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Photoisomerized bilirubin in blood from infants receiving phototherapy

(fluorescence/photobiology/neonatal hyperbilirubinemia)

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ABSTRACT **A pigment different from (Z,Z)bilirubin-IXα was detected by fluorometric methods in blood specimens from newborn infants undergoing blue-light therapy for unconjugated hyperbilirubinemia; it was not detected in specimens from infants not under therapy. The phototherapy-associated pigment has fluorescence, solubility, and photochemical properties that are identical to those exhibited by what are thought to be configurational ($Z \rightarrow E$) isomers of bilirubin. It is concluded that isomerized bilirubin in the blood of neonates under phototherapy can reach as high as 15% of the total.**

Phototherapy is commonly administered to newborn infants with hyperbilirubinemia in order to decrease the body burden of neurotoxic bilirubin (1). The decrease in serum bilirubin associated with phototherapy was thought to be due to oxidative destruction of the pigment (2, 3). However, substantial quantities of unconjugated bilirubin (BR) are found in the bile when phototherapy is administered (4, 5). This fact and the failure to find much of the expected photooxidation products in excreta of infants or experimental animals after phototherapy (6) led to the abandonment of the idea that photodegradation is a major pathway (7, 8). In the absence of light, excretion of BR by the liver is insignificant. McDonagh first suggested that the apparent hepatic excretion of BR during phototherapy proceeds via the formation of an isomer of BR that would be excreted with good efficiency but might revert to the natural BR in the bile (9–11).

McDonagh and Ramonas (12) supplied evidence for the *in vivo* photoisomerization of BR based upon kinetics of biliary excretion of the pigment in irradiated Gunn rats. Ostrow and coworkers (13–15) showed that BR is transformed to more polar, water-soluble, isomeric products when irradiated in organic solvents and that some of these products could be excreted by the Gunn rat liver. That the first photoproducts formed *in vitro* are derived from configurational isomerization about exocyclic (*meso*) carbon-carbon double bonds in the molecule—i.e., $Z \rightarrow E$ isomerization— is best demonstrated by the data of Lightner *et al.* (16–18) and by observation of such isomerization in model compounds (19). However, primarily because of the instability of these compounds in solution, unequivocal structure determination has not yet been accomplished for any of these bilirubin photoisomers. Following McDonagh *et al.* (7, 16), we call these *E* isomers "photobilirubin (PBR)."

McDonagh and coworkers recently detected PBR in the blood of jaundiced rats irradiated with blue light and demonstrated that this isomerized bilirubin is excreted into the bile of the animals (7). We report here the detection and assay of PBR in blood specimens from newborn infants receiving phototherapy.

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METHODS

Specimens. Specimens from infants were obtained by heel stick or via an umbilical catheter. Required authorization was obtained before collection of each specimen. Heparin was the anticoagulant in all specimens. Phototherapy lamps were off during blood collection. Direct diazo-reacting bilirubin was <0.5 mg/100 ml in all specimens studied.

Artificially "jaundiced" blood and human serum albumin were prepared from adult blood according to reported procedures (20). Except when noted, the buffered saline was 0.15 M NaCl/0.01 M Tris·HCl, pH 7.4.

The hematofluorometer measurements were made immediately upon collection of the specimen. The remainder of each specimen was centrifuged at once and the plasma fraction was frozen and kept dark until further use. Manipulations of specimens containing bilirubin were carried out in dim light or under red photographic safe light.

Fluorometric Measurements. Albumin-bound bilirubin and total unconjugated blood bilirubin were determined as described (20) by using an automated filter fluorometer (hematofluorometer) (21, 22). Fluorometric measurements, made with a Perkin–Elmer MPF4 spectrofluorometer or with a photon-counting spectrofluorometer, used a 3-mm-square cuvette in "right-angle" geometry for diluted plasma specimens and a special cuvette (23) in "front-face" geometry for whole blood. Measurements were made at 23 ± 2°C except for those with the hematofluorometer, in which specimens were kept at 37°C.

Specimens were irradiated in the spectrofluorometers with simultaneous monitoring of fluorescence or in a separate apparatus using a 150-W xenon arc and a 10-nm-bandwidth interference filter centered at 430 nm. Light fluxes never exceeded 50 μW cm^{-2} and were usually lower.

Preparation of Photoisomerized Bilirubin. Four hundred milliliters of purified (17) BR (10 mg/100 ml) in $CHCl_3$ (washed with 5% sodium carbonate, dried, and distilled) was irradiated with blue light (425–435 nm) while being rapidly stirred at 0°C under argon bubbling. When not more than 5% of the BR was isomerized, the cold solution was extracted with 10 ml of buffered saline (pH 8.1). Two milliliters of a solution (3 g/100 ml) of human serum albumin in buffered saline (pH 7.4) was added at once to the yellow extract. The photoproduct–albumin complex could be stored (dark, 4°C) for at least 2 days without change (assessed spectrally).

Extraction of Plasma with Chloroform. Plasma (50 μl) was diluted 1:1 with buffered saline, gently mixed for several minutes with an equal volume of buffer-washed chloroform, and

Abbreviations: BR, unconjugated (4Z,15Z)bilirubin-IXα; PBR, photobilirubin or *E* isomers of bilirubin-IXα; CD, circular dichroism.

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centrifuged. After extraction with a second volume of chloroform, the specimen was used for fluorescence measurements.

RESULTS AND DISCUSSION

Effects of Phototherapy of Fluorometric Bilirubin Assays. When bound to its primary binding site on human serum albumin, BR is fluorescent. Unbound BR and BR bound elsewhere in blood are negligibly fluorescent. Thus, it is possible to assay the albumin-bound bilirubin (B) on the basis of the fluorescence signal from whole blood (20). Bilirubin fluoresces when sequestered in micelles of dimethyldodecylamine oxide (Ammonyx-LO), which can extract BR from all blood binding sites. Therefore, it is possible to assay total whole blood bilirubin (T) from the fluorescence signal of a blood specimen made 3% in dimethyldodecylamine oxide (20). These assays can be performed rapidly with the use of the hematofluorometer (22, 24).

A value considered central to the assessment of risk for bilirubin encephalopathy is the concentration of BR not bound to albumin, T-B (20, 22, 24). We have found that T-B and (T-B)/T, the fraction of BR not bound to albumin, correlate well with other measures of BR binding and with factors associated with gestational immaturity. (T-B)/T is called Δ.

In the course of a study of relationships between hematofluorometer assay values and clinical observations (to be published elsewhere), we discovered that infants receiving phototherapy averaged higher apparent values of Δ than did infants not being treated therapy (Table 1). Otherwise healthy full-term jaundiced infants with birth weights >2500 g had high BR binding capacities and usually had Δ values <0.1. However, an average value of 0.2 was observed for such a population of infants receiving phototherapy.

That this observation reflected an effect of the phototherapy *per se* rather than a therapy-associated selection of populations was made evident by the following observations. The average value of Δ for a population of infants before therapy was 0.17. The average Δ for the same group after 4–24 hr of therapy was 0.29. In addition, a 3-year-old girl with Crigler–Najar syndrome (I) [congenital chronic hyperbilirubinemia (25)] was followed through three sessions of phototherapy within a 6-month period; this patient had a chronic total blood bilirubin level of about 24 mg/100 ml. Δ increased during therapy on all three occasions. A maximal increase of about 0.12 was observed after about 3 hr and persisted during further therapy.

Fluorescence Properties of Photoisomerized Bilirubin. The most quantum efficient reaction that occurs when albumin-bound BR is irradiated with blue light is associated with a reversible, oxygen-independent, increased absorbance in the region 470–530 nm (17, 26, 27) (Fig. 1). If the light intensity is kept low and mixing of the sample is good, a photostationary state can be achieved (17, 18, 28). Concomitant with the spectral

Table 1. Effect of phototherapy on Δ

Population	Δ		Change in Δ	*P*
	No phototherapy	Phototherapy		
All babies in study	0.10 ± 0.10 (n = 159)	0.24 ± 0.11 (n = 171)	0.14	<0.005
All babies with birthweight >2500 g and no complications	0.07 ± 0.07 (n = 26)	0.20 ± 0.14 n = 11	0.13	<0.005
Same infants before and during therapy* (n = 55)	0.17 ± 0.10	0.29 ± 0.11	0.12	<0.01

Results are shown as mean ± SEM.
* Specimens were obtained from these infants before and again during therapy.

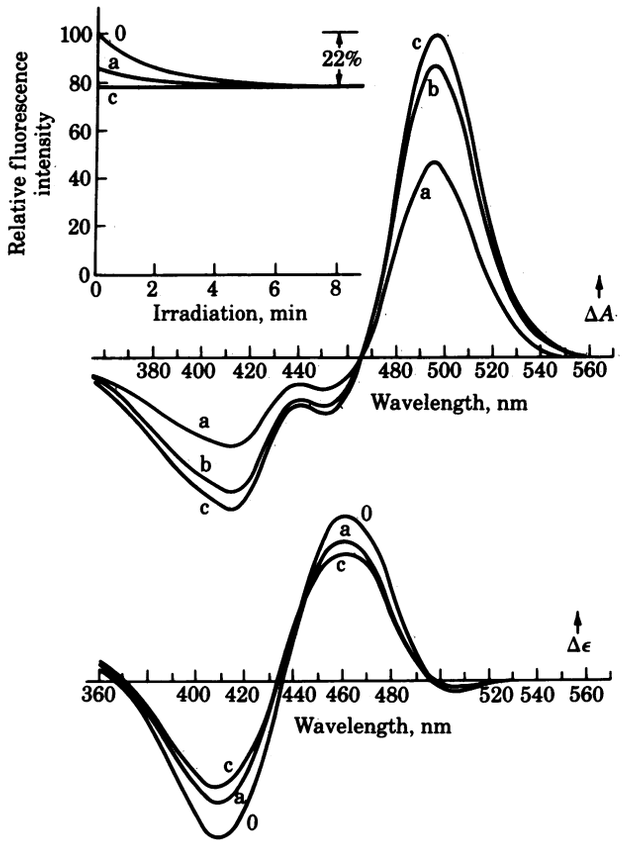

FIG. 1. Spectral changes accompanying the irradiation of BR/human serum albumin mixture with blue light (460–470 nm). Specimens 0, a, b, and c had been irradiated in a separate apparatus for 0, 4, 10, and 20 min, respectively. Results for 30-min irradiation were virtually identical to those at 20 min. The total bilirubin concentration was 5 mg/100 ml; albumin was 1 g/100 ml. (*Top*) Fluorescence intensity (at 520 nm) as the specimens were irradiated (at 465 nm) in the fluorometer. (*Middle*) Difference absorption spectra, with an unirradiated specimen as the reference. (*Bottom*) Circular dichroism (CD) spectra of irradiated specimens.

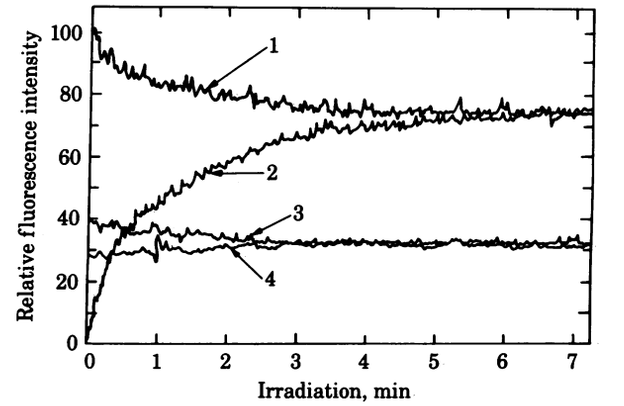

FIG. 2. Fluorescence intensity (at 520 nm) as a function of the dose of excitation light (425–435 nm) for buffered solutions of BR/albumin (curve 1) and PBR/albumin (curve 2) matched for absorbance at 465 nm and the curves obtained for similarly matched solutions to which 3% dimethyldodecylamine oxide was added (curve 3, BR; curve 4, PBR).

changes observed in absorbance, there was a decrease in the intensity of the fluorescence from the sample (with no change in spectrum) and an alteration of its circular dichroism (CD) spectrum (28) (Fig. 1). Both the fluorescence intensity and the CD spectrum reached stationary values simultaneously with the absorption spectrum. When photostationary, the fluorescence intensity was reduced by 25 ± 2% and 22 ± 2% (mean ± SEM) from the original intensity for excitation light centered at 430 and 465 nm, respectively (28).

From chloroform solutions of bilirubin irradiated with low doses of blue light, we extracted a product composed of one or more bilirubin isomers. This isomeric bilirubin binds tightly to human serum albumin (28). Based on its spectral and photochemical properties, as well as observations by others (16–18), we conclude that the new species is configurationally isomerized bilirubin which we call PBR.§

We observed that the PBR–albumin complex could be efficiently converted to a photostationary mixture with the identical absorption, CD, and fluorescence (see below) properties observed for the mixture obtained upon irradiation of BR/albumin (27).¶ These and other observations lead us to conclude that irradiation of BR/albumin produces the same isomerized bilirubin species as obtained by irradiation of BR in $CHCl_3$. Details of these studies will be reported elsewhere.

Solutions of BR/albumin and PBR/albumin, matched for absorbancy at 465 nm ($\Delta A = 0$ point in the difference absorption spectrum) showed identical photostationary fluorescence intensities after correction for scattered excitation light (Fig. 2), consistent with a BR/albumin $\rightleftarrows$ PBR/albumin photoequilibrium. Solutions of PBR/albumin carefully kept dark exhibited initial fluorescence yields not greater than 10% of those of BR/albumin.

In marked contrast, the fluorescence emissions from matched solutions of BR/albumin and PBR/albumin exhibited nearly the same intensities upon addition of 3% dimethyldodecylamine oxide (Fig. 2). In this case, the fluorescence yield of BR was about one-third that of BR/albumin. The detergent may have two effects on PBR: it may greatly enhance the fluorescence yield of PBR over that of PBR/albumin, approaching that of BR in detergent, or it may cause PBR to undergo rapid reversion to BR.

In summary, the product(s) (PBR) of the highest quantum yield photoreaction of bilirubin, either in chloroform solution or bound to human serum albumin—undoubtedly configurational *E* type isomers—binds strongly to HSA and, in this bound state, exhibits a fluorescence yield not greater than 10% that of albumin-bound BR. Addition of 3% dimethyldodecylamine oxide to BR/albumin and PBR/albumin causes them to exhibit fluorescence with nearly equal yields. [By using CD spectroscopy, we have determined that the affinity of human serum albumin for PBR in buffered saline is about one-fourth that for BR (unpublished data).]

We propose that the increase in the observed Δ associated with phototherapy is due to the presence of PBR in the specimens rather than to an increase in the fraction of bilirubin not bound to albumin. When a fraction of the BR is converted to PBR, the low fluorescence yield of PBR/albumin leads to an apparent lower value for albumin-bound bilirubin as determined by using the hematofluorometer. However, the value for total blood bilirubin is hardly affected because of the action of the detergent. Furthermore, because the fluorescence yield of PBR/albumin is negligible compared with that of BR/albumin, the change in Δ should be a good estimate of the fraction of isomerized bilirubin (PBR).

To test the hypothesis that change in Δ is associated with the presence of PBR, we devised two fluorometric methods, one of which yields estimates of the amount of PBR present.

Fluorescence Decrement Method. Irradiation of plasma containing BR with narrow-band light centered at 465 nm causes a decrease in the fluorescence intensity due to photoisomerization of BR. With careful control, a plateau can be observed at about 78% of the original intensity. The fluorescence yield of PBR in plasma is small compared to that of BR, and essentially only the remaining BR contributes to the fluorescence. Therefore, the stationary mixture contains approximately 22% PBR. Comparison of the CD spectra of BR/albumin and PBR/albumin (not shown) with the spectrum of the mixture leads to the same conclusion. If PBR is already present

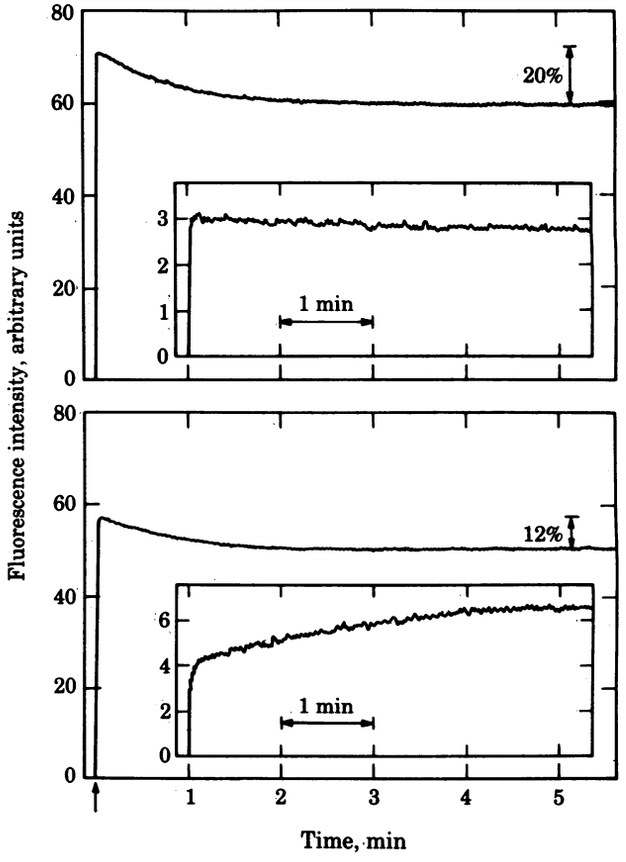

FIG. 3. Fluorescence decrement and increment (*Insets*) tests for PBR on plasma specimens from the Crigler–Najar patient. (*Upper*) Specimen collected after more than 6 hr without phototherapy. (*Lower*) Specimen collected after 3 hr of phototherapy. ↑, Shutter opened.

§ Our experimental conditions for photoisomerization of BR in $CHCl_3$ were different from those of Stoll *et al.* (15). They used high-intensity unfiltered light from a mercury arc, and (we estimate) a dose between 100 and 1000 times that necessary to achieve maximal production of the simple configurational isomers. In addition, most of the light was in the wavelength range 300–400 nm, leading to different photochemical paths [Lightner and Cu (29); K. Schaffner and A. Holzwarth, personal communication]. It appears to us that Stoll *et al.* isolated isomers of bilirubin that were probably not the initial *E* isomers. In any event, the products reported by them have spectral and solubility properties different from those of the product we have isolated.

¶ There are at least three reactions (photooxidation, photoaddition of BR to albumin, and other rearrangements) that occur upon irradiation of BR/albumin in addition to isomerization (25). These processes have much smaller quantum yields than does $Z \rightarrow E$ isomerization (G. Jori, personal communication; unpublished data). However, because these low-yield processes are essentially irreversible, their products eventually accumulate.

Table 2. Assay of PBR in plasma specimens

Patient	Phototherapy* duration, hr	Change in Δ†	PBR in plasma: Fluorescence decrement method, % of total bilirubin	PBR in plasma: Fluorescence increment method
Ca	0	0	<2	No
	8	0.21	18	Yes
Gl	0	0	<2	No
St	24	0.16	10, 10	Yes, yes
Pa	0	0	<2	No
Za	12	0.1–0.2	15	Yes
Ha	0	0	<2	No
Sh	8	0.49‡	6	No
Do	0	0	<2	No
Cr	0	0	<2	No
	4	0§	7	Yes
Rh	0	0	<2	No
	4	¶	6	Yes
Te	0	0	<2	No
	4	¶	14	Yes
C-N‖	Yes + >6 hr dark	0	<2, <2, <2	No, no, no
	1.5	0.04	6, 10	Yes, yes
	2	0.07	7	Yes
	2.8	0.08	9	Yes
	3	0.12	11, 10	Yes
	3 + 1 hr dark	0.10	7	Yes
	3 + 2.3 hr dark	0.04	4	No
	2.8 + 3.8 hr dark	0	<2	No

* Not standardized; various blue and white light phototherapy units were used.
† Defined as 0 before phototherapy.
‡ Exceeds expected limit of 0.22; probably not due to phototherapy.
§ The prephototherapy Δ was very high, 0.44.
¶ Not determined.
‖ The Crigler–Najar syndrome (I) patient.

in the plasma specimen, the fluorescence decrement measured should be smaller than 22%. This is apparent in the fluorescence curves of Fig. 1 for bilirubin–albumin solutions and was also observed for artificially "jaundiced" adult plasms (spectra not shown). If there is no interfering fluorescence, an estimate of the percentage PBR already present is given by 22 minus the observed percentage decrease in fluorescence intensity upon irradiation (465 nm) of the specimen until photoequilibrium is achieved.

Results of fluorescence decrement measurements for two plasma specimens from the Crigler–Najar syndrome patient are shown in Fig. 3. Plasma obtained from the patient at least 6 hr after phototherapy showed a 20% decrement. Plasma obtained immediately after 3 hr of blue-light therapy exhibited a decrement of 12% (i.e., 10% PBR). The change in Δ value was 0.12. Results for other specimens from the Crigler–Najar syndrome patient, as well as from several neonates, are given in Table 2.

Fluorescence Increment Method. If a mixture of BR and PBR in plasma contains more than 22% PBR, irradiation with 465-nm light causes an increase in the fluorescence as PBR is converted to BR. The presence of PBR in plasma may be demonstrated in this way if the BR present can be selectively removed, so that the remaining pigment is more than 22% PBR (e.g., by chloroform extraction (7). Curves of fluorescence intensity as a function of irradiation at 465 nm for plasma treated with chloroform are shown in Fig. 4. The total bilirubin concentration was 10 mg/100 ml. The specimen containing no PBR gave a slightly decreasing fluorescence signal upon irradiation. The specimens containing PBR exhibited an increasing fluorescence signal. The increment in fluorescence was greatest for the specimen originally showing 22% PBR by the fluorescence decrement method.

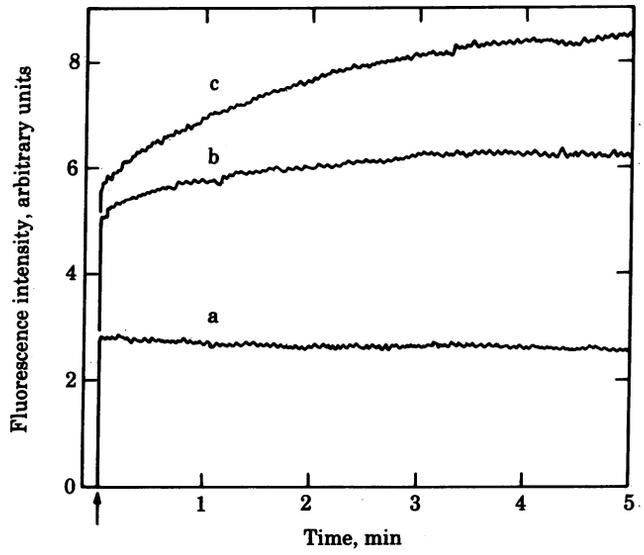

FIG. 4. Fluorescence intensity (at 520 nm) as a function of the dose of exciting light (460–470 nm) for three chloroform-extracted specimens of plasma containing bilirubin. The plasma, obtained from an adult male, was artificially "jaundiced" and separated into three portions, two of which were irradiated to produce PBR. Curves a, b, and c refer to specimens that contained 0%, 10%, and 22%, respectively, of PBR as judged by the fluorescence decrement method. ↑, Shutter opened.

This method cannot be used to quantitate the PBR because the efficiency of chloroform extraction is variable and, more importantly, chloroform treatment leads to a substantial and varied scattered light background in the fluorescence measurement. However, the fluorescence increment method does appear to be a valid way to detect the presence of PBR in plasma.

Results for specimens from the Crigler–Najar syndrome patient and for specimens from several neonates are given in Table 2. Agreement of the results among the various methods is excellent.

Conclusions. Our results show that blood specimens from infants receiving phototherapy for hyperbilirubinemia contains a pigment different from (Z,Z)bilirubin-IXα. This pigment, not detected in blood from infants not receiving therapy, has fluorescence, solubility, and photochemical properties identical to those of configurational $Z \rightarrow E$ isomer(s) of bilirubin that can be prepared by irradiation of the pigment *in vitro*. The fraction of isomerized bilirubin in blood specimens from neonates receiving phototherapy appears to reach as high as 15%.

The high quantum yield, about 0.2 (27), for the production of PBR in itself predicts its presence in infants receiving phototherapy. Indeed, McDonagh *et al.* (7) detected PBR in the blood of Gunn rats given phototherapy and demonstrated that PBR is excreted into the bile of these animals. It is therefore not surprising to find PBR in human neonates who are receiving phototherapy. However, it remains to be demonstrated that PBR is excreted by human infants. The high levels of PBR (up to 15%) found appears to argue against its efficient excretion when compared with the much lower steady-state levels observed in the Gunn rat (7). However, after phototherapy was

halted, PBR disappeared from the blood of the Crigler–Najar syndrome patient with a half-life of 1.5 hr, about 8 times faster than the *in vitro* thermal reversion of PBR to BR at 37°C (unpublished data) and probably reflects excretion.

While this paper was in preparation, Onishi *et al.* (31, 32) reported the detection of a phototherapy-associated pigment in plasma specimens from human infants by reversed-phase high-pressure liquid chromatography. The new pigment has chromatographic properties identical to those of PBR produced by irradiation of BR/albumin *in vitro*. The range of plasma PBR levels found by Onishi *et al.* agrees well with our observations.

We emphasize that conversion of a fraction of BR to PBR in blood does not alter the value of total blood bilirubin determined with the hematofluorometer. As explained above, the apparent value of albumin-bound bilirubin is reduced in proportion to the quantity of PBR present. However, with phototherapy this quantity does not appear to exceed about 15% of the total bilirubin, so that only small errors, insignificant for purposes of clinical management, are introduced into the instrumental assays for albumin-bound bilirubin and bilirubin-binding capacity.

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