

Excretion of Administered and Endogenous Photobilirubins in the Bile of the Jaundiced Gunn Rat

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ABSTRACT Radiolabeled photobilirubins, prepared *in vitro* by anaerobic illumination of [¹⁴C]bilirubin, were injected intravenously into homozygous jaundiced Gunn rats with an external bile fistula. With the animals kept in darkness, the labeled photobilirubins were excreted rapidly in bile. Photobilirubins IA and IB were excreted primarily as unconjugated bilirubin, whereas photobilirubin II was excreted primarily as photobilirubin II and not converted into bilirubin. Bile of Gunn rats given no exogenous pigments, but undergoing phototherapy, contained a large proportion of photobilirubin II and, if collected in liquid nitrogen, traces of photobilirubins I; neither was found in bile when these rats were kept in the dark. Because there is prior evidence that these photobilirubins are isomers of bilirubin, these experiments indicate that the major mechanism of phototherapy is photoisomerization of bilirubin. Photobilirubin II is the unidentified major photoderivative described previously, whereas formation of photobilirubins IA and IB, and their reversion to bilirubin-IX α , account for the remarkably increased output of the parent pigment during phototherapy.

INTRODUCTION

The formation of oxidative bilirubin derivatives by photosensitized singlet oxygen attack was initially postulated as an important mechanism for increased

catabolism and clearance of bilirubin during phototherapy of the Gunn rat (1-3). This is of considerable clinical relevance, since the Gunn rat is a good model for neonatal jaundice, which is now usually treated by phototherapy rather than the more hazardous exchange transfusion (4). However, it was soon appreciated that this mechanism could not explain excretion in bile of either unconjugated bilirubin *per se* (3, 5-8), or its major photoproduct (6), during phototherapy of the Gunn rat.

In the past 2 yr, we (9) and Onishi et al. (10, 11) have reported the preparation and characterization of two pairs of polar photobilirubins, formed by anaerobic illumination of bilirubin dissolved in chloroform, dimethyl sulfoxide (9), or human albumin solution (10, 11). The properties of these photobilirubins suggested that they were bilirubin isomers, and that they corresponded to the major bilirubin derivatives formed during phototherapy of jaundice *in vitro*. Possibly related *in vitro* photoproducts have been reported by Lightner et al. (12-14), though direct comparisons are not possible since they were unable to isolate and purify their derivatives (16). This paper supports the role of the photobilirubins in the catabolism of bilirubin during phototherapy of the Gunn rat by showing that they are excreted rapidly in bile when given intravenously to the Gunn rat, and by showing that they appear endogenously in bile collected during phototherapy of these jaundiced animals. In view of prior *in vivo* studies (6, 8, 10, 11, 13-16), this work suggests also that the major mechanism of increased bilirubin catabolism during phototherapy is probably photoisomerization, rather than photo-oxidation of bilirubin.

METHODS

Normal rat serum was prepared by centrifugation (900 g for 20 min) of blood drawn by cardiac puncture of 450-600 g male Sprague-Dawley rats under light ether anesthesia. All chemicals were analytical reagent grade.

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Preparation of bile pigments

[¹⁴C]Bilirubin was prepared biosynthetically from [4-¹⁴C] aminolevulinic acid (New England Nuclear, Boston, Mass.) administered intravenously to Sprague-Dawley rats (300–350 g) with a bile fistula. The labeled bilirubin was isolated from bile, crystallized, and its specific activity determined as described by Ostrow et al. (17). Specific activities ranged from 1,600 to 5,550 dpm/μg. The term "bilirubin," as used in this paper, refers to the natural IX α isomer in the 4Z, 15Z configuration (18).

Irradiated bilirubin. [¹⁴C]Bilirubin (500 μg), dissolved in 5 ml chloroform containing a trace of EDTA, was purged with 99.99% nitrogen for 10 min and then illuminated with a mercury lamp for 5 min in the apparatus described previously (9) for preparation of photobilirubins. After illumination, the solution was evaporated to dryness in a stream of nitrogen at 40°C, and the residue used for studies. Photobilirubins IA, IB, and II, both ¹⁴C-labeled and unlabeled, were isolated as described (9) by thin-layer chromatography of the products formed by anaerobic illumination of [¹⁴C]- or unlabeled bilirubin in chloroform or dimethyl sulfoxide solution, using the 100-W mercury spot lamp.

Solutions of bile pigments for injection

Bilirubin and irradiated bilirubin. The ¹⁴C-labeled pigment (250–350 μg), dissolved in dimethyl sulfoxide (200 μl), was added slowly with continuous mixing to 1.0 ml normal rat serum, and the clear yellow solution (1.2 ml) administered intravenously.

Photobilirubins I (A and/or B). Normal rat serum (1.0 ml) containing a trace of EDTA, was added to photobilirubin(s) I, freshly prepared from 1 mg unlabeled or [¹⁴C]bilirubin. After sonication for 5 min at 55 kHz and 125 W in an ultrasonic cleaner (model B-220, Branson Ultrasonics Corp., Shelton, Conn.), the suspension was centrifuged at 900 g for 5 min to remove undissolved material, and the clear yellow supernate administered immediately intravenously.

Photobilirubin II. 80–180 μg photobilirubin II was dissolved in 1 ml oxygen-free sodium phosphate buffer (0.1 M, pH 7.40) containing 1 mM disodium EDTA, and given intravenously without further preparation.

Studies with Gunn rats

Female, albino, homozygous, jaundiced Gunn rats, 250–400 g, were purchased from the Animal Colony, Skin and Cancer Hospital, Temple University Health Sciences Center, Philadelphia, Pa. Under anesthesia with intraperitoneal pentobarbital (5 mg/100 g), polyethylene catheters (PE-10, Intramedic, Clay-Adams Co., New York, N. Y.) were placed in the femoral vein for infusions and injections, and in the common bile duct for collection of bile. The catheters were painted black and encased in aluminium foil to shield the emerging bile from light. The rats were then placed in Bollman-type restraining cages, and infused with 2.5% dextrose-0.45% NaCl (wt/vol) at 1.8 ml/h. For experiments to study the excretion of exogenous photobilirubins, the rats were maintained in darkness. To study the excretion of endogenously formed photoderivatives, the rats were placed under a phototherapy unit (6, 8) which contained six Daylite fluorescent lamps (General Electric Co., Cleveland, Ohio), that delivered 3.2 mW/cm² of radiant flux at the skin surface in the range of wavelengths from 400–500 nm.

To attain a stable minimum biliary excretion of bile salts and bilirubin, bile flow was diverted for 15–18 h before commencement of the experiments (6). Bile, 1–2 ml/h, protected from light, was collected in iced tubes or directly into

liquid nitrogen as required. Collection tubes were changed every 1.5–2.0 h and kept on ice in the dark until analyzed on the same day. Serum was obtained by centrifugation of clotted blood withdrawn by incision of the tail vein during bile collections, and by cardiac puncture at the conclusion of each experiment.

Analysis of serum and bile pigments

Total diazo-reactive pigments were determined by the Michaelsson method (19), using 0.2 ml of bile or extracts, and 25 μl of serum. In serum, this was almost entirely unconjugated bilirubin (6, 8).

Unconjugated bilirubin in bile was determined by modified Folch solvent partition at pH 5.8 (2), followed by thin-layer chromatography of the lower (chloroform) phase on silica gel H (Analtech, Inc., Newark, Del.), developed with chloroform-acetic acid, 100:1 by vol (20). The bilirubin band was removed and quantitated as described by Boonyapisit et al. (21), by *in situ* reaction with diazotized *p*-iodoaniline, followed by spectrophotometric determination at 530 nm of the azopigments extracted into *n*-butyl acetate. Losses were corrected by external standardization.

Total labeled photobilirubins and their derivatives were extracted from bile into the lower phase of a Folch solvent partition (2), modified as a single extraction at pH 3.0. The upper phase was then extracted twice with 4 vol chloroform. The combined lower phases were washed with 1 vol water, and then taken to dryness under nitrogen at 40°C. The pigment residue was dissolved in chloroform-methanol (2:1 by vol) and applied to 0.5-mm thin layers of silica gel G (Analtech, Inc.). After development with chloroform-methanol-formic acid, 30:3:1 by vol (2), pigment bands were eluted individually with chloroform-methanol-water, 40:9:1 by vol, except for the origin material, which was eluted with methanol-aqueous ammonia, 9:1 by vol.

Photobilirubin I was extracted from Gunn rat bile drained directly into liquid nitrogen during phototherapy. After addition of chloroform (4 vol) containing 0.1 vol acetic acid and a trace of disodium EDTA, pigments were extracted by vigorous shaking and then centrifugation at 900 g for 2 min. The chloroform layer was washed twice with 1.2 vol water, and evaporated to dryness under nitrogen at 40°C. The residue, dissolved in chloroform-methanol (2:1 by vol), was applied to 0.5-mm thin layers of Camag, Inc. (New Berlin, Wis.) silica gel D-O containing disodium EDTA (100 mg/60 g dry gel), and developed with chloroform-methanol-water (40:9:1 by vol). The photobilirubin I band was eluted with the development solvent.

Radioassay. Eluates of bile pigments were placed in scintillation vials and dried under nitrogen at 40°C. Azopigment extracts were first decolorized by illumination with the mercury lamp after addition of 1 drop 30% H₂O₂, and then similarly taken to dryness. These residues, bile (200 μl), or serum (50 μl), were blended with 0.2 ml of 1 M hyamine in methanol (New England Nuclear, Boston, Mass.), and then 3.0 ml ethanol, 10 ml diphenyloxazole (8 g/liter) in toluene, and 2 drops of glacial acetic acid were added sequentially. Counting was performed to 10 K in a Packard Tricarb model 2650 liquid scintillation spectrometer at 87% efficiency, as determined by an external standard and channel ratios.

RESULTS

Excretion of exogenous photobilirubins by Gunn rats

Injection of labeled bilirubins (Figs. 1 and 2). Two test rats were each injected with 1.2 ml of serum con-

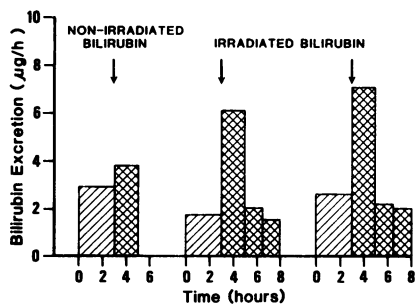


FIGURE 1 Excretion of bilirubin in the bile of Gunn rats given irradiated or unirradiated [^{14}C]bilirubin. After a 3-h base-line collection period, each animal received, as a single bolus, 350 μg i.v. of the labeled pigment, dissolved in 1.2 ml normal rat serum. Animals kept in dark throughout.

taining 350 μg irradiated [^{14}C]bilirubin, and one control rat with a comparable solution of unirradiated [^{14}C]bilirubin (5,500 dpm/ μg sp act for each). Thin-layer chromatography revealed that the irradiated bilirubin contained 81.7% bilirubin, 6.1% photobilirubins I, and 12.2% miscellaneous photoproducts. The injections raised the serum bilirubin levels by <23% above base-line values.

In the two test rats, 76 and 96% of the label injected as nonbilirubin photoproducts were recovered in the bile excreted during the next 5 h. Of this, 19 and 24% were converted to bilirubin, resulting in an increase in bilirubin outputs during only the 1st 2 h to 2.5 and 3.0 times the base-line excretion rates (Fig. 1). In the control rat, only 0.6% of the injected ^{14}C was excreted, of which only 4% was bilirubin, and bilirubin excretion increased by only one-third. During the 1st 2 h after injection, the specific activity of the bilirubin in the bile of the two test animals was 14 and 17 times that of the control animal, with values 70 and 86% of the

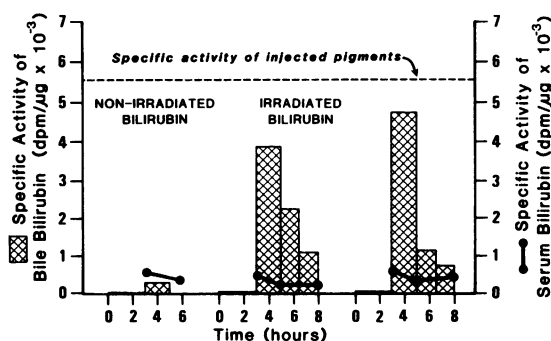


FIGURE 2 Specific activity of bilirubin in the bile of Gunn rats given irradiated or unirradiated [^{14}C]bilirubin (5,500 dpm/ μg sp act). Animals are the same as in Fig. 1. For each collection period, the specific activity of the bilirubin isolated from the bile (bars) is compared with the specific activity of the relevant serum bilirubin, and the injected bilirubin.

specific activity of the administered [^{14}C]bilirubins, but declined rapidly thereafter (Fig. 2). Moreover, the specific activity of the initial bile bilirubin of the test animals was at least 10 times greater, whereas that of the control animal was less, than the specific activity of the corresponding serum bilirubin.

Injection of unlabeled photobilirubins I (Fig. 3). During the 1st h after intravenous administration to a single Gunn rat of 1.0 ml rat serum containing 100 μg photobilirubins I, excretion of diazo-reactive compounds increased to 5.7 times, and of bilirubin to 148 times their respective base-line outputs in bile. These outputs returned to base-line values by 3 h after injection. By this time, correcting for the base-line excretion, 49% of the injected diazo-reactive pigment was recovered in the bile, with >90% of this in the form of bilirubin. During the 1st h, two-thirds, and during the 2nd and 3rd h, all the excess diazo-reactive compounds excreted were represented by bilirubin. The succeeding experiment with ^{14}C -labeled photobilirubins I showed that the injected material contained a proportion of bilirubin and traces of other polar products.

Injection of labeled photobilirubin IA and IB individually (Table I). One Gunn rat each was given 1.0 ml i.v. normal rat serum containing a purified preparation of [^{14}C]photobilirubin IA or IB, isolated by preparative thin-layer chromatography of irradiated [^{14}C]bilirubin. However, rechromatography of the administered pigments revealed that, during elution, evaporation, and dissolution in serum, all but 22.4% of photoisomer IA and 10.9% of isomer IB had reverted to [^{14}C]bilirubin and <2% of other polar products.

Of the total administered ^{14}C -pigments, 46.8% of preparation IA and 61.8% of preparation IB were re-

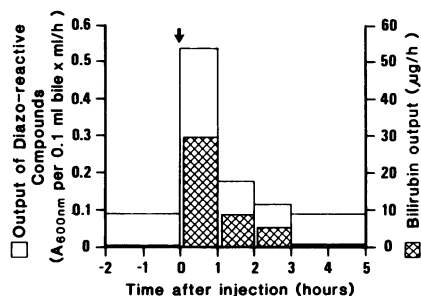


FIGURE 3 Excretion of bile pigments after administration of a preparation of photobilirubins I to a Gunn rat. Photobilirubins I, 100 μg , were given as an intravenous bolus (arrow) at time 0, after a 2-h base-line bile collection. Output of diazo-reactive compounds (left scale, open bars), determined by the Michaelsson method (19), is expressed as $A_{600\text{ nm}}$ per 0.1 ml bile \times bile volume (ml/h). It is scaled to be roughly equivalent to the bilirubin output (right scale, shaded bars), were all the diazo-reactivity due to bilirubin (for bilirubin, diazo A_{600} per 0.1 ml bile \times 9.61 = micrograms bilirubin per 0.1 ml bile).

TABLE I
Administration of [¹⁴C]Photobilirubins IA
and IB Intravenously to Gunn Rats

| | Preparation injected | |
|---|----------------------|-------|
| | IA | IB |
| Pigment administered, μg | 105.6 | 80.3 |
| As photobilirubin, μg | 23.7 | 8.75 |
| As photobilirubin, % | 22.4 | 10.9 |
| Specific activity, $\text{dpm}/\mu\text{g}$ | 1,606 | 1,606 |
| Pigment recovered in bile: | | |
| During first 2 h | | |
| Total, % injected ¹⁴ C | 30.7 | 43.5 |
| [¹⁴ C]Bilirubin, μg | 16.3 | 5.7 |
| % Excreted ¹⁴ C | 50.1 | 16.3 |
| % Injected photobilirubin | 68.8 | 65.4 |
| Specific activity, $\text{dpm}/\mu\text{g}$ | 1,201 | 1,197 |
| During 24 h | | |
| Total, % injected ¹⁴ C | 46.8 | 61.8 |
| [¹⁴ C]Bilirubin, μg | 16.8 | 5.84 |
| % Excreted ¹⁴ C | 34.0 | 11.8 |
| % Injected photobilirubin | 70.9 | 66.8 |
| Serum bilirubin specific activity, $\text{dpm}/\mu\text{g}$ | 14 | 11 |

Each preparation, dissolved in 1.0 ml normal rat serum given as an intravenous bolus to one rat at time 0. Animals kept in dark throughout study.

covered in the bile during the subsequent 24 h, of which approximately two-thirds was excreted during the 1st 2 h. Of the radioactivity administered as photoisomer per se, approximately two-thirds was recovered in the bile as [¹⁴C]bilirubin, virtually all of which was excreted during the 1st 2 h. The specific activity of the [¹⁴C]bilirubin in the bile of each rat was approximately three-fourths that of the administered pigment, but almost 100 times that of the corresponding serum bilirubin. None of the administered photoisomer was recovered unchanged. The label not recovered as bilirubin was present as polar compounds that were not further characterized but were chromatographically similar to those described in Gunn rat bile during phototherapy (6).

Injection of unlabeled photobilirubin II. Two Gunn rats each received a bolus of 180 μg photobilirubin II dissolved in 1.0 ml phosphate buffer. During the 1st 50 min after injection, output of diazo-reactive material in bile increased to 2.6–2.8 times base-line values, but returned nearly to base line thereafter (Table II). The azopigments formed with the postinjection bile sample exhibited the same atypical, orange-red color given by diazotization of photobilirubin II (9). In both animals, inspection of the thin-layer chromatograph of the Folch extracts of the post-injection bile revealed a marked outpouring of a yellow, nonfluorescent pigment, C-9, that was not visible in the

TABLE II
Excretion of Diazo-reactive Compounds in the Bile after
Bolus Administration of Photobilirubin II
to Gunn Rats

| Bile collection | Output of diazo-reactive compounds* | | |
|-----------------|---|----------|------------|
| | Basal | 0–50 min | 50–130 min |
| | $A_{600\text{ nm}}/0.1\text{ ml bile} \times \text{ml/h}$ | | |
| Rat A | 0.104 | 0.268 | 0.115 |
| Rat B | 0.054 | 0.150 | 0.060 |

At time 0, each rat received 180 μg i.v. photobilirubin II dissolved in 1.0 ml 0.1 M sodium phosphate buffer, pH 7.4, containing a trace of EDTA (diazo $A_{600\text{ nm}}$ of 0.1 ml = 0.189). Animals kept in the dark throughout.

* Diazo reaction performed with the method of Michaelsson. For reference bilirubin standard, $A_{600\text{ nm}} \times 9.61 \pm$ micrograms bilirubin/0.1 ml bile.

basal bile. Pigment C-9 corresponded in appearance and R_f value to the injected photobilirubin II, chromatographed concurrently, either after dissolution directly in chloroform, or after addition to basal bile followed by Folch extraction. The initial postinjection bile also exhibited moderate increments in pigments C-5 (dihydroxybilirubin) and C-14 (the brownish, poorly soluble origin material), along with small increases in C-1 (bilirubin), the hydroxybilirubins (C-6, -7, and -13), and biliverdin (C-11). Of these products, traces of C-7 and C-11 contaminated the administered photobilirubin II, whereas only bilirubin, C-5, -6, -7, and -14 were present in basal bile. A significant increase in C-14 occurred during Folch extraction of a mixture of photobilirubin II and basal bile, suggesting that this material was in large part produced by chemical degradation of photobilirubin II during extraction of bile.

Injection of labeled photobilirubin II as a bolus (Table III). After administration of 1.0 ml of a buffered solution of [¹⁴C]photobilirubin II (79 μg , sp act 1,606 $\text{dpm}/\mu\text{g}$) 90.3% of the radioactivity was excreted in the bile of the Gunn rat in 3 h, almost all in the 1st h. On Folch extraction at pH 3.0, 12.1% of the radioactivity of the bile collected during the 1st h, remained in the upper, aqueous phase. Chromatography of the lower phase revealed a pattern similar to that seen with the unlabeled pigment, with half the excreted label recovered as unchanged photobilirubin II (C-9) and 6.5% as dihydroxybilirubin (C-5). Small amounts of ¹⁴C appeared also in the other dihydroxyderivatives (C-6 and -7) and the immobile brown pigment at the origin (C-14). In contrast to the results after injection of photobilirubins IA or IB, <0.2% of the injected photobilirubin label was recovered as bilirubin. The remaining one-fourth of excreted radioactivity was scat-

TABLE III
Excretion of Labeled Bile Pigments after Bolus
Administration of [¹⁴C]Photobilirubin II
to a Gunn Rat

| | R _f value | Quantity μg | Percent injected pigment* |
|----------------------------|----------------------|----------------|---------------------------------|
| Total bile samples | | | |
| 0-1 h | | 67.8 | 85.8 |
| 1-2 h | | 2.7 | 3.4 |
| 2-3 h | | 0.8 | 1.0 |
| Pigments in 0-1 h bile‡ | | | |
| Folch upper phase | — | 8.2 | 12.1 |
| C-1, bilirubin | 0.91 | 0.12 | 0.2 |
| C-5, dihydroxybilirubin | 0.58 | 4.4 | 6.5 |
| C-6/7, dihydroxybilirubins | 0.43/0.32 | 0.97 | 1.4 |
| C-9, photobilirubin II | 0.24 | 35.2 | 51.9 |
| C-14, original material | 0.00 | 1.2 | 1.8 |
| Remaining bands | — | 17.7 | 26.1 |

[¹⁴C]Photobilirubin II, 79 μg, 1,606 dpm/μg sp act, dissolved in 1.0 ml 0.1 M sodium phosphate buffer, pH 7.4, administered intravenously at time 0 to a Gunn rat with a bile fistula, kept in the dark throughout the study.

* For pigments in 0-1-h bile, values are for excreted pigment.

‡ Thin-layer chromatography of Folch lower phase extract performed as in reference 6, with designated bands eluted and quantitated by radioassay.

tered among the other pigment bands, which have not yet been identified.

Infusion of unlabeled photobilirubin II (Table IV). A single Gunn rat, kept in the dark, was infused with photobilirubin II in buffer at a rate of 45 μg/h, approximately the normal rate of bilirubin production in the Gunn rat (22). As revealed by visual comparison of the chromatograms, the bile collected before infusion contained no detectable C-9 and only traces of bilirubin. With infusion of photobilirubin II for 3 h, the output of diazo-reactive materials increased to twice

the base-line rate, the C-9 bands became intensely yellow, and a slight increase in bilirubin was detected also. After 3 h, with the infusion of photobilirubin II continued, phototherapy was administered. During this period, excretion of C-9 appeared qualitatively undiminished, but there was a further increment in diazo-reactivity to approximately three times that of base line, largely due to a markedly enhanced output of bilirubin. After 4.75 h, with phototherapy continued, the photobilirubin infusion was halted; this resulted in a marked decrease in the excretion of C-9, while the output of bilirubin remained high. Throughout the study, less striking changes in the output of C-5 and C-14 paralleled those of C-9.

Identification of endogenously formed photobilirubins excreted in bile during phototherapy. Pooled bile, collected on ice and in the dark from two Gunn rats exposed to phototherapy for 9 h, was subjected to sequential Folch partition at pH 7 and 3, as described previously (2, 6). Chromatography of the lower phases confirmed prior reports (2, 6) that the pH 7 extract contained only bilirubin and porphyrins, whereas the pH 3 extract contained bilirubin and C-9 as the major components. The endogenous C-9 photopigment was eluted from the chromatogram and compared with authentic photobilirubin II, prepared in vitro by anaerobic illumination of bilirubin in dimethyl sulfoxide (9). The two compounds, studied simultaneously, exhibited identical properties as follows: (a) visible absorption maxima at 441 nm in chloroform and 429 nm in methanol; (b) rapid and almost complete reversion to bilirubin on anaerobic illumination in chloroform; (c) reaction with diazotized sulfanilic acid in methanol to yield an atypical, orange-red azopigment; (d) stability in chloroform, methanol, dimethyl sulfoxide and 0.1 M NaOH, as long as light and oxygen were excluded; (e) formation of poorly soluble brown products (C-14) on exposure to acetic acid or Folch extraction at pH 3.0; (f) formation of a plum-red color, but no biliverdin, on reaction with 2,3-dichloro-5,6-dicyano-

TABLE IV
Excretion of Pigments in Gunn Rat Bile during Infusion of Photobilirubin II*

| Collection period | -1.5-0 h | 0-1.5 h | 1.5-3.0 h | 3.0-4.75 h | 4.75-6.0 h |
|-------------------------------------|----------|---------|-----------|------------|------------|
| Photobilirubin II infusion | No | Yes | Yes | Yes | No |
| Phototherapy | No | No | No | Yes | Yes |
| Output of diazo-reactive compounds‡ | 0.091 | 0.126 | 0.172 | 0.251 | 0.260 |
| Photobilirubin II excreted in bile§ | None | 4+ | 4+ | 4+ | 2+ |
| Bilirubin in bile§ | Trace | 1+ | 1+ | 4+ | 4+ |

* Photobilirubin II infused in phosphate buffer (see Table II) at 45 μg/h.

‡ As in Table II, diazo-reactive compounds expressed as A_{600 nm}/0.1 ml bile × ml/h; for bilirubin, A_{600 nm} × 9.61 = μg bilirubin per 0.1 ml bile.

§ Semiquantitative assessment from visual intensity of band on thin layer chromatography plate.

p-benzoquinone in dimethyl sulfoxide (*g*) identical R_f values on thin-layer chromatography in three different solvent-gel systems (9).

Because the unstable photobilirubins I were not identified in the above study, we attempted to stabilize this pigment in bile by collecting the bile directly into liquid nitrogen in the dark, and extracting and chromatographing the bile pigments after only 2 h of bile collection. Phototherapy bile thus obtained contained abundant bilirubin and photobilirubin II, but also small amounts of a pigment, with the appropriate R_f value, that shared all the properties described previously (9) for photobilirubin I. No such pigment was found in the bile of an unilluminated Gunn rat, collected and processed in identical fashion.

DISCUSSION

In 1971, McDonagh (1) produced evidence that photo-oxidation via autosenitized addition of singlet oxygen might constitute an important mechanism of bilirubin photodegradation. However, the major photoproducts predicted by this mechanism, 5,5'-diformyldipyrromethane and propentdyopents (23), were not found among the bilirubin derivatives excreted in Gunn rat bile during phototherapy (6), and the two major pigments excreted during phototherapy, bilirubin itself and a yellow, acid-labile, diazo-positive pigment called C-9 (6), were not reproducible by photo-oxidative singlet oxygen reactions. McDonagh (7) subsequently postulated that photoisomerization might produce tetrapyrroles that were sterically incapable of the internal hydrogen bonding of bilirubin IX α (18), and thus would be sufficiently polar to be excreted in bile without conjugation.

Recently, Lightner et al. (13, 14) reported the formation of three photobilirubins during illumination of bilirubin in a variety of solvents under aerobic conditions, but were unable to purify these derivatives because of their thermal instability (16). By illumination of bilirubin under anaerobic conditions that precluded oxidative reactions, we (9) and Onishi et al. (10, 11) produced and purified chromatographically a pair of unstable (I) and a pair of stable (II) photobilirubins. All the reported photobilirubins were more polar than the parent bilirubin IX α , being easily soluble in water and methanol. They also reverted to bilirubin IX α when reilluminated briefly with visible light, attaining a photoequilibrium in which <10% of the pigment was in the form of the photobilirubins. Mass spectrometry (9, 24) and azopigment analysis (10, 11) indicated that these derivatives were neither photoadducts nor the III α , XIII α , IX β , IX γ , or IX δ isomers of bilirubin. Based on these properties, all three groups postulated that the photobilirubins resulted from configurational (geometric, Z to E) photoisomerization at the $\Delta 4,5$ and/

or $\Delta 15,16$ methene bridges of bilirubin IX α , which are normally in the Z configuration (18). Unfortunately, these photobilirubins have not yet been prepared with sufficient quantity or stability to permit nuclear magnetic resonance spectroscopic analysis, which could provide rigorous proof of these tentative structural assignments.

All three groups have reported that the less polar photobilirubins (our IA and IB) are thermally unstable in the dark, reverting rapidly to bilirubin, especially in polar solvents (e.g., bile) (9–11, 14). The reversion is retarded greatly by 1 mM EDTA, serum albumin, and nonpolar solvents such as chloroform (9–11). These properties suggested that formation and excretion of these polar photobilirubins could explain the enhanced appearance of bilirubin IX α in bile during *in vivo* phototherapy (8). Strong indirect evidence for this thesis has been the demonstration that the photobilirubins accumulate in the serum of Gunn rats (16) and human infants (11, 15) during phototherapy, and correlate temporally with the enhanced excretion of bilirubin IX α in their bile (15). Moreover, when photobilirubin-enriched serum from an illuminated donor Gunn rat was administered intravenously to another Gunn rat kept in the dark, large amounts of the natural bilirubin IX α ,Z,Z isomer appeared in the bile of the recipient rat (16).

Our present studies with purified photobilirubins prepared *in vitro* provide more direct evidence of their role in this phenomenon. Administration to unilluminated Gunn rats of a mixture of photobilirubins, or of isolated photobilirubins IA and IB, led to >100-fold increments in bilirubin IX α ,Z,Z in their bile. Unfortunately, during the isolation and dissolution in serum of the photobilirubins IA and IB, the majority reverted to bilirubin and a small proportion degraded to other polar products. Moreover (Table I), a larger proportion of the ^{14}C excreted was in the form of other polar products, presumably formed by metabolism or degradation of some of the administered photobilirubins during passage through the animal or processing of the bile. However, since the injected ^{14}C pigments contained <2% of these other products, the 33% of excreted ^{14}C in bilirubin IX α could have derived only from the injected [^{14}C]photobilirubin. Against the possibility that the metabolites might have been intermediates in this process are the following observations: (a) only photobilirubins I revert to bilirubin IX α *in vitro*; (b) excretion of [^{14}C]bilirubin was virtually completed within 2 h, yet output of other labeled derivatives, representing approximately one-third of the excreted radioactivity, continued for the remainder of the 24-h period after injection of photobilirubins IA or IB; (c) in studies reported previously (6), injection of ^{14}C -labeled photoproducts, isolated from Gunn rat bile during phototherapy, did not augment the excretion of

bilirubin by recipient Gunn rats. This last observation is also against the possibility that endogenous bilirubin "piggy-backed" into bile in complexes with excreted photoproducts. Moreover, in the present studies, the similarity of the specific activity of the injected photobilirubins and the excreted bilirubin indicates that the latter is derived from the former, and not from the endogenous plasma bilirubin of vastly lower specific activity. The control experiment (Figs. 1 and 2), in which only [¹⁴C]bilirubin was injected and virtually no [¹⁴C]bilirubin was excreted, eliminates the contaminating bilirubin IX α as the source of the bilirubin found in bile during the injection of the photobilirubin I preparations.

Of special importance was the demonstration, by appropriate precautions during collection and excretion of the bile, that photobilirubins IA and IB per se were excreted during phototherapy, but were not found in the bile from an unilluminated Gunn rat that was processed simultaneously. These observations support the proposal that these photobilirubins pass into bile before reversion to bilirubin IX α . The predicted in vivo stabilization of the photobilirubins IA and IB was evidenced also by the continued appearance of excess bilirubin in the bile for 2–3 h after the bolus injection of these photoisomers, and the recovery as [¹⁴C]bilirubin in the bile of two-thirds of the label administered as photobilirubin IA or IB per se. Thus, our experiments suggest strongly that photobilirubins IA and IB are the precursors of the bilirubin IX α that appears in bile during phototherapy, but it is appreciated that some means of prolonged stabilization of the photobilirubins I in pure form is essential to achieve unequivocal documentation of both their in vivo behavior and their structural identification.

Much clearer was our demonstration that, after intravenous administration, the more stable photobilirubin II is excreted in the bile and corresponds to the other major photoproduct (C-9) that appears in the bile of the Gunn rat (6) and human infant (15) during phototherapy. It may correspond also to the "430 pigment" of Kapitulnik et al. (25). Over half of injected [¹⁴C]photobilirubin II was recovered as such in the bile, and virtually none reverted to bilirubin IX α ,Z,Z in vivo. Photobilirubin II appeared to serve also as the in vivo precursor of the dihydroxylated bilirubins, C-5, -6 and -7 (2, 6), presumably via pathways not involving secondary photoreactions, since the recipient Gunn rats were maintained in the dark. Because only C-7 was in part produced from C-9 during extraction procedures, C-5 and C-6 are clearly natural derivatives resulting from biochemical reactions of photobilirubin II. By contrast, C-14, the insoluble brown pigment at the origin of the chromatogram (Table III), is apparently in large part an artefact created by degradation of photobilirubin II during the Folch extraction at low

pH. Since C-5 and C-6 are found in the bile of unilluminated Gunn rats, but increase during phototherapy, it is probable that photobilirubin II is more susceptible to hydroxylation than the parent bilirubin IX α ,Z,Z. Alternatively, as suggested by McDonagh et al. (16), small amounts of other conformers of bilirubin IX α ,4Z,15Z may exist at equilibrium even in the dark, and serve as precursors for the dihydroxybilirubins found in Gunn rats not subjected to phototherapy.

Although each individual experiment was reported in only one or two animals, the major qualitative phenomena were observed in a larger number of animals, including those described here and other rats that received less satisfactory photobilirubin preparations during the developmental stages of this work. Thus, the appearance of bilirubin IX α in bile after administration of irradiated bilirubins or photobilirubins I is reported in five animals, and the excretion of intact photobilirubin II in four animals.

Considered together, all published studies suggest that the photobilirubins are geometric (Z,E) isomers of bilirubin IX α , whose formation may account for the major pathways of bilirubin photocatabolism in the Gunn rat and human neonate. These photoisomers presumably are formed rapidly when light penetrates to the bilirubin deposits in the skin and subcutaneous tissue, then partitioned to the plasma, taken up by the liver, and excreted into the bile. In bile, the thermally unstable photobilirubins IA and IB, believed to be the E,Z and Z,E isomers, rapidly revert to the Z,Z configuration, accounting for the augmented excretion of bilirubin during phototherapy of both Gunn rats (8) and human neonates (15, 26). The more stable photobilirubin II remains mostly intact and constitutes the major polar photoproduct, but is in part hydroxylated at the methene bridges, by photo-independent reactions, to yield other minor derivatives. Together, these photoisomerizations and secondary reactions apparently account for >80% of the accelerated bilirubin catabolism during phototherapy (6, 15).

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