Urobilinogen-i is a major derivative of bilirubin in bile of homozygous Gunn rats

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INTRODUCTION
In mammals and rodents, haem is catabolized predominantly to unconjugated bilirubin-1Xa. The pigment is taken up by hepatocytes and conjugated with glucuronic acid, glucose or xylose, and the glycosides are excreted in bile. Subsequent deconjugation and partial conversion into uroobilinogens is catalysed by enzymes released by the anaerobic colonic flora, mainly present in the large intestine. The bile pigments are then eliminated in the stools to a small extent as unconjugated bilirubin and largely as urobilinogens; a small part undergoes absorption with enterohepatic circulation and re-secretion in bile or excretion in urine (Fevry & Blanckaert, 1990).

When a deficiency of microsomal bilirubin UDP-glucuronosyltransferase prevents conjugation and efficient biliary excretion (Blanckaert et al., 1977a), the unconjugated bilirubin accumulates in the organism, as is seen in the Crigler–Najjar syndrome, in neonatal jaundice or in Gunn rats. The levels of hyperbilirubinemia in Gunn rats remain quite constant over a long period of time suggesting that bilirubin is removed from the organism at the same rate as it is formed.

Alternative pathways of pigment metabolism and disposition thus seem to play an important role in this species. Previous investigations suggested that bilirubin is converted mainly into more polar and diazo-negative metabolites (Schmid & Hammaker, 1963), which are excreted predominantly in bile and to a lesser degree in urine (Schmid, 1963). In addition, small amounts of hydroxylated bilirubins have been detected in bile (Berry et al., 1972; Blanckaert et al., 1977b). Furthermore some bilirubin might be broken down by mitochondrial bilirubin oxidase (Brodersen & Bartels, 1969; Yokosuka & Billing, 1987) or by a microsomal system (De Matteis et al., 1989). The nature of the polar diazo-negative bilirubin derivatives remained largely unknown. Direct transport of unconjugated bilirubin across the intestinal wall into the faeces has also been suggested as an alternative pathway (Schmid & Hammaker, 1962).

Clarification of the alternative route would be useful for the understanding of both the normal and disturbed bilirubin metabolism. Therefore the chemical characterization of the so-far-undetermined alternative bilirubin metabolites excreted in bile of Gunn rats was the subject of the present study.

MATERIALS AND METHODS

Chemicals

[14C]Bilirubin was prepared from 5-amino [14C]laevulinic acid by the method of Ostrow et al. (1961). Labelled bilirubin was recrystomatographed in t.l.c. solvent system B (see below under 'T.l.c. analysis') before injection. The colourless uroobilinogen (Scheme 1) was prepared by reduction of the pinkish-yellow or orange urobilin-i with 20% (w/v) FeSO4 in 2.5% (w/v) NaOH (Henry et al., 1961). Bilirubin IX and urobilin-i were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Vitamin B-2 was from Merck A.G. (Darmstadt, Germany). Other chemicals used were of pro analysis or h.p.l.c.-grade purity.

Animals

Male homozygous Gunn (j/j) and Wistar RA (J/J) rats (Leyten et al., 1986) weighing 250–350 g were used. The activity of bilirubin UDP-glucuronosyltransferase in these Gunn rats is undetectable (Fevry et al., 1972; Leyten et al., 1986) and the concentration of unconjugated bilirubin in serum is about 170 µmol/l (Blanckaert et al., 1977b). Under sodium pentobarbital anaesthesia (6 mg/100 g body wt. given intraperitoneally) the rats were provided with an external biliary catheter and a jugular-vein catheter. The animals were placed in restraining cages and kept in an incubator to keep their body...
temperature constant at 37 °C. Bile was collected in 2 h periods for 2 days, in tared tubes placed on ice in the dark. The oxidation of pigments was prevented by collection of the bile under a layer of paraffin oil and an Ar atmosphere. Samples were processed immediately after collection. Biliary loss of water and electrolytes was compensated for by intravenous infusion of 5% (w/v) glucose in 0.15 M-NaCl. Some rats were injected with a solution of [14C]bilirubin (90 nmol with specific radioactivity 13000 d.p.m./nmol) sterilized through a 0.22 μm-pore-size Millex-GV filter (Millipore Corp., Bedford, MA, U.S.A.) 16 h after operation. The labelled pigment was firstly dissolved in 0.1 ml of 0.1 M-NaOH before addition to 2 ml of Wistar-rat serum. Approx. 2% of the injected label was recovered in the first 2 h bile collection. In experiments to suppress the gut microflora, neomycin (10 mg/100 g body wt. per day) was administered in a chew for 6 days. To obtain a more complete suppression of the gut microflora a combination of bacitracin and neomycin was used (1 g of each per litre of drinking water) and the rats were kept in sterile cages provided with sterile bedding, water and food. In another attempt to confirm the intestinal origin of urobilinogen two Gunn rats each provided with an external biliary catheter and a jugular-vein catheter were kept in restraining cages. After 12 h bile collections were started over 1 h periods. After 3 h the distal part of the small intestine and the total large intestine were ligated and resected. In these animals bile collection proceeded for a further 3 h under continuing anaesthesia.

Methods

As far as possible all manipulations were done under dim light in the dark.

Quantitative determination of urobilinogen. Urobilinogen was oxidized by 0.05 M-I2 in the presence of 1% (w/v) zinc acetate in dimethyl sulphoxide. The absorption spectrum of the zinc complex of urobilin formed was measured in a spectrophotometer in the region 440–540 nm (P. Kotal & J. Fevery, unpublished work).

H.p.l.c. analysis. The apparatus was purchased from Waters Associates (Milford, MA, U.S.A.), with exception of the integrator (M390A, from Hewlett-Packard, Avondale, U.S.A.). It was composed of an automated gradient controller (M680), solvent delivery system (M501 and M510), intelligent sample-

Scheme 1. Structures of bile pigments

Reductive conversion of bilirubin (Mr, 584) into urobilinogen-i (or mesobilirubinogen; Mr, 592) and dehydrogenation to urobilin-i (Mr, 590) is shown. Side chains consist of methyl (M), vinyl (V), ethyl (E) and propionic acid (P) groups.

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For analytical separation a 3 μm-particle-size C18 column with a length of 83 mm and an internal diameter of 4.6 mm was used. For preparative separation a 5 μm-particle-size Altex Ultrasphere ODS column (Beckman Instruments, Fullerton, CA, U.S.A.) with an internal diameter of 10 mm and a length of 250 mm was used (Spivak & Yue, 1986). A guard column packed with 35–70 μm-particle-size μBondapak C18 and an LC pre-column filter (Walters Associates) were fitted to the column.

Bile was injected without preparation in amounts up to 2 ml, and the pigments were separated by eluting the column at a rate of 2 ml/min with a linear gradient starting off with 25% (v/v) solution A (0.04 M-sodium acetate in methanol) and 75% (v/v) solution B (1% sodium acetate in water, pH 4.5), to 40% solution A in 5 min and from 40% solution A to 100% solution A in 30 min, and then maintaining the solution at 100% solution A for an additional 5 min (gradient I). Alternatively, a gradient of 0% solution A/100% solution B to 100% solution A in 20 min was used, followed by maintenance of 100% solution A for an additional 5 min (gradient II). The column was equilibrated for 5 min before each separation. Fractions (1 mm) were collected and used for radioactivity counting or for spectral analysis.

Extraction of pigment. Fractions from 20 h.p.l.c. separations (40 ml of crude bile) were collected and evaporated under vacuum at ambient room temperature. The residue was re-extracted with chloroform in the presence of glycine/HCl buffer (0.4 M-HCl adjusted to pH 2.4 with solid glycine) with a 93% recovery. This sample was used for chromatographic and spectral determination. Methyl esters of pigments were prepared with diazomethane (Fuhrkop & Smith, 1975).

T.l.c. analysis. The organic extracts obtained by h.p.l.c. were applied to t.l.c. plates (DC-Kieselgel F254, 5715/0025; Merck A.G.) and the pigments were separated by development with pyridine/butan-1-ol/water (1:1:1, by vol.) (system A), with methanol/water/pyridine (4:1:4, by vol.) (system B) (Lester & Schmid, 1965), with toluene/ethanol (25:2, v/v) (system C) (Stoll & Gray, 1970) or with chloroform/methanol/conc. NH3 (1000:150:7, by vol.) (system D) (Vermeir et al., 1984).

After development, silica-gel bands were scraped off the t.l.c. plates and the pigments were eluted with 2 ml of methanol (Fevery et al., 1983). The eluates were used for radioactivity counting or for spectral analysis.
Identification of urobilinogen in Gunn-rat bile

Paper chromatography. The organic extracts were applied on a strip of Whatman no. 1 paper and the pigments were separated by development with octan-1-ol/ethyl acetate (1:1, v/v) (system E) saturated with NH₄O₂ or with methanol/butan-1-ol/conc. NH₄O₂ (1:3:2, by vol.) (system F). The urobilins are characterized by the following \( R_f \) values: urobilin-d, \( R_f \) 0.92 (system E) and \( R_f \) 0.87 (system F); urobilin-1 (stercobilin), \( R_f \) 0.44 (system E) and \( R_f \) 0.81 (system F); urobilin-2 \( R_f \) 0.02 (system E) and \( R_f \) 0.58 (system F) (Royer et al., 1964).

Spectral analysis. Absorption spectra of pigments were measured on an SP8-250 UV/VIS spectrophotometer (Pye-Unicam, Cambridge, U.K.) and fluorescent spectra on an SPF-500 Ratio Spectrofluorometer (Aminco, Silver Spring, MD, U.S.A.) with excitation at 506 nm (Lloyd & Weston, 1982).

Radioassays. Preparation of crude samples of excreta for measurement of \(^{14}C\) radioactivity were carried out as described by Blanckaert (1980), and h.p.l.c. fractions were directly mixed with 10 ml of scintillation cocktail (Insta-Gel; Packard Instrument Co., Downers Grove, IL, U.S.A.).

RESULTS

Characterization of the main bilirubin derivative as urobilinogen-1

From Gunn-rat bile a variety of pigments can be directly separated by this newly developed reverse-phase h.p.l.c. (Fig. 1), but only few have an absorption maximum about 400 nm (Figs. 1b and 1c). Some of these can be considered as alternative bilirubin metabolites, assuming that they contain at least one conjugating link between two pyrrole units. A yellow pigment giving green-yellow fluorescence (\( R_f \) 17.2 min), was isolated. Its absorption spectrum was identical with that of riboflavin (vitamin B-2), as also observed previously by Ostrow et al. (1974). Its identity was further verified by injection of standard vitamin B-2 in the h.p.l.c. system. It occurs in both Gunn-rat and Wistar-rat bile, and its yellow-green fluorescence is characteristic for crude bile. It may be noted that there were no substantial differences between the chromatograms of Gunn-rat and RA-rat bile (results not shown) during monitoring at various wavelengths from 220 to 500 nm, with exception of the huge peak formed by bilirubin conjugates in bile of RA rats and an unidentified peak monitored at 490 nm in bile of Gunn rats (Fig. 1c).

The distribution of radioactivity in fractions separated from bile obtained from Gunn rats loaded with labelled bilirubin is depicted in Fig. 2. The results represent the means for ten injections of bile samples collected over 2 h periods from two Gunn rats within 2 days after the injection of \(^{14}C\)bilirubin. The labelled pigment appeared already in bile within the first 2 h collection after bilirubin injection, and there were no substantial changes either in bile-pigment composition or its concentration over the 2 days of collection. Most of the radioactivity was recovered in one pigment fraction (peak at 34 min). This fraction had an extreme instability, as can be seen from Fig. 3, depicting

Fig. 1. Preparative h.p.l.c. separation of bile pigments from Gunn rats

A 2 ml portion of crude freshly collected bile was directly injected in every run. Flow rate was 2 ml/min, gradient I. Detection was at (a) 275 nm, (b) 430 nm and (c) 490 nm. Standards used to identify some peaks consisted of vitamin B-2 (B-2; \( R_f \) 17.1), urobilin-i (U; \( R_f \) 33.5) and unconjugated bilirubin (UCB; \( R_f \) 45.7)

Fig. 2. Distribution of \(^{14}C\) radioactivity in h.p.l.c. fractions

Samples of crude bile obtained after injection of \(^{14}C\)bilirubin into a Gunn rat were analysed by h.p.l.c. (gradient I). Approx. 60% of radioactivity injected into the h.p.l.c. system was recovered in the fractions collected at 32-36 min.

Fig. 3. Distribution of \(^{14}C\) radioactivity (a) and pigments detected at 275 nm (b) in h.p.l.c. fractions

A sample of crude bile obtained as in Figs. 1 and 2 was thawed three times during the storage period and finally analysed by h.p.l.c. (gradient II).
the distribution of radioactive label in h.p.l.c. fractions (gradient II) of bile thawed three times within a 2 months storage period at −20 °C. The radioactivity was shifted to several fractions with shorter retention times, suggesting decomposition of the former pigment into various more polar derivatives.

The former fraction containing the major radioactivity was further identified. After spontaneous oxidation it showed a broad absorption maximum at 450 nm in neutral milieu (methanol), which was shifted after acidification with HCl (3% HCl in methanol) to a sharp maximum at 490.5 nm. Extraction with chloroform led to a maximum at 495 nm. These absorption properties are characteristic for urobilin-i XIa (Lightner, 1979; Billing, 1986). Re-subjection of the isolated fraction to h.p.l.c. revealed the same Rf. T.l.c. of this fraction obtained from preparative h.p.l.c. showed that more than 90% of the loaded radioactivity moved with the pigment absorbing at 490 nm (Table 1).

Addition of ZnII ions (5% zinc acetate in methanol) to the isolated pigments caused a further shift to 505.5 nm. The ZnII complex revealed a strong green fluorescence with a maximum at 512 nm, which could be suppressed by adding HCl. All these characteristics are similar to those of standard urobilin (Royer et al., 1964; Lester & Schmid, 1965; Lester & Klein, 1966; Lightner, 1979; Stoll, 1982).

Further identification was carried out by various chromatographic techniques, utilizing 73 μg of urobilin isolated from 40 ml of Gunn-rat bile by preparative h.p.l.c. in acidified methanol. This was evaporated and extracted into chloroform for subsequent analysis. Urobilin, secreted in its reduced and colourless form, i.e. urobilinogen, is partially oxidized during bile collection and during isolation. The separated fraction of the now oxidized urobilin is relatively stable. It is pinkish-yellow or orange owing to the presence of the central dipyrrmethene moiety (Fig. 1). The Rf of the secreted compound was identical with that of standard urobilinogen prepared by urobilin (Rf, 33.5 min) (Fig. 1c). The isolated fraction as well as its methyl ester had the same mobility as the commercial standard in all systems used (Table 2). Two systems of paper chromatography also showed that the isolated pigment had the same Rf as urobilin-i (Table 2). Urobilin-i is a racemic mixture and can even contain four stereoisomeric forms (Lightner, 1979).

Mechanisms of urobilinogen-i formation in Gunn rats

In further experiments the origin of biliary urobilinogen was investigated. Peroral administration of neomycin to Gunn rats decreased the biliary excretion of urobilinogen to 50% of control values. Total suppression of urobilinogen excretion was obtained by treating animals for 5 days with the combination of neomycin and bacitracin and keeping them in sterile conditions. Resection of the gut also stopped biliary excretion of urobilinogen (Fig. 4), though the excretion of bilirubin itself was only slightly influenced.

DISCUSSION

Previous investigators (Schmid, 1963; Schmid & Hammaker, 1962, 1963) observed diazo-negative labelled derivatives in bile and to some extent also in urine of Gunn rats after injection of radioactive bilirubin but these compounds escaped full identification. With our experience in both t.l.c. (Blanckaert et al., 1977b; Favery et al., 1983) and h.p.l.c. (Muraca et al., 1987) of tetrapyrrole analysis, we decided to tackle the pigments present in Gunn-rat bile. First a method for direct analysis of crude bile was developed in order to avoid decomposition of possible alternative metabolites of bilirubin. Since these metabolites proved to be extremely unstable in bile (e.g. compare Figs. 3 and 4), only a prompt separation using a non-oxidative method could give reliable results. H.p.l.c. techniques permit exclusion of light
and of O₂ and direct injection of crude bile without preceding preparation (Spivak & Yuey, 1986). The pronounced instability observed might at least partially explain the difficulty experienced previously in arriving at full identification (Schmid & Hammaker, 1962, 1963).

Analysis of bile of Gunn rats loaded with labelled bilirubin revealed one major labelled peak, which represents a more polar metabolite of bilirubin. This colourless compound rapidly underwent spontaneous oxidation to a pinkish-yellow pigment. Using preparative h.p.l.c. we prepared sufficient amounts of this pigment for subsequent characterization. Spectral analysis of the fully oxidized isolated fraction proved identity in absorption and fluorescence spectra with standard urobilin-i. Also, the chromatographic behaviours of the pigment and its methyl ester were identical with those of urobilin-i and its methyl ester in seven different chromatographic systems. Re-separation of the labelled fraction obtained by preparative h.p.l.c. allowed recovery of almost all radioactivity at the same Rₘ as the urobilin standard in a t.l.c. system. The compound secreted in bile thus corresponds to urobilinogen-i, which undergoes rapid and spontaneous oxidation to urobilin (Billing, 1986).

In normal rats urobilinogen is formed from biliary conjugated bilirubins predominantly in the large intestine (Stoll, 1982; Billing, 1986), but in unconjugated hyperbilirubinemia another pathway could be present. To confirm the intestinal origin of biliary urobilinogen, the gut microflora, supposed to be the source of enzymes catalysing urobilinogen formation, were suppressed by antibiotics; in addition, the intestine as a reservoir of urobilinogen was surgically withdrawn. Since no urobilinogen was excreted in bile after treatment with antibiotics or after resection of the intestine, an extra-intestinal source of urobilinogen seemed to be excluded.

The polar diazo-negative bilirubin derivatives previously observed in bile in unconjugated hyperbilirubinemia were considered to be the main alternative metabolites of bilirubin disposition (Schmid & Hammaker, 1963). Our study, however, showed that urobilinogen-i represents the major bilirubin derivative in freshly analysed bile. Our observation is supported by the so-far-unexplained observation by Schmid & Hammaker (1963), who described a strong diminution of bile secretion of diazo-negative derivatives in rats treated with neomycin. Since neomycin suppresses the formation of urobilinogen, a decrease in the enterohepatic circulation and thus of the biliary excretion of the bilirubin-derived urobilinogen can explain this observation.

Urobilinogen-i undergoes spontaneous oxidation to urobilin and, especially in bile, to other polar breakdown products (Fig. 3). The oxidation product urobilin is diazo-negative owing to the presence of the conjugating link between the central pyrrole units; a similar situation can be assumed for the other oxidation products of urobilinogen. Thus decomposition products of urobilinogen could represent a substantial part of the diazo-negative bilirubin derivatives found in bile in earlier studies (Schmid & Hammaker, 1962, 1963), especially if we take into account the fact that non-oxidative direct analytical methods were not available at that time.

Urobilinogen is formed in the large intestine from bilirubin, and subsequently a small fraction is absorbed and re-excreted, predominantly by the liver (Lester & Schmid, 1965). In Gunn rats there is no limit for the biliary excretion of urobilinogen, in contrast with the situation with bilirubin (Lester & Klein, 1966).

Since less than 2% of the radioactivity injected into Gunn rats was recovered over a 2 h period as urobilinogen, this route cannot account for an efficient elimination of pigment. Other routes of bilirubin disposal should thus be looked for besides biliary excretion. A direct passage of plasma bilirubin into the intestinal lumen as described by Schmid & Hammaker (1963) can at least partially represent such a route.

In conclusion, our study showed that urobilinogen represents a major metabolite of bilirubin in the bile in unconjugated hyperbilirubinemia of Gunn rats. It might also be present in other conditions of glucuronoyltransferase deficiency existing in man or in experimental animals.

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