.58	30	1.02*	
3	0.022mM-Bilirubin	20	0.60	0.79*
	0.045mM-Bilirubin	20	0.77	0.82*
	0.134mM-Bilirubin	20	1.00*	
4	2.6mM-UDP-glucuronic acid	10	1.00*	
		20	0.91*	
	12.8mM-UDP-glucuronic acid	10	0.82*	1.11*
		20	0.89*	0.96*
	17.9mM-UDP-glucuronic acid	10	1.00*	1.20*
		20	0.94*	0.94*

With UDP-glucose as the sugar donor the behaviour was similar with respect to changes in the reaction parameters (Table 6). The mean R value was 1.01 ± 0.18 (S.D.; $n = 34$).

Considering the nature of the diazo-reaction of bile pigments (Scheme 1) and the precautions taken in determining the ratio R it is reasonable to conclude that bilirubin β-D-monoglycosides were synthesized under the incubation conditions described. Because of the rather wide range of the results minor diconjugate formation cannot be excluded. By making use of individual R values, the percentages of conjugated bile pigments were calculated as $98.3 \pm 3.9\%$ (S.D.; $n = 32$) of bilirubin β-D-monoglucuronoside and $99.9 \pm 8.7\%$ (S.D.; $n = 34$) of bilirubin β-D-monoglucoside. Jansen & Stoll (1971) suggest that glucuronyl transfer to either of the carboxylic acid groups of bilirubin may occur *in vitro*.

With UDP-xylose as the sugar donor the situation is more complex (Figs. 2–4). Monoxyloside formation predominated at pH7.4, considerable fractions of diconjugate being formed when the pH was decreased to 6.4 (Fig. 2). Dixyloside formation was stimulated by prolonging the incubation (Figs. 2–4), by decreasing the concentration of bilirubin (Fig. 3) and by increasing the concentration of the sugar nucleotide (Fig. 4). The changes were well outside the estimated limits of error. Qualitatively, the observed behaviour is compatible with the synthesis sequence: bilirubin → bilirubin monoxyloside → bilirubin dixyloside.

General comment

As noted above, bile of the normal rat contains mainly the mono- and di-glucuronoside of bilirubin. The rather large amounts of monoconjugate (about 73% of total conjugated bile pigment) reported by Van Roy & Heirwegh (1968) were probably caused by collection of bile from rats under ether anaesthesia. Indeed, Van Damme *et al.* (1971) demonstrated that during recovery from anaesthesia the percentage of monoconjugate gradually decreased to 39%. Liver slices and isolated liver preparations from rat (Schoenfield & Bollman, 1963) and rat hepatoma cells (Rugstad *et al.*, 1970) appear to be capable of transforming bilirubin into the diglucuronoside and it is surprising that it has not been possible to obtain demonstrable diconjugate formation with homo-

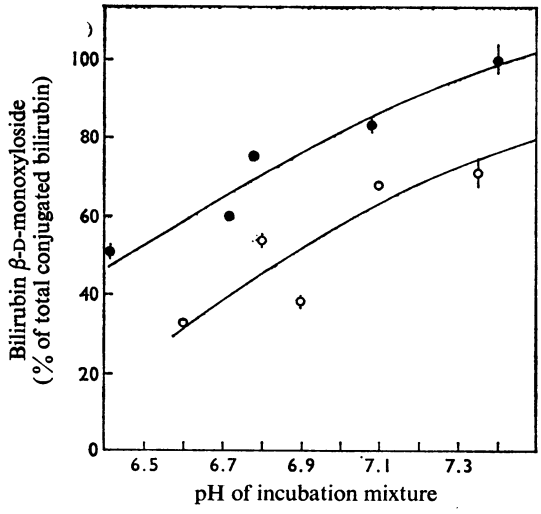


Fig. 2. *Effects of pH and incubation time on the synthesis in vitro of the mono- and di-xyloside of bilirubin*

Digitonin-activated microsomal material was incubated at 37°C with albumin-solubilized bilirubin and UDP-xylose. The incubation times were 30min (●) and 60min (○). Different pH values were obtained by varying the concentration of triethanolamine in the buffer used. Duplicate incubation mixtures prepared in parallel (UDP-xylose omitted) were used to determine the pH. Vertical bars indicate ranges of experimental error.

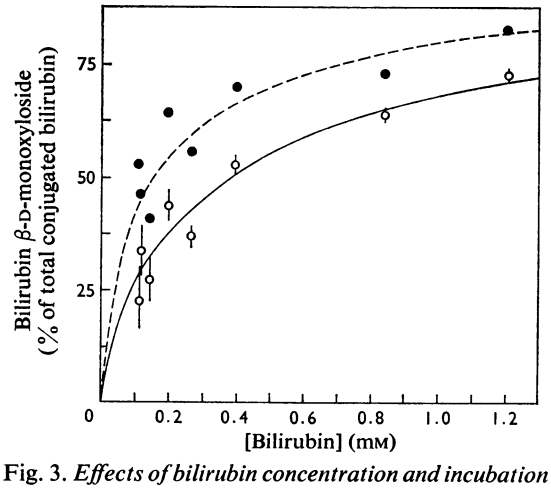


Fig. 3. *Effects of bilirubin concentration and incubation time on the synthesis in vitro of the mono- and di-xyloside of bilirubin*

Digitonin-activated microsomal material was incubated at 37°C at pH7.1 with albumin-solubilized bilirubin and UDP-xylose. The incubation times were 4min (●) and 8min (○). Vertical bars indicate ranges of experimental error.

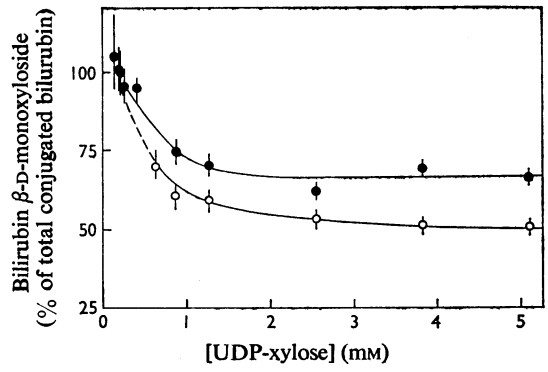


Fig. 4. *Effects of UDP-xylose concentration and incubation time on the synthesis in vitro of the mono- and di-xyloside of bilirubin*

Digitonin-activated microsomal material was incubated at 37°C at pH7.1 with albumin-solubilized bilirubin and UDP-xylose. The incubation times were 10min (●) and 20min (○). Vertical bars indicate ranges of experimental error.

genate or microsomal material from rat liver (Table 5).

With the rat liver systems exhaustion of UDP-glucuronic acid is very unlikely (Table 5). It is suggested that rat liver may contain a factor essential for diglucuronoside formation that either is present at too low a concentration to be effective in the more dilute incubation mixtures or is destroyed during fractionation of tissue. The requirements may be less stringent for the synthesis of dixyloside.

We thank Dr. J. Vandenbroucke and Dr. J. De Groote for their encouragement and Dr. F. Compernolle for valuable discussions. The financial support of the Fonds voor Wetenschappelijk Geneeskundig Onderzoek of Belgium and the receipt of a post-doctoral fellowship from the Nationaal Fonds voor Wetenschappelijk Onderzoek of Belgium by J. F. are much appreciated.

References

Adlard, B. P. F. & Lathe, G. H. (1970) *Biochem. J.* **119**, 437–445

Black, M., Billing, B. H. & Heirwegh, K. P. M. (1970) *Clin. Chim. Acta* **29**, 27–35

Brown, A. K., Zuelzer, W. W. & Burnett, H. H. (1958) *J. Clin. Invest.* **37**, 332–340

Compernolle, F., Jansen, F. H. & Heirwegh, K. P. M. (1970) *Biochem. J.* **120**, 891–894

Compernolle, F., Van Hees, G. P., Fevery, J. & Heirwegh, K. P. M. (1971) *Biochem. J.* **125**, 811–819

Fevery, J., Van Hees, G. P., Leroy, P., Compernolle, F. & Heirwegh, K. P. M. (1971) *Biochem. J.* **125**, 803–810

Fevery, J., Leroy, P. & Heirwegh, K. P. M. (1972) *Biochem. J.* **129**, 619–633

Grodsky, G. M. & Carbone, J. V. (1957) *J. Biol. Chem.* **226**, 449–458

Heirwegh, K. P. M., Van Hees, G. P., Leroy, P., Van Roy, F. P. & Jansen, F. H. (1970) *Biochem. J.* **120**, 877–890

Heirwegh, K. P. M., Meuwissen, J. A. T. P. & Fevery, J. (1971) *Biochem. J.* **125**, 28P–29P

Heirwegh, K. P. M., Van de Vijver, M. & Fevery, J. (1972) *Biochem. J.* **129**, 605–618

Jansen, F. H. & Stoll, M. S. (1971) *Biochem. J.* **125**, 585–597

Krebs, K. G., Heusser, D. & Wimmer, H. (1967) in *Dunnschicht-Chromatographie, ein Laboratoriumshandbuch* (Stahl, E., ed.), 2nd. edn., pp. 813–859, Springer Verlag, Berlin, Heidelberg and New York

Lathe, G. H. & Walker, M. (1958) *Biochem. J.* **70**, 705–712

Menken, M., Barrett, P. V. D. & Berlin, N. I. (1966) *Clin. Chim. Acta* **14**, 777–785

Ostrow, J. D. & Murphy, N. H. (1970) *Biochem. J.* **120**, 311–327

Pogell, B. M. & Leloir, L. F. (1961) *J. Biol. Chem.* **236**, 293–298

Rugstad, H. E., Robinson, S. H., Yannoni, C. & Tashjian, A. H. (1970) *J. Cell Biol.* **47**, 703–710

Schmid, R., Hammaker, L. & Axelrod, J. (1957) *Arch. Biochem. Biophys.* **70**, 285–288

Schoenfield, L. J. & Bollman, J. L. (1963) *Proc. Soc. Exp. Biol. Med.* **112**, 929–932

Van Damme, B., Fevery, J. & Heirwegh, K. P. M. (1971) *Experientia* **26**, 27–28

Van Roy, F. P. & Heirwegh, K. P. M. (1968) *Biochem. J.* **107**, 507–518

Wong, K. P. (1971*a*) *Biochem. J.* **125**, 27–35

Wong, K. P. (1971*b*) *Biochem. J.* **125**, 929–934