Biochem. J. (1977) **164**, 737–746
Printed in Great Britain

Comparison in Different Species of Biliary Bilirubin-IXα Conjugates with the Activities of Hepatic and Renal Bilirubin-IXα–Uridine Diphosphate Glycosyltransferases

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(*Received* 10 *December* 1976)

The bilirubin-IXα conjugates in bile and the activities of bilirubin-IXα–UDP-glycosyltransferases in liver and kidney were determined for ten species of mammals and for the chicken. 1. In the mammalian species, bilirubin-IXα glucuronide was the predominant bile pigment. Excretion of neutral glycosides was unimportant, except in the cat, the mouse, the rabbit and the dog, where glucose and xylose represented 12–41% of total conjugating groups bound to bilirubin-IXα. In chicken bile, glucoside and glucuronide conjugates were of equal importance. They probably represent only a small fraction of the total bile pigment. 2. The transferase activities in liver showed pronounced species variation. This was also apparent with regard to activation by digitonin, pH optimum and relative activities of transferases acting on either UDP-glucuronic acid or neutral UDP-sugars. 3. Man, the dog, the cat and the rat excrete bilirubin-IXα largely as diconjugated derivatives. In general, diconjugated bilirubin-IXα could also be synthesized *in vitro* with liver homogenate, bilirubin-IXα and UDP-sugar. In contrast, for the other species examined, bilirubin pigments consisted predominantly of monoconjugated bilirubin-IXα. Synthesis *in vitro* with UDP-glucuronic acid, UDP-glucose or UDP-xylose as the sugar donor led exclusively to the formation of monoconjugated bilirubin-IXα. 4. The transferase activities in the kidney were restricted to the cortex and were important only for the rat and the dog. No activity at all could be detected for several species, including man. 5. Comparison of the transferase activities in liver with reported values of the maximal rate of excretion in bile suggests a close linkage between conjugation and biliary secretion of bilirubin-IXα.

In mammals, conjugation of exogenous compounds with sugars leads in most cases to the formation of monoglucuronides (Dutton, 1966, 1971). In contrast, some steroids (Layne, 1970) and bilirubin-IXα (Heirwegh *et al.*, 1975) may yield various mono- and di-conjugates of glucuronic acid and of other sugars.

Bilirubin-IXα is the major end-product of haem catabolism (Gray *et al.*, 1972; Lathe, 1972). In normal situations, excretion of the bile pigment in urine is negligible (Fevery *et al.*, 1968; With, 1968). In the bile of various animals it occurs almost exclusively in conjugated forms containing sugar residues (Fevery *et al.*, 1972*c*; Thompson & Hofmann, 1973; Boonyapisit *et al.*, 1974). Small amounts of bilirubin sulphate have been detected in bile of man, dog and rat (Noir & Nanet, 1974). The IIIα- and XIIIα-isomers, which could arise from bilirubin-IXα by dipyrrole exchange (McDonagh & Assisi, 1972; Jansen, 1973), have not been detected in biological fluids (Kuenzle, 1970; McDonagh & Assisi, 1972; Jansen, 1974; Heirwegh *et al.*, 1975), but small amounts of the non-α-isomers of bilirubin-IX occur in body fluids (Petryka, 1966; O'Carra & Colleran, 1970; Tipton & Gray, 1971; Blanckaert *et al.*, 1977). Biliverdin has been demonstrated in fowl bile (With, 1968; Cornelius *et al.*, 1975) and in the bile of amphibia (Lester & Schmid, 1961); its presence in mammalian bile has not been documented unequivocally. Therefore studies of the conjugation of bile pigments in normal mammals can be focused largely on the synthesis and biliary secretion of glycosidic conjugates of bilirubin-IXα.

Before undertaking detailed investigations of the conjugative metabolism of bilirubin-IXα it is desirable to know whether or not clearly different conjugation patterns are found among animal species. Scattered observations support this view (Fevery *et al.*, 1972*c*; Wong, 1972; Thompson & Hofmann, 1973; Cornelius *et al.*, 1975). In the present study of 11 species, biliary bile-pigment composition showed striking differences with regard to the ratio of mono- to di-conjugated bilirubin-IXα and to the types and relative amounts of conjugating groups. Assays in liver and kidney homogenates of the activities of

bilirubin-IXα–UDP-glycosyltransferases also demonstrated considerable species variation and suggested correlation with the composition of biliary bile pigments.

Materials and Methods

Animals

All animals were adult males. Rats from the Wistar R/A strain, NMR1 mice, DHP guinea pigs and mongrel cats and dogs were used. Two rabbits were from the Small Hollander strain and one from the Flemish Giant strain. The other animals were those available at the local market.

Bile analysis

Samples of gall-bladder bile from pigs, sheep and oxen were obtained at the slaughterhouse and stored immediately at −20°C. In smaller animals, gall-bladder bile was obtained during surgery under Pentothal or ether anaesthesia, and analysed as soon as possible, or stored at −20°C in the dark for 1–2 days.

Hepatic bile was collected after catheterization of the common bile duct. For the rat, only the fraction delivered after normalization of the body temperature (Fevery *et al.*, 1972*c*) was used. Samples of bile were collected in dark bottles placed on ice. They were analysed immediately or stored at −20°C for 1–2 days. Before analysis, the samples were diluted 10–100-fold with 0.9% NaCl and treated at room temperature (20–25°C) for 30min with diazotized ethyl anthranilate at pH2.7 (Van Roy & Heirwegh, 1968).

Determination of the azopigment composition

The azopigment extracts were submitted to t.l.c. and the relative amounts of azopigments α_0, α_1, α_2, α_3, γ and δ were determined by densitometry (Fevery *et al.*, 1971). The azopigments obtained from dog bile served as a chromatographic reference mixture (Table 1; Fevery *et al.*, 1971). The ratio of monoconjugated to total conjugated bilirubin-IXα was calculated from the ratio of azopigment α_0 to the sum of azopigments α_0, α_1, α_2, α_3, γ and δ (Heirwegh *et al.*, 1974).

Enzyme assays

Liver tissue from normal man and from dogs was obtained during surgery. The livers of other species were excised immediately after killing and exsanguination. Homogenates containing 250mg of liver/ml of suspension were prepared at 0°C in 0.25M-sucrose containing 1mM-EDTA (disodium salt). The enzyme preparations were incubated as such or were previously activated by treatment for 30min at 0°C with 1vol. of digitonin solution (20mg/ml for the mouse, 10mg/ml for man, and 12mg/ml for the other species). In the present work the optimal digitonin concentration for human liver was one-half of the value reported previously (Black *et al.*, 1970). This different behaviour may be because the preparation of digitonin at present in use could be solubilized completely by heating on a water bath, whereas that used previously was only obtained as a fine suspension.

With bilirubin-IXα as the acceptor substrate UDP-glucuronyltransferase activity (Heirwegh *et al.*, 1972) and UDP-glucosyl- and UDP-xylosyl-transferase activities (Fevery *et al.*, 1972*a*) were assayed by the procedures described in these references. With UDP-glucuronic acid the pH of incubation was 7.4, except for rat and mouse, where it was 7.8. The incubation time was 10 and 15min with UDP-xylose, 15 and 20min with UDP-glucuronic acid and 30 and 40min with UDP-glucose. The final concentrations, in the incubation mixtures, of UDP-glucuronic acid, UDP-xylose and UDP-glucose were 2.54, 2.54 and 23.7mM respectively. After enzymic incubation the reaction mixtures were treated with diazotized ethyl anthranilate and the azopigment colour was measured photometrically (Heirwegh *et al.*, 1972). T.l.c. of the azopigments followed by densitometric scanning was applied for evaluating the ratio of monoconjugated bilirubin-IXα to total conjugates synthesized (Fevery *et al.*, 1972*b*). When the absorption difference (A_{test}–$A_{control}$) was smaller than 0.010 the complete extract (2ml) was applied as a single spot to a thin-layer plate and analysed by t.l.c. to ascertain whether

Table 1. *Structures of ethyl anthranilate azo derivatives*

Symbol	Structure	Source	References
α_0	Azodipyrrole	Bilirubin-IXα; human and dog bile	Compernolle *et al.* (1970, 1971); Jansen & Stoll (1971)
α_2	Azodipyrrole D-xylopyranoside	Dog bile	Compernolle *et al.* (1971)
α_3	Azodipyrrole β-D-glucopyranoside	Dog bile	Compernolle *et al.* (1971); Gordon *et al.* (1974)
		Human bile	J. Fevery (unpublished work)
δ	Azodipyrrole β-D-glucopyranuronoside	Rat bile	Compernolle *et al.* (1970, 1977)
		Human bile, dog bile	Gordon *et al.* (1976)

any conjugated azopigment could be detected or not. Renal enzymes were assayed as for liver enzymes, but incubation periods of 1–2h had sometimes to be used. Usually, only material from the cortical zone was investigated.

Results and Discussion

To allow a proper appreciation of the aspects of bilirubin-IXα metabolism investigated, a brief assessment of the experimental methods is desirable.

Observations on experimental methods

Bile-pigment composition of bile and of enzymic incubation mixtures. Bile has been shown to contain a variety of mono- and di-conjugates of bilirubin-IXα (Heirwegh *et al.*, 1975; Noir, 1976). Ideally, analysis should proceed by direct separation of the tetrapyrrolic pigments. Unfortunately quantitative determination is not yet feasible (Thompson & Hofmann, 1973; Heirwegh *et al.*, 1975). Alternative analysis of dipyrrolic azo derivatives, in general, does not allow one to calculate the composition of the parent bile pigments. However, two important indices can be obtained: (*a*) the relative amounts of conjugating groups, and (*b*) the ratio of monoconjugated bilirubin-IXα to total conjugates.

(*a*) Formation of the ethyl anthranilate azopigments proceeded at 20–25°C at pH2.7. They were extracted with pentan-2-one and separated by t.l.c. with neutral solvent systems. These relatively mild conditions minimize the risk of hydrolysis of conjugating bonds.

The chromatograms were evaluated primarily in terms of the chromatographic components α_0, α_1, α_2, α_3, γ and δ (Table 2). The structures of the predominant azopigments α_0, α_2, α_3 and δ are known for the species man, dog and rat (Table 1). For the other species, the four azopigments mentioned had the same colours and R_F values as the corresponding reference compounds. Enzymic incubation of liver homogenates, from any of the species examined, with bilirubin-IXα and UDP-xylose, UDP-glucose or UDP-glucuronic acid yielded azopigments α_2, α_3 and δ respectively.

The structure of azopigment α_1 is of little concern, as this component was obtained only in trace amounts (Table 2). The γ-azopigment from human bile is an ester conjugate of azodipyrrole containing hexuronic acid (Heirwegh *et al.*, 1970). Other azopigments, such as β_x, which are derived from non-α-isomers of bilirubin-IX (Blanckaert *et al.*, 1976, 1977), have been disregarded. No attempt was made to measure azodipyrrole sulphate, which might be present in azopigment δ (Noir & Nanet, 1974).

(*b*) The ratio of monoconjugated bilirubin-IXα to total conjugates is calculated from the fraction of azopigment α_0 expressed as the percentage of the sum of unconjugated (α_0) and conjugated azodipyrroles (α_1, α_2, α_3, γ and δ) (Table 2). The analytical requirements for these determinations have been discussed in some detail elsewhere (Heirwegh *et al.*, 1973, 1974). One particular point may be mentioned: if azopigment α_0 is derived exclusively from monoconjugated bilirubin-IXα, then the α_0-fraction must lie between the values 0% (only diconjugates) and 50% (only monoconjugates). The most likely source of additional azopigment α_0 is bilirubin-IXα. It cannot arise from non-α isomers of bilirubin-IX (Blanckaert *et al.*, 1976). After enzymic incubation, t.l.c. of the control mixtures allows correction for azodipyrrole derived from the larger excess of acceptor substrate (Fevery *et al.*, 1972*b*). The uncertainty, by this method, of the calculated ratio of monoconjugated bilirubin-IXα to total conjugates is 5–10%. In analysing bile samples, straightforward correction is not possible. Fortunately, in fresh hepatic bile of normal man and rats the concentration of unconjugated bilirubin-IXα is so low that even complete reaction with the diazo reagent would not measurably increase the fraction of azodipyrrole (Fevery *et al.*, 1972*c*). Semiquantitative assays for a number of other animals also indicated low ratios of unconjugated to conjugated bilirubin-IXα (Thompson & Hofmann, 1973).

In general, the values of the α_0-fraction were within the theoretical range 0–50%, except for gall-bladder bile of the chicken, the guinea pig and the sheep, for which the α_0-fraction values were 54, 53 and 57% respectively (Table 2). Slight overestimation for gall-bladder bile of the α_0-fraction is suggested by parallel analyses on both hepatic and gall-bladder biles (cat, chicken, guinea pig). This could be due to partial deconjugation in the gall bladder and promotion of diazo reaction by substances such as bile salts. Their concentration in diluted hepatic bile could be too low to be effective.

Enzymic conjugation of bilirubin-IXα. Both mono- and di-conjugates can be formed. The diazotization procedure used in the present work assays the total amount of conjugates. Synthesis *in vitro* of the diconjugates probably occurs via the monoconjugates (Heirwegh *et al.*, 1973):

$$\text{Bilirubin-IX}\alpha \xrightarrow{1} \text{bilirubin-IX}\alpha\text{ monoconjugate} \xrightarrow{2} \text{bilirubin-IX}\alpha\text{ diconjugate}$$

If so, one would measure the rate of step 1. Partial reconversion of monoconjugate into bilirubin-IXα would lead to underestimation of the velocity. The ratio of diconjugate to total conjugate synthesized reflects the relative importance of step 2.

Comparison of enzymic activities as in the present work is limited by the fact that the assay conditions used may not have been optimal for each species

Table 2. *Azopigment composition obtained for bile samples of various animal species*

The structures of the major azopigments (α_0, α_2, α_3 and δ) are defined in Table 1. BM, the percentage of conjugated bilirubin-IXα which is monoconjugated is obtained by multiplying the amount of unconjugated azodipyrrole, α_0 (expressed as a percentage of the sum of azopigments α_0, α_1, α_2, α_3, γ and δ) by a factor of two. Samples from individual animals were analysed, except for the mouse, where a pool of 30 bile samples was tested. When more than two analyses are available the percentage of total azo colour is expressed as the average value ±1 S.D. tr, Trace amounts; n.d., not detected.

Source of bile				Percentage of total azo colour						Percentage of total conjugated azopigment				
Species	No. of samples	BM	Azopigment ...	α_0	α_1	α_2	α_3	γ	δ	α_1	α_2	α_3	γ	δ
Duodenal bile														
Man	8	22		11±4	tr	1±0.5	4±1	9±3	75±6	tr	1	4	11	83
Hepatic bile														
Rat	6	38		19±1	tr	tr	tr	6±1	72±2	tr	tr	tr	8	92
Cat	1	16		8	tr	0.4	11	20	59	tr	0.4	12	22	65
Guinea pig	3	90		45±4	tr	n.d.	2±1	10±2	43±3	tr	n.d.	4	17	78
Chicken	1	84		42	tr	6	20	11	20	tr	11	35	19	35
Gall-bladder bile														
Dog	10	18		9±2	2±1	9±2	28±2	9±2	43±4	2	10	31	10	47
Rabbit	1	84		42	tr	6	17	6	29	tr	10	29	10	50
Mouse	30	88		44	n.d.	n.d.	7	tr	49	n.d.	n.d.	12	tr	88
Ox	3	100		50±3	n.d.	n.d.	n.d.	16±1	34±3	n.d.	n.d.	n.d.	32	68
Pig	4	100		50±6	tr	n.d.	2±1	15±5	33±8	tr	n.d.	4	30	66
Sheep	2	110, 116		55, 58	n.d.	n.d.	n.d.	3, 3	41, 39	n.d.	n.d.	n.d.	7	93
Cat	2	22, 24		11, 12	3, 4	0, 1	20, 21	16, 14	49, 45	4	1	24	17	54
Guinea pig	1	106		53	tr	n.d.	2	10	35	tr	n.d.	3	22	75
Chicken	2	106, 108		53, 54	3, 2	8, 2	16, 14	8, 15	12, 14	5	10	30	23	26

and tissue examined. Indeed, except with regard to pH, the incubation conditions were those established for rat liver (Fevery *et al.*, 1972*a*; Heirwegh *et al.*, 1972). However, optimal conditions for assaying bilirubin-IXα–UDP-glucuronyltransferase activity of human (Black *et al.*, 1970) and rat liver are similar. In the present comparative assays, minor quantitative differences may escape attention, qualitative and major quantitative differences being evident. It should be stressed that t.l.c. of the azopigment extracts is frequently necessary to ascertain whether conjugates have really been formed.

Biliary bilirubin-IXα conjugates

Conjugating groups. Clear-cut species differences were demonstrated with regard to the nature and amount of conjugating groups (Table 2). In all species examined, azopigment δ predominated, except for the chicken, where azopigment α_3 was about equally important. For man, dog and rat, azopigment δ corresponds nearly exclusively to azodipyrrole β-D-monoglucuronide (Table 1).

Azopigment α_3 obtained from human or dog bile has been identified as azodipyrrole β-D-monoglucoside (Table 1). It amounted to 29–35% of the conjugated azopigment for bile of the chicken, the dog and the rabbit, 12–24% for bile of the cat and the mouse, and only 0–4% for bile of the other species. According to Cornelius *et al.* (1975), azopigment α_3 is the only conjugated azodipyrrole obtained from snake bile. Appreciable amounts of azopigment α_2 (azodipyrrole monoxyloside; dog bile) were only obtained from bile of the chicken, the dog and the rabbit in a molar ratio azopigment-α_2/azopigment-α_3 close to 1:3. The sum of both azo derivatives, presumably containing only neutral glycosides in all cases, ranged from 0 to 46% of total conjugated azopigment.

Azopigment α_1 was of minor importance. Various γ-azopigments, assayed together, amounted to 0–20% of total azo colour. A greenish pigment, probably biliverdin (Garay *et al.*, 1965), was present in bile of the chicken, the turkey and the rabbit. According to Lin *et al.* (1974) bilirubin and biliverdin represent 6 and 94% respectively of biliary bile pigments in the chicken.

Mono- and di-conjugated bilirubin-IXα. In bile of normal adults, and of the dog and the cat, monoconjugated bilirubin-IXα represented only about 20% of total conjugated bilirubin-IXα (Table 2). About twice as much was found in normal rat bile, and 80–90% in fresh hepatic bile of the chicken and the guinea pig. Similar values were obtained when gall-bladder bile of other species was examined. As explained above, the latter analyses may have yielded somewhat high values, owing to a possible systematic error affecting the assays with gall-bladder bile. However, there can be little doubt that seven species (chicken, guinea pig, mouse, ox, pig, rabbit, sheep) out of eleven excrete bilirubin-IXα predominantly as monoconjugates.

Bilirubin-IXα–UDP-glycosyltransferase activities

Activities of hepatic transferases. In general, enzyme activities were comparable with, or slightly higher than, those reported by other workers (for a review see Fevery *et al.*, 1976). Quantitative differences among species were quite important (Table 3). For the rat, the activity of digitonin-activated UDP-glucuronyltransferase was 50–60-fold higher than for the chicken. The very low values found for the latter species appear to be biologically significant, as small amounts of conjugated bilirubin-IXα are present in bile (Table 2; Lin *et al.*, 1974; Cornelius *et al.*, 1975). A striking species difference appeared when the ratio activated enzyme/untreated enzyme was determined for four species (Table 4). The value was 14 ± 3 (S.D., $n=6$) for the rat and only 2.6 ± 0.5 (S.D., $n=4$) and 1.8 ± 0.5 (S.D., $n=3$) for the mouse and the guinea pig respectively. In two assays with human liver the ratio was 3.8. As within this group of four species the activities of untreated enzyme were comparable, digitonin apparently activates bilirubin-IXα–UDP-glucuronyltransferase in rat liver rather specifically. The value of the pH optimum distinguished the rat and mouse from several other species. In activated homogenates from liver of man, and of guinea pig, the pig, the horse, the ox and the sheep, UDP-glucuronyltransferase activity was higher at pH7.4 than at pH7.8. For the mouse and the rat the pH optimum was at 7.8 (the present work) and at 7.96 (Heirwegh *et al.*, 1972) respectively.

Although still important, species variation was less pronounced when either UDP-glucose or UDP-xylose was used as the sugar donor instead of UDP-glucuronic acid (Table 3). Apparently this is due, at least to a large extent, to the fact that digitonin caused relatively unimportant stimulation of the glucosyl- and xylosyl-transfer rates with homogenates from liver of the rat (Table 4). Similar activation ratios were found for liver preparations from the mouse and the guinea pig, in contrast with the assays with UDP-glucuronic acid. The ratio of glucuronyl/glucosyl transfer rates varied from 1 for the rabbit, the chicken and the cat to about 20 for the sheep (Table 3).

Formation in vitro of mono- and di-conjugated bilirubin-IXα. With UDP-xylose as the sugar donor. diconjugate formation was important when digitonin-activated liver homogenates from man, and from the dog, the cat and the rat were tested (Table 5), but could not be demonstrated for the other species. Qualitative agreement with the predominance in bile of either monoconjugates or mixtures of mono-

Table 3. *Bilirubin-IXα–UDP-glycosyltransferase activities in digitonin-activated liver homogenate*
The enzyme activities were determined as outlined in the Materials and Methods section. When three or more liver samples were assayed, results are given as the average values (±S.D.) for the numbers of samples given in parentheses. For mouse liver homogenate the final digitonin concentrations in the incubation mixture were 6mg/ml (*) and 10mg/ml (†).

	Bilirubin conjugated (nmol/10min per g of liver)			Bilirubin conjugated (nmol/min per g of protein)			Ratio of transfer rates (glucuronyl/ glucosyl/ xylosyl)
Sugar donor ... / Source of liver	UDP-glucuronic acid	UDP-glucose	UDP-xylose	UDP-glucuronic acid	UDP-glucose	UDP-xylose	
Rat	731±116 (6)	213±37 (3)	318±38 (3)	424±63	124±19 (3)	209±28 (3)	1:0.3:0.4
Man	530±125 (3)	37±5 (3)	330±38 (3)				1:0.07:0.6
Mouse	154±51 (3)*	73, 76	82, 99	79, 124	44, 56	60, 60	1:0.5:0.6
	242±61 (4)†			167±47 (4)			
Horse	193	176	397				1:0.9:2
Pig	164±11 (3)	42±3 (3)	72±3 (3)	141±10 (3)	35±3 (3)	61±3 (3)	1:0.3:0.4
Ox	124, 192	16, 26	72, 106	84, 110	10, 16	45, 66	1:0.14:0.6
Sheep	117±14 (3)	6±1 (3)	37±5 (3)	76±10 (3)	4±0.5(3)	24±3 (3)	1:0.05:0.3
Guinea pig	94±38 (4)	6, 11	10, 15	32, 40	6, 8	3, 8	1:0.1:0.1
Rabbit							
Flemish Giant	156	211	304	97	71	138	1:1.3:1.9
Small Hollander	69, 82	59, 79	110, 129				1:0.9:1.6
Calf	54, 66	2, 6	36, 40	42, 60	2, 5	31, 36	1:0.08:0.6
Dog	59±1 (4)	25±6 (4)	59±5 (4)	34±1 (4)	15±3 (4)	35±2 (4)	1:0.4:1
Cat	40, 42	35, 52	92, 107	39, 34	26, 39	68, 83	1:1.1:2.4
Chicken	7, 21	5, 21	5, 28	4, 11	3, 11	3, 15	1:1:1

Table 4. *Bilirubin-IXα–UDP-glycosyltransferase activities in liver homogenate at various concentrations of digitonin*
Homogenates containing 250mg of liver/ml of suspension were diluted with 1 vol. of sucrose solvent or of digitonin solution and kept for 30min at 0°C before the enzyme assays (see the Materials and Methods section). The sucrose solvent contained 0.25M-sucrose in 1mM-EDTA (disodium salt). The concentration of digitonin in the homogenate/digitonin mixtures is given in the Table. When three or more livers were assayed, results are given as the average values (±1S.D.) for the numbers of samples given in parentheses.

Species	[Digitonin] (mg/ml)	Sugar donor ...	Bilirubin conjugated (nmol/10min per g of liver) UDP-glucuronic acid	UDP-glucose	UDP-xylose	Ratio of transfer rates (glucuronyl/ glucosyl/xylosyl)
Rat	0		55±10 (6)	83±22 (3)	149±36 (3)	1:1.5:2.7
	6		731±116 (6)	213±37 (3)	318±38 (3)	1:0.3:0.4
Mouse	0		93±20 (4)	67, 72	85, 157	1:0.7:1.2
	6		154±51 (3)	74, 77	83, 99	1:0.5:0.6
	10		242±61 (4)			
Guinea pig	0		53±18 (3)	11, 15	6	1:0.2:0.1
	6		55, 133	10, 11	14	1:0.2:0.2
	10		94±38 (4)	32, 32	11, 15	1:0.3:0.1
Man	0		86, 162	54, 66	176, 286	1:0.5:1.9
	5		324, 622	30, 47	227, 340	1:0.1:0.6

and di-conjugates of bilirubin-IXα thus was excellent. Except for the rat, the parallelism persisted when UDP-glucuronic acid was used as the donor substrate. Our apparent inability to synthesize significant amounts of bilirubin-IXα diglucuronide with liver preparations from the rat of our inbred colony (Wistar R/A rats) is intriguing. Attempts with a variety of activation and incubation conditions have failed so far (Van Roy & Heirwegh, 1968; Fevery *et al.*, 1972*b*), although in similar assays synthesis of diconjugate was achieved for another inbred strain of Wistar rats (Strebel & Odell, 1971) and for Fisher rats (Halac *et al.*, 1972). By storage experiments, Halac *et al.* (1972) demonstrated that the diconjugate-forming enzyme activity is much more labile than the activity responsible for synthesis of monoconjugate. We therefore suggest that in our experiments, during preparation and/or incubation of

Table 5. *Monoconjugated bilirubin-IXα present in bile and synthesized in vitro by digitonin-activated liver homogenate from species that excrete predominantly diconjugates*

For synthesis *in vitro*, ranges are indicated, as the results depend on incubation time and substrate concentration (Fevery *et al.*, 1972*b*). For bile, results are given as average values (±1 S.D.) (for the number of samples in parentheses) when more than two samples were analysed. DB, duodenal bile, HB, hepatic bile, GB, gall-bladder bile.

		Monoconjugate (expressed as a percentage of total conjugate)			
		Enzymic synthesis			
Species	Sugar donor ...	UDP-glucuronic acid	UDP-glucose	UDP-xylose	Bile
Man (DB)		60–80	90	60–70	22±8 (8)
Dog (GB)		20–60	90	0–60	18±4 (10)
Cat (HB)		46–75	85	85	16
(GB)					22,24
Rat (HB)		90–100	90–100	60	38±2 (6)

Table 6. *Digitonin-activated bilirubin-IXα–UDP-glucuronyltransferase activity in kidney sections and in liver*

Homogenates from various parts of the kidney (50mg of tissue/ml of suspension) were treated with 1 vol. of digitonin solution (10mg/ml). The mixtures were then kept for 30min at 0°C and then assayed as described in the Materials and Methods section. Liver homogenates were treated with digitonin and assayed enzymically as described in the same section.

	Bilirubin-IXα conjugated (nmol/10min per g of tissue)			
		Kidney		
Species	Liver	Subcortical area	Medulla area	Papilla region
Dog	51	40	12	0
Rat	530	265	37	0

liver homogenates, an essential part of the diconjugate-forming apparatus is lost or destroyed.

Renal UDP-glycosyltransferase(s). By assays *in vitro*, bilirubin-IXα–UDP-glucuronyltransferase activity has been detected in tissues from kidney, digestive tract and brain of the guinea pig and the rat (Grodsky & Carbone, 1957; Tenhunen & Torsti, 1959; Arias *et al.*, 1960; Stevenson & Dutton, 1962). Homogenates from the cortical, medulla and papilla areas of the kidney, and also liver homogenates, obtained from one dog and one rat were assayed enzymically (Table 6). In the kidney, UDP-glucuronyltransferase activity was confined largely to the cortical region. Histological examination showed that the sample assayed consisted mainly of proximal-tubule cells. In twelve species, homogenates from liver and from the subcortical region were assayed in parallel for conjugation with glucuronic acid, glucose and xylose (Table 7). Except for preparations from kidney of the dog and the rat, conjugation was barely measurable. No activity could be detected in homogenates of kidney cortex from man, and from the calf, the cat and the ox. With preparations from rat kidney (two experiments), synthetic xyloside contained 42 and 46% as diconjugated material, but no significant amounts of diglucoside or diglucuronide were formed. In contrast, with cortex preparations from dog kidney the diglucuronide amounted to 56%±8 (S.D., $n = 3$) of total glucuronide synthesized.

General comments

Analysis of biliary bile pigments offers a simple and reasonably accurate method for comparing the conjugation of bilirubin-IXα in animal species. The near-absence of conjugated bilirubin-IXα from normal urine and serum suggests that, after synthesis in the hepatocyte, the conjugates are excreted directly into the next compartment, the bile canaliculus. Therefore the bile-pigment composition is expected to reflect rather closely the relative transferase activities *in vivo*, provided that the bile pigments are not altered after their synthesis or re-absorbed preferentially in the bile canaliculus.

In our survey of eleven species, seven excreted 80% or more of conjugated bilirubin-IXα as monoconjugates. In contrast, monoconjugates represented only 20% in bile of man, the dog and the cat and 38% in rat bile. The parallelism with the composition of conjugates synthesized *in vitro* with liver homogenates (Table 5) suggests that the conversion of mono- to di-conjugates *in vivo* is largely inoperative in some species.

The results shown in Fig. 1 illustrate the tendency of the glucoside/glucuronide ratio for bile to increase with the ratio of glucosyl/glucuronyl transfer rates found for liver. In particular, the data for species that form predominantly monoconjugates (Fig. 1, full

Table 7. *Digitonin-activated UDP-glycosyltransferase activities in subcortical material from the kidney*

For each animal, homogenates from liver and from subcortical material of the kidney (250mg of tissue/ml of suspension) were preincubated at 0°C for 30min with 1vol. of digitonin solution (12mg/ml) and assayed enzymically (see the Materials and Methods section). For preparations from the kidney, enzyme activities are given as average values (±1S.D.) when more than two animals were tested. Similar ratios were found for three rats when the bilirubin-IXα concentration in the incubation mixtures was 0.26mM instead of 0.13mM used in the present work. 0, Absence of conjugated bilirubin-IXα verified by t.l.c.

Species	No. of animals	Sugar donor	Bilirubin-IXα conjugated (nmol/10min per g of kidney) ... UDP-glucuronic acid	UDP-glucose	UDP-xylose	Ratio of enzyme activities (kidney/liver) UDP-glucuronic acid	UDP-glucose	UDP-xylose
Rat	4		58±14	45±5	36±7	0.27	0.34	0.26
Dog	2		40, 59	23, 28	37, 40	0.95	0.50	0.35
Rabbit	1		7	5.6	10	0.04	0.02	0.03
Horse	1		8	20	22	0.04	0.10	0.05
Sheep	1		0.4	0.08	0.15	0.004	0.01	0.004
Chicken	2		0	10, 11	9, 11	0	0.50	0.33
Guinea pig	6		5.6±0.2	0	0			
Pig	1		0.07	0	0			
Cat	3		0	0	0			
Man	4		0	0	0			
Calf	2		0	0	0			
Ox	2		0	0	0			

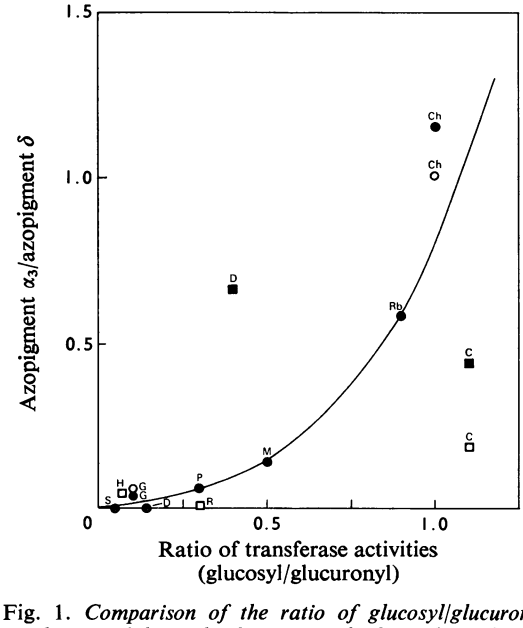

Fig. 1. *Comparison of the ratio of glucosyl/glucuronyl residues in bile with the ratio of glucosyltransferase/glucuronyltransferase activities in liver*

Species: C, cat; Ch, chicken; D, dog; G, guinea pig; H, human adult; M, mouse; ○, ox; P, pig; R, rat; Rb, rabbit; S, sheep. Predominant excretion of monoconjugated (○, ●) or diconjugated bilirubin-IXα (□, ■); hepatic bile (○, □) or gall-bladder bile (●, ■). The values were calculated from Tables 2 and 3 respectively.

line) support the idea that the biliary composition is closely related to the enzyme activities. Obviously, in the dog, the rabbit and the chicken, excretion of nearly 40% of bilirubin-IXα conjugates as neutral glycosides (Table 2) demonstrates that conjugation with neutral sugars may be quite important in animals. Predominant excretion of bilirubin-IXα glucoside in snake bile is suggested by work of Cornelius *et al.* (1975). This pathway is not unique for bilirubin-IXα, as steroid glucosides have been isolated from rabbit urine (Layne, 1970) and as some foreign substances are excreted largely in the form of glucosides by the dog (Millburn, 1976).

Several observations with enzyme preparations from rat liver are compatible with the catalysis of transfer of glucosyl and glucuronyl residues to bilirubin-IXα by different enzymic sites. Differences in pH optimum and in stabilization by albumin have been reported (Fevery *et al.*, 1972*a*). Treatments perturbing lipid structure (Marniemi, 1974; Abou-El-Makarem & Bock, 1976), including the effect of digitonin reported in the present work, also demonstrate a marked difference between both transferase activities. However, a single enzymic site with affinities that differ for the UDP-sugars and that are affected differently by perturbation of the surrounding lipid would not contradict the observations.

The significance of renal conjugation of bilirubin-IXα remains difficult to assess. The dog and the rat showed the highest enzyme activities *in vitro* (Table 7), but in general, renal conjugation *in vivo* is found

Table 8. *Comparison of the biliary transport maximum of bilirubin-IXα with the digitonin-activated bilirubin-IXα–UDP-glycosyltransferase activities in liver*

The enzyme activities are taken from Table 3. The values of T_m, the biliary transport maximum of bilirubin-IXα, have been reported in the literature. Both variables are expressed as nmol of bilirubin-IXα conjugated or excreted/10min per g of liver.

Species	Sugar donor ...	Enzyme activity: UDP-glucuronic acid	Enzyme activity: UDP-xylose	T_m	References
Rat		731	318	538	Van Damme & Desmet (1969)
Mouse		242	91	147	Roberts & Plaa (1969)
Sheep		117	37	26	Upson *et al.* (1970)
Dog		59	59	62	Goresky *et al.* (1974)
Chicken		14	16	9	Lin *et al.* (1974)

under quite abnormal conditions. These involved the use of the isolated kidney or of the whole animal after hepatectomy (Schoenfield *et al.*, 1961; Royer *et al.*, 1965, 1974; De Schepper & Van der Stock, 1972; Franco *et al.*, 1972) or of transplantation of normal rat kidneys into the Gunn rat (Foliot *et al.*, 1975). Under these conditions, urinary secretion of conjugated bilirubin-IXα has been demonstrated in the dog (Barac, 1969; Heirwegh & Barac, 1970; De Schepper & Van der Stock, 1972), but not in the rat. Whether or not renal conjugation of bilirubin-IXα occurs at normal serum concentrations cannot be decided as yet. However, it is likely at increased serum concentrations of unconjugated bilirubin-IXα. It is noteworthy that both ligandin (Kirsch *et al.*, 1975) and the transferases (Table 6) seem to be confined to the proximal-tubule cells of the kidney and that these cells show marked changes and accumulation of bile pigment in experimental bile-duct ligation (De Vos *et al.*, 1972*a*, *b*).

Several authors have proposed that biliary secretion is rate-limiting at high bilirubin loads because of reflux of conjugated bilirubin-IXα into serum (Weinbren & Billing, 1956; Arias *et al.*, 1961; Bloomer & Zaccaria, 1976). Robinson *et al.* (1971) compared the 'apparent' bilirubin transport maximum (T_m) for bilirubin-IXα with the activity in liver of bilirubin-IXα–UDP-glucuronyltransferase for a number of Sprague–Dawley (*JJ*) and heterozygous Gunn rats (*jJ*). A linear relationship was found over a wide range of values when the individual T_m values were plotted against the corresponding transferase activities. Obviously, as noted by Robinson *et al.* (1971), conjugation and biliary secretion of bilirubin-IXα must be closely related. Comparison of the reported T_m values for various animals with UDP-glucuronyltransferase activity (the present work) suggests that a similar relationship holds (Table 8).

We are indebted to Dr. J. De Groote for his constant support, to Dr. R. Kerremans, Dr. F. Penninckx, Dr. E. Fossion, Dr. M. Dochez, Dr. J. De Schepper and Dr. J. Van der Stock for providing liver and bile samples, to Dr. B. Van Damme for help with histological examinations, and to Mr. P. Leroy for skilful technical assistance. We also thank the Nationaal Fonds voor Wetenschappelijk Geneeskundig Onderzoek for financial support.

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