Comparison of the Biliary Excretion of the Four Isomers of Bilirubin-IX in Wistar and Homozygous Gunn Rats

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The biliary excretion of the four isomers of bilirubin-IX was studied in Wistar rats (JJ) and homozygous Gunn rats (jj). Synthetic preparations of ^{14}C -labelled pigments were used. 1. After intravenous administration, the α -isomer was rapidly excreted in conjugated form in bile of Wistar rats. In Gunn rats excretion was insignificant. In contrast, both rat species promptly excreted the non- α -isomers at rates that were comparable with that found for bilirubin-IX α in Wistar rats. 2. In normal rats about 16% of the β - and δ -isomers and at least 50% of the γ -isomer were excreted as ester conjugates of the injected parent bile pigments. Conjugation of the β - and δ -isomers had occurred exclusively at the carboxyl groups of pyrrole ring D and C respectively. For bilirubin-IX γ no preference for any carboxyl group could be established. 3. In homozygous Gunn rats the non- α -isomers were apparently excreted chemically unaltered. This suggests that, as for bilirubin-IX α , conjugation of the non- α -isomers is also deficient in Gunn rats.

In most mammals degradation of haem leads predominantly to the formation of bilirubin-IX α (Gray *et al.*, 1972; Lathe, 1972) with trace amounts of the β -, γ - and δ -isomers (Petryka, 1966; O'Carra & Colleran, 1970; Blanckaert *et al.*, 1977). Apparently, efficient excretion of the α -isomer is possible only after previous transformation into more polar conjugates (Heirwegh *et al.*, 1976). Even the Gunn rat, which shows a severe conjugation defect with regard to bilirubin-IX α (Carbone & Grodsky, 1957), with concentrations in blood that are about 100-fold higher than in normal rats, excretes only a minor fraction of degraded haem as unconjugated bilirubin-IX α (Schmid & Hammaker, 1963; Blanckaert *et al.*, 1977). Apparently, this behaviour is related to its apolar character, but is unexpected, since two carboxyl groups are present in the bile pigment. Probably this originates primarily from the involvement of these groups in the formation of multiple intramolecular hydrogen bonds (Blauer *et al.*, 1972; Kuenzle *et al.*, 1973; Manitto *et al.*, 1974; Knell *et al.*, 1975; Bonnett *et al.*, 1976) inhibiting their ionization.

Practical synthesis of the non- α -isomers has become possible only recently, and the chromatographic and solvent distribution properties of these compounds indicate that they are more polar than bilirubin-IX α (Blanckaert *et al.*, 1976). This suggested that the mode of excretion of the non- α -isomers of bilirubin-IX may be different from that of bilirubin-IX α . This possibility was tested by synthesizing all four isomers from ^{14}C -labelled haemin and injecting them individually into Wistar rats and homozygous Gunn rats.

Our results showed that all non- α -isomers, in contrast with bilirubin-IX α , were excreted rapidly and at comparable rates by Wistar and Gunn rats and that whereas the latter excreted these isomers exclusively in unconjugated forms, in Wistar rat bile both conjugated and unconjugated derivatives of these isomers were found.

Materials and Methods

Chemicals

Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co. (Eastbourne, Sussex, U.K.) and δ -amino-[4- ^{14}C]laevulinic acid (53 mCi/mmol) from The Radiochemical Centre (Amersham, Bucks., U.K.). The other chemicals were as specified in Blanckaert *et al.* (1976).

Animals

Male Wistar R/A rats (JJ) and homozygous Gunn rats (jj) weighing 300–400 g were used. The latter animals are characterized by an apparently complete hereditary deficiency of bilirubin-IX α UDP-glucuronyltransferase activity (Carbone & Grodsky, 1957). Only minute amounts of conjugated bilirubin-IX α could be detected in Gunn rat bile (Blanckaert *et al.*, 1977). Further details about our local colony have been given in the same paper.

Preparation of radioactively labelled compounds

Work with bile pigments was done in dim light. ^{14}C -labelled haemin was prepared biosynthetically by incubating δ -amino-[4- ^{14}C]laevulinic acid with

reticulocyte haemolysates from phenylhydrazine-treated chick (Zaman & Akhtar, 1976). Immediately before administration to rats the four [^{14}C]-bilirubin-IX isomers were synthesized from [^{14}C]-labelled haemin and purified by t.l.c. by the procedures of Blanckaert *et al.* (1976) with the following modifications. (a) Reduction of biliverdin dimethyl esters to bilirubin dimethyl esters with NaBH_4 was performed in methanol solution containing 0.2–0.25 ml of acetic acid or 1–2 ml of glycine/HCl buffer [prepared by adjusting a 0.4M-HCl solution to pH2.7 with solid glycine at room temperature (20–22°C)]; (b) if the green colour of biliverdin reappeared after alkaline hydrolysis of bilirubin dimethyl esters, a freshly prepared solution of NaBH_4 (5–6 mg) in 1 ml of methanol was added to the incubation mixture 15–30 s before acidification.

Purified pigment bands obtained by t.l.c. were eluted with methanol which was evaporated *in vacuo* to near-dryness. Finally the still 'wet' residue was dissolved in 1–2 ml of a solution of bovine serum albumin (10 mg/ml) in iso-osmotic 0.9% NaCl. After a brief centrifugation at 1000 $g_{av.}$, 0.1 ml of the supernatant solution was withdrawn for measuring radioactivity and a known volume (0.7–1.9 ml) of the remainder was injected into rats.

Of the four bilirubin-IX isomers, the extinction coefficient of only the α -isomer is known (Fog, 1964). Therefore the specific radioactivity of this compound alone was determined (219 d.p.m./nmol). For the remaining isomers it was assumed that owing to their identical content of [^{14}C] radioactivity and their identical molecular weight, the specific radioactivities of all the isomers would be the same as that of bilirubin-IX α .

Preparation of experimental animals

Administration of radioactively labelled bilirubin and collection of bile samples. The rats were anaesthetized by intraperitoneal injection of pentobarbital (sodium salt; Nembutal from Abbott, Saint Rémy sur Avre, France; 4–5 mg/100 g body wt.). The left jugular vein and the common bile duct were cannulated with a polyethylene catheter (internal diam. 0.3 mm; length 40 cm). A flat contact thermometer (Thermistor 421 in conjunction with a Telethermistemp 73 TK, from Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) was inserted subcutaneously with the receptor site against the abdominal muscle wall. After surgery the animals were transferred to restraining cages and placed in containers with thermostatically controlled humidified air. Throughout the experimental period the body temperature of the rats was kept at $38 \pm 0.5^\circ\text{C}$. When the animals had recovered fully from the anaesthesia (usually after 4–6 h) a solution of the [^{14}C]-labelled pigment (40–50 nmol) was injected rapidly (within

60 s) into the jugular vein. Bile was collected in pre-weighed tubes placed on ice. Collection proceeded over 20 min periods for 1 h and over 30 min periods for the next 2 h. Over the next 8–13 h, bile was collected in a single tube. Bile flow was determined gravimetrically.

Radio assays

A portion (0.1 ml) of bile contained in polyethylene counting vials was diluted with water (0.25 ml) to prevent complete evaporation and the samples were bleached under eight 20 W Vita-Lite fluorescent lamps mounted in a reflecting canopy at a distance of 20 cm for 10 h. After the addition of 10 ml of Instagel (Packard Instrument Co., Warrenville, IL, U.S.A.) the vials were capped, shaken vigorously and kept overnight in darkness at 4°C . The samples were counted for radioactivity for 10 min or for 100 000 counts in a Packard Tri-Carb liquid-scintillation spectrometer model 2450, and the observed count rates were corrected by use of a quench curve. The standard error of the count rates varied between 1 and 4% and the efficiency of counting was 80–87%.

Localization and estimation of the relative amounts of radioactive material in the chromatograms were done as follows. The chromatograms were divided into consecutive rectangular zones of 1 cm $\times$ 2 cm. The silica gel in these zones was scraped into 10 ml centrifuge tubes and eluted with 3 $\times$ 1 ml of methanol. Each time, after brief centrifugation, the supernatants were removed and combined in corresponding vials. The contents of the vials were evaporated to about 0.5 ml and after addition of 10 ml of Instagel they were counted for radioactivity in the liquid-scintillation spectrometer.

Analysis of bile pigments

After samples for measurement of radioactivity had been taken, the remaining material was combined and stored at -20°C for a maximum of 1 week. Azo derivatives were formed by treatment with diazotized ethyl anthranilate at pH 6.0 (Blanckaert *et al.*, 1977). The approaches used to establish the structures of unconjugated (Blanckaert *et al.*, 1976) and conjugated bilirubin-IX pigments (Heirwegh *et al.*, 1974) have been described in detail in the publications mentioned. For the present purpose it will suffice to summarize briefly some essential points.

(1) The purity of the synthetic [^{14}C]-labelled bilirubin-IX isomers was established by t.l.c. with respect to structurally known reference compounds. A single yellow component moving with the expected R_F values was observed in each case. Subsequently,

Scheme 1. Structures of bilirubin-IX isomers and of their dipyrrolic ethyl anthranilate azo derivatives. A typical pair of dipyrrolic azo derivatives is obtained by treatment of each of the isomers of bilirubin-IX with diazotized ethyl anthranilate. The nomenclature used is as described previously (Blancaert *et al.*, 1976). Compounds IA and IB correspond respectively to the so-called vinyl and isovinyl isomers of azodipyrrole derived from bilirubin-IX α (Jansen & Stoll, 1971). R represents H in the unconjugated pigments, CH_3 in the methyl ester derivatives and a conjugating residue in the conjugated pigments. The letters A, B, C and D in the five-membered rings of the bilirubin-IX isomers refer to the nomenclature used for the parent protoporphyrin-IX.

formation of the characteristic pairs of dipyrrolic azo derivatives (Scheme 1) was also demonstrated.

(2) The structures of the excreted bile pigments were verified only by azopigment analysis. Two major points have been considered: (a) whether the labelled azo derivatives contained the dipyrrole moieties expected from the formulae of the injected parent tetrapyrroles (Scheme 1); and (b) if any of them were present in conjugated forms.

By t.l.c. with respect to known reference compounds establishment of the structures of unconjugated azopigments is straightforward. Azopigments IID and IVG obtained from bilirubin-IX β and -IX δ respectively were rechromatographed in two discriminating solvent systems: benzene/ethyl acetate (9:1, v/v) and benzene/hexane (1:1, v/v). Owing to their pronounced lability (Blanckaert *et al.*, 1976; Compennolle *et al.*, 1976) and small amounts, further derivative formation was not feasible. The identity of the other azopigments was established by t.l.c., both as the free acids and as the methyl ester derivatives (Blanckaert *et al.*, 1976).

Ester conjugates of dipyrrolic azopigments are easily detected by treatment with methanolic NaOH (Heirwegh *et al.*, 1974), the conjugating residues being replaced by methyl ester groups. T.l.c. of the reaction products then establishes (a) the nature of the dipyrrolic moiety, and (b) the point(s) of attachment of conjugating residues. Reference preparations of the various methyl esters of the azopigments shown in Scheme 1 are available (Blanckaert *et al.*, 1976).

Results and Discussion

Rates of excretion of the isomers of [¹⁴C]bilirubin-IX in Wistar and Gunn rats

Intravenous administration of [¹⁴C]bilirubin-IX α (100–350 nmol) to Wistar rats led to a rapid excretion of most of the label within 2 h. After 11 h about 90% of the injected label could be accounted for in the bile. This is in marked contrast with Gunn rats which under parallel conditions excreted only 3–6% of total radioactivity in 11 h (Fig. 1a). These results are in good agreement with previously published data (Lester & Klein, 1966).

The non- α -isomers were excreted readily by both Wistar and Gunn rats at rates that were comparable with those found for bilirubin-IX α in the Wistar rat (Fig. 1). In experiments done in parallel, similar patterns were found for both species when each of the non- α -isomers was compared (Figs. 1b–d). After 11–16 h of excretion, recovery of label obtained after injection of β -, γ - and δ -isomer equalled 80–100%. The curves indicate delayed excretion of the β -isomer.

Characterization of excreted bile pigments

The amount of labelled bile pigments excreted during these experiments was small and it is well established that bile pigments are labile. Therefore bile was treated with diazotized ethyl anthranilate to yield dipyrrolic azopigments which in addition to being stable are well suited for analysis on a micro-scale (Heirwegh *et al.*, 1974). The reaction was carried out under mild conditions at near-neutral pH, thus rendering losses of groups bound in acid-labile linkage unlikely. However, modification of the tetrapyrrole skeleton rendering either of the two dipyrrole halves diazo-negative will not be detected by azopigment analysis.

The distribution of radioactivity in the chromatograms is shown in Figs. 2(a)–2(d) and Table 1. It was found that after injection of each of the bilirubin-IX isomers into Wistar or Gunn rats, the bile from corresponding rats gave the expected pattern of azopigments as predicted from Scheme 1. In Wistar rats, after injection of bilirubin-IX α , 21% of the excreted radioactivity was contained in the unconjugated isomeric compounds IA and IB (Scheme 1) which are derived from the unconjugated dipyrrole halves of monoconjugated bilirubin-IX α (Heirwegh *et al.*, 1976), whereas the greatest amount of radioactivity (73%) was located in the conjugated fraction.

After administration of the β - and δ -isomers, the azopigment pattern obtained from both Wistar and Gunn rats was identical, except that in Wistar rats a small percentage of both isomers (Table 1) was excreted in conjugated forms. Alkaline methanolysis of the conjugated azopigment fractions yielded exclusively monomethyl esters of compound IIC (ring D) and IVC (ring C). Therefore the parent

Fig. 1. Biliary excretion of radioactivity after intravenous administration of ¹⁴C-labelled bilirubin-IX isomers to Wistar (○) and Gunn rats (●)

(a), (b), (c) and (d) Excretion of radioactivity after injection of ¹⁴C-labelled bilirubin-IX α , -IX β , -IX δ and -IX γ respectively. The mean $\pm$ s.d. (vertical bars) and the number of experiments for each of the curves are given in parentheses. In Wistar rats, 83 $\pm$ 6 (3) and 80 $\pm$ 6 (3) % of injected α - and β -isomers respectively was recovered in bile after 11 h; for the γ - and δ -isomers recoveries after 16 h were 99 $\pm$ 1 (3) and 93 $\pm$ 6 (4) % respectively. In Gunn rats 4.5 $\pm$ 0.8 (4) and 80 $\pm$ 13 (3) % of administered α - and β -isomer respectively were excreted in bile after 11 h; after 16 h recoveries of 101 $\pm$ 2 (3) and 90 $\pm$ 11 (4) % of injected label were found respectively for the γ - and δ -isomers.

Fig. 2. Distribution of ${}^{14}\text{C}$ radioactivity in the chromatograms of azo derivatives prepared from bile of Wistar and Gunn rats after intravenous injection of [${}^{14}\text{C}$]bilirubin-IX

Ethyl anthranilate azo derivatives were prepared from bile and separated by t.l.c. The chromatograms were divided into zones of $1\text{ cm} \times 2\text{ cm}$ and the radioactivity in the methanol eluate of the silica gel from each zone was determined. (a), (b), (c) and (d) Results for animals injected with bilirubin-IX α , -IX β , -IX δ and -IX γ respectively. Open bars, Wistar rats; shaded bars, Gunn rats. The unconjugated azopigments are denoted by symbols defined in Scheme 1, the conjugated pigments by f_1 and/or f_2 . In the case of injection of bilirubin-IX α into the Wistar rat, f_1 and f_2 correspond to the previously defined azopigments γ and δ respectively (Heirwegh *et al.*, 1974). The application line is indicated by S, and the solvent front by F.

tetrapyrroles were ester conjugates of bilirubin-IX β and -IX δ containing a single conjugating residue bound specifically to the carboxyl group of ring D and C respectively (Scheme 1). From the ratio of conjugated to unconjugated azopigments IIC (0.17) and IVC (0.15) it follows that about 16% of bilirubin-IX β and -IX δ were present in monoconjugated forms. On the other hand, whereas the γ -isomer was excreted unconjugated in Gunn rats, in Wistar rats it was excreted to a large extent in conjugated form (Table 1). Here again an ester linkage was

demonstrated by alkaline methanolysis. Since both isomeric methyl esters of azopigments IIIE and IIIF were obtained in comparable amounts, conjugation of either of the two carboxyl groups must have occurred. The fact that the ratio of conjugated/unconjugated azopigment is about 1 suggests excretion of predominantly monoconjugated bilirubin-IX γ or of a nearly equimolecular mixture of unconjugated and diconjugated bilirubin-IX γ .

In normal Wistar rats bilirubin-IX α is conjugated exclusively with glucuronic acid (Heirwegh *et al.*,

Table 1. Radioactivity in the various azopigment fractions obtained from the bile of rats after intravenous administration of [^{14}C]bilirubin-IX

The preparation of the chromatograms and the quantification of the ^{14}C radioactivity were done as described in the legend to Fig. 2. The symbols starting with Roman numerals refer to the formulae of unconjugated azopigments or to the aglycones* of conjugated azopigments (Scheme 1).

		Percentage of total radioactivity on t.l.c. plate							
Chromatographic compound ...	Nature of azopigment ... Bilirubin isomer	Unconjugated		Unconjugated			Conjugated*		Sum
		IID	IVG	(IA+IB)	(IIIE+IIIF)	IIC(IVC)	f ₁	f ₂	
Strain									
Wistar	IX α	—	—	21	—	—	13 (IA+IB)	60 (IA+IB)	94
Wistar	IX β	19	—	—	—	53	—	11 (IIC)	83
Gunn	IX β	18	—	—	—	58	—	—	76
Wistar	IX γ	—	—	—	44	—	25 (IIIE+IIIF)	22 (IIIE+IIIF)	91
Gunn	IX γ	—	—	—	64	—	—	—	64
Wistar	IX δ	—	36	—	—	40	—	7 (IVC)	83
Gunn	IX δ	—	33	—	—	50	—	—	83

1976). Because of lack of material it was not possible to identify the conjugating groups demonstrated in the non- α -isomers.

Recovery of excreted radioactivity in azopigments

Recovery of excreted radioactivity in the azopigment extracts from bile of both strains was estimated to be more than 80%. When this radioactivity was applied to the thin-layer plates, 79–91% was recovered in the major azopigment fractions (Table 1). These values may represent lower estimates. Probably, in the β - and δ -isomers, part of the radioactivity present in the labile divinyl-substituted dipyrrole halves was not accounted for. Treatment of each isomer of bilirubin-IX with diazotized ethyl anthranilate yields two dipyrrolic azopigments (Scheme 1). Since ^{14}C radioactivity is equally distributed in all pyrrolic moieties of the bilirubins, it is expected that each dipyrrolic azopigment would contain 50% of the total radioactivity in the parent compound. Poor recoveries were observed for azopigments IID (18–19%) and IVG (33–36%) which are derived from the IX β and IX δ isomers respectively. This discrepancy could be explained by the pronounced lability (Blanckaert *et al.*, 1976; Compernelle *et al.*, 1976) and lower reactivity (during diazo-coupling) of the dipyrrole residues containing two vinyl and two methyl groups as β -substituents. The reason for the relatively poor recovery (64%) as azopigment of radioactivity in bile of Gunn rats injected with bilirubin-IX γ is unclear.

General comments

The biliary excretion of low-molecular-weight organic substances is determined by biological

factors such as species, sex and pathophysiological conditions and by the chemical nature of the excreted compounds. A relatively high molecular weight (above 300 in the rat) and an amphiphilic structure appear to be essential requirements for extensive biliary excretion (Millburn, 1976). The latter two rules have been established by studying a large number of xenobiotics and appear to apply as well to natural substances such as bile acid salts and steroid conjugates.

Considering the formulae of the four bilirubin-IX isomers (Scheme 1), efficient biliary excretion would be expected. As shown in the present work the β -, γ - and δ -isomers of bilirubin-IX are indeed excreted promptly into the bile of Wistar and Gunn rats. However, only a small fraction of the injected bilirubin-IX α appeared in the bile of Gunn rats, thus confirming previous results (Lester & Klein, 1966). In Wistar rats excretion is rapid, but only after previous conjugation (Lester & Klein, 1966), predominantly with glucuronic acid (Talaftant, 1956; Billing *et al.*, 1957; Schmid, 1957; Heirwegh *et al.*, 1976). As Gunn rats show a severe deficiency in the glycosidic conjugation of bilirubin-IX α (Carbone & Grodsky, 1957; Fevery *et al.*, 1972; Blanckaert *et al.*, 1977), it seems evident that the α -isomer must be conjugated in order to be excreted rapidly.

In accordance with the relatively pronounced polarity of the non- α -isomers (Blanckaert *et al.*, 1976), their carboxyl groups are probably completely ionized at physiological pH values. The surprising finding that bilirubin-IX α cannot be excreted efficiently without previous conjugation can be understood if one considers the conformation of the isomers. Multiple intramolecular hydrogen bonds in the α -isomer causing efficient shielding of the polar

character of the carboxyl groups and of the NH-CO groups of pyrrole rings A and B have been strongly suggested by the work of Blauer *et al.* (1972), Kuenzle *et al.* (1973), Manitto *et al.* (1974), Knell *et al.* (1975) and Bonnett *et al.* (1976). On the other hand, model building showed that such bonds cannot be formed for the non- α -isomers. In contrast with the non- α -isomers the introduction of a polar group in bilirubin-IX α would thus be required to open the hydrogen-bonded structure and to acquire the amphiphilic character appropriate for biliary excretion.

Lack of excretion of mesobilirubin-IX α in Gunn rats and the need for conjugation before excretion in Wistar rats (Lester & Klein, 1966) probably reflect the same tendency to hydrogen-bonding of the unconjugated pigment as has been documented for bilirubin-IX α . Although spatial models of i-urobilin and mesobilirubinogen are not available, it is likely that their rapid excretion in unconjugated form, both by Gunn rats and Wistar rats (Lester & Klein, 1966), is due to the absence of hydrogen-bonding of the carboxyl groups.

Although conjugation apparently is not in itself required for efficient biliary excretion, a significant fraction of the non- α -isomers of bilirubin-IX was excreted as ester conjugates in Wistar rats (Table 1). Specific conjugation of only one of both carboxyl groups of the β - and δ -isomers supports an enzymic process. For the α -isomer a close link between conjugation and secretion is suggested by the linear relationship between the activity of hepatic bilirubin-IX α UDP-glucuronyltransferase and the maximum rate of excretion (Robinson *et al.*, 1971). Apparently, for the non- α -isomers such a link is absent. Similarly, dissociation of metabolic conversion and secretion has been demonstrated for xenobiotics (Levine *et al.*, 1970) and cholic acid (O'Maille *et al.*, 1965).

Conjugated derivatives of non- α -isomers could not be detected in bile from Gunn rat. This suggests that the hereditary inability to form glycosidic conjugates of bilirubin-IX α also extends to the non- α -isomers.

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