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CONFIGURATIONAL ISOMERIZATION OF BILIRUBIN AND THE MECHANISM OF JAUNDICE PHOTOTHERAPY

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SUMMARY Bilirubin-IXα and its dimethyl ester, which have the Z configuration at the *meso* bridge double bonds, undergo rapid and reversible photoisomerization on irradiation with visible light giving Z-E, E-Z and E-E isomers. At photoequilibrium [Z-Z] >> [E-Z] ≈ [Z-E] > [E-E]. The non-Z-Z isomers of bilirubin-IXα are less lipophilic and more polar than the natural Z-Z isomer. The reaction probably is important *in vivo* during phototherapy of neonatal jaundice and responsible for the biliary excretion of unconjugated pigment associated with it.

INTRODUCTION

Due to its intraverted structure (1,2) and poor aqueous solubility (3,4,5), unconjugated bilirubin (BR)* IXα (1, Figure 1) cannot be excreted by mammals to to any marked degree (6). It must first be converted enzymatically to more water-soluble cholephilic conjugates (6). Yet during treatment of neonatal jaundice with phototherapy, considerable amounts of unconjugated BR are excreted into the bile (7). This is both fortunate and useful since the aim of phototherapy is to rid the jaundiced infant of unconjugated BR, which is toxic. But it is also puzzling, and it has never been shown how phototherapy enables BR to slip through the liver and into the bile without conjugation. It has long been recognized, however, that some conformational or configurational modification of the pigment is probably responsible because covalent bonds are neither formed nor destroyed in the overall photobiological process (8,9,10). Recently, we reported that brief irradiation of BR-IXα in many solvents yields a photostationary mixture containing mostly BR-IXα and a small proportion of a more polar material which we called *photobilirubin* (PRB)* (11). As shown in Figure 2, the

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* Abbreviations used, BR: bilirubin, PBR: photobilirubin, HPLC: high performance liquid chromatography, TLC: thin layer chromatography.

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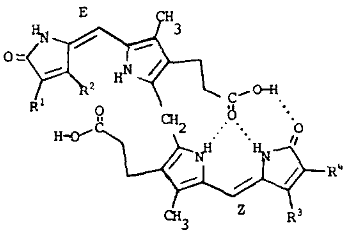
reaction can readily be followed by absorbance difference measurements, which show reciprocal synthesis and loss peaks near 500 and 460 nm respectively as the photostationary state is approached. Formation of PBR from BR occurs rapidly; it does not involve solvent or oxygen; and it is reversible photochemically, thermally and catalytically (11). We concluded that PBR is a mixture containing three geometric isomers of BR-IXα (the Z-E, E-Z and E-E isomers, Figure 1) with different configurations about the 5,15 *meso* double bonds. However, chromatography and purification of PBR was frustrated by its tendency to revert to BR, especially on silica, and we were unable to resolve it completely into the three putative components. We now present evidence, obtained with dimethyl ester derivatives of BR, that consolidates our previous structural interpretation. In addition, we show that the solubility of PBR and its rate of formation suggest a simple explanation for the curious excretion of unconjugated BR that occurs during jaundice phototherapy.

MATERIALS AND METHODS

Bilirubin (Sigma or Koch-Light) was purified as previously described (12) or by washing a $CHCl_3$ solution 3x with 5% aq. $NaHCO_3$ and crystallization from $CHCl_3$-CH_3OH (∿1:1). Purified BR contained less than 5% of the IIIα and XIIIα isomer impurities (13) by thin layer chromatography (TLC) (13) or by high performance liquid chromatography (HPLC) using $CHCl_3$-0.75% ethanol-1% acetic acid (14). Bilirubin-IXα dimethyl ester was prepared by reaction of 120 mg (0.2 mmol) of BR-IXα with diazomethane (15) or 1-methyl-3-*p*-tolyltriazene (16). The crude reaction product was chromatographed on a 50 cm x 1 cm column of Act. II-III neutral alumina (E. Merck) to isolate the ester from overmethylated products using 1:1 $CHCl_3$-pet. ether, $CHCl_3$, and 20:1 $CHCl_3$-CH_3OH. Further purification by preparative thick layer chromatography on silica gel F (M. Woelm) using benzene-10% ethanol gave 30 mg (25%) of BR dimethyl ester: λ_{max} = 398 nm (ε 59,800) and 450 nm (sh), 10^{-5} M in $CHCl_3$; λ_{max} = 413 (ε 48,500) and 450 (sh), 10^{-5} M in N-methylformamide; reported (16) λ_{max} = 400 in $CHCl_3$. (Note. Bilirubin-IXα dimethyl ester does not obey Beer's Law. The λ_{max}, ε_{max} and band shape are solvent and concentration dependent.) This material contained 80% IXα, 8% IIIα and 12% XIIIα isomers by HPLC on silica using toluene-5% ethanol (14). Pure BR-IXα dimethyl ester, free from IIIα and XIIIα isomers, was obtained similarly following isomerization of 500 mg of BR-IXα (17) to a mixture of isomers (51% IXα, 24% IIIα and 25% XIIIα) and methylation of the mixture.

Three different light sources were used for photochemical experiments. Source A was a Bausch and Lomb monochromator (Model 33-86-07) equipped with a 200 W Hg lamp emitting 10 or 20 nm bandpass monochromatic light (450 or 410 nm). Source B was an electronic photoflash unit (Kako Hi-beam III or Focal 320 BS). Source C was a 20 W Westinghouse F20T12/BB special blue fluorescent tube. Except where noted otherwise, solutions were irradiated in standard 1 cm pathlength quartz cuvettes. Analytical and preparative scale experiments on BR esters were carried out using 1.0 x 10^{-4} M solutions in $CHCl_3$ or toluene/95% ethanol (5% v/v, filtered through paper to remove excess water).

HPLC analyses were performed on a DuPont Zorbax-SIL column (25 x 0.46 cm) using a Perkin-Elmer Series 3 liquid chromatograph. Photoproducts from BR esters were analyzed by HPLC using the same solvent (toluene/95% ethanol, 95:5 v/v) used for photochemistry. Photoproducts from BR were analyzed by TLC on Cheng-Chin 50 μ polyamide sheets (Gallard-Schlesinger, methanol-1% conc. ammonium hy-



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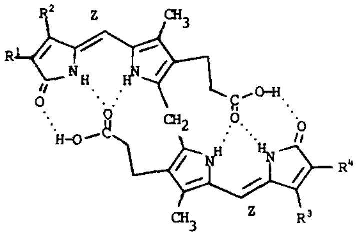

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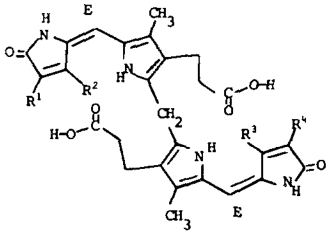
IXα: $R^1=R^3=CH_3$, $R^2=R^4=CH=CH_2$

IIIα: $R^1=R^4=CH=CH_2$, $R^2=R^3=CH_3$

XIIIα: $R^1=R^4=CH_3$, $R^2=R^3=CH=CH_2$



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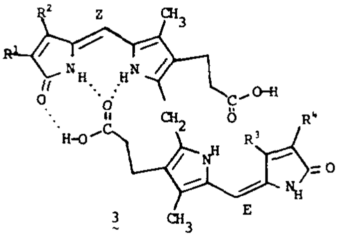

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Figure 1 Configurational isomers of BR showing geometric isomerism about the 5,15 *meso* double bonds: **1** = Z-Z, **2** = E-Z, **3** = Z-E and **4** = E-E. The Z-Z configuration (**1**) is the stable ground state structure of BR (**1**). The E-Z (**2**), Z-E (**3**) and E-E (**4**) structures are the photochemically accessible isomers which, in the case of BR-IXα, we call collectively PBR. The E-Z (**2**) and Z-E (**3**) isomers are identical for (symmetrical) BR-IIIα and BR-XIIIα but not for BR-IXα. In the corresponding BR dimethyl esters, the propionic acid groups become methyl propionate groups.

droxide). Non-photochemical manipulations were carried out under subdued light or a photographic safelight when appropriate.

Absorbance and absorbance difference measurements were done on a Cary 118 or 219 spectrophotometer. Solvents were analytical reagent grade or purified and redistilled reagent grade.

Formation and reversion of PBR (Figure 5). A solution of BR-IXα (2.5 mg) in $CHCl_3$ (10 ml) in a 10 ml Pyrex Erlenmeyer flask was purged with argon (10 min) and irradiated for 15 min, with continuous argon bubbling, with light Source C placed horizontally beneath the flask. A sample (1 ml) was removed and evaporated to dryness (Residue 1). The remaining solution was evaporated to dryness and the residue was mixed rapidly at 0° C with ice-cold CH_3OH (6 ml). The mixture was centrifuged at 4° for 2 min and the clear yellow supernatant was separated. One portion (2 ml) of this solution was added to 0.02 ml conc. NH_4OH and evaporated to dryness at once (Residue 2). A second portion (2 ml) was purged with argon in the dark (10 min), then irradiated under argon with light Source C

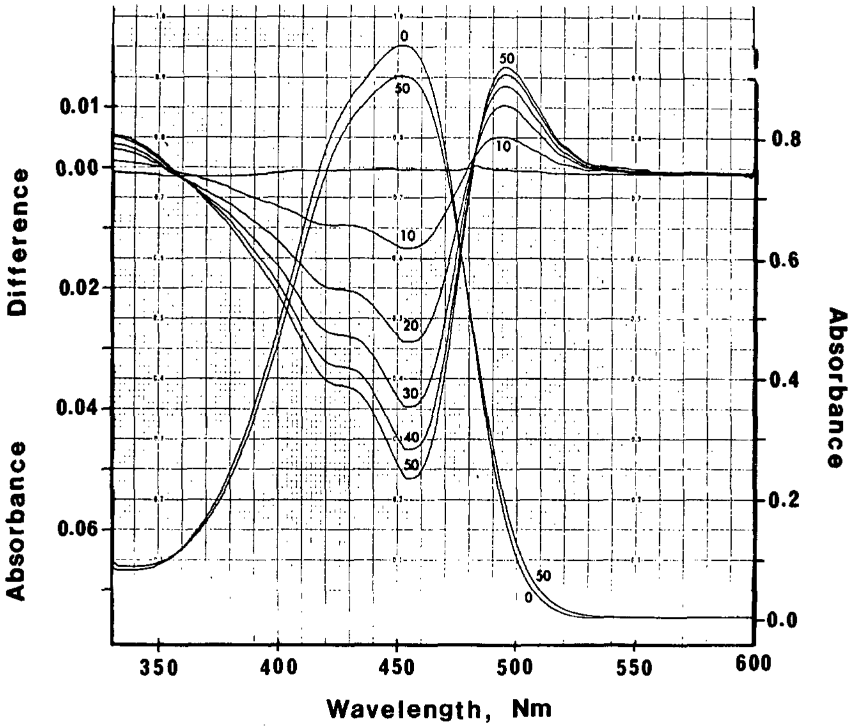

Figure 2 Absorbance difference spectra (sigmoid) obtained from irradiation of a 1.5 x 10^{-5} M solution of BR-IXα in $CHCl_3$-1% ethanol-10% triethylamine using Source A at 450 nm (10 nm bandpass). The cumulative irradiation time (sec) is indicated on each scan. Absorption curves of the sample solution before and after irradiation are superimposed on the absorbance difference spectra.

for 10 min and finally evaporated to dryness (Residue 3). The remaining CH_3OH solution was heated to 50° for 30 min and then evaporated to dryness (Residue 4). All four residues were dissolved in 1% conc. NH_4OH-CH_3OH (0.07-0.3 ml) and samples (6 λ) were chromatographed on polyamide (Figure 5). All glass vessels were silanized on the inside and solutions were evaporated rapidly at room temperature under reduced pressure. During irradiation or warming of the clear CH_3OH supernatant a precipitate formed. This was shown by TLC, in separate experiments, to be BR-IXα. For a control experiment a solution of BR-IXα (2.5 mg) in $CHCl_3$ (10 ml) was evaporated to dryness and the residue was mixed with ice-cold CH_3OH. The supernatent obtained after centrifuging this mixture was almost colorless.

RESULTS AND DISCUSSION

Irradiation of BR-IXα dimethyl ester should yield three additional geometric isomers, an E-E and a Z-E/E-Z pair (Figure 1). In contrast, each of the two symmetrically substituted compounds, BR-IIIα dimethyl ester and BR-XIIIα dimethyl ester, should yield only two: a Z-E (= E-Z) and an E-E isomer (Figure 1). When BR-IXα dimethyl ester was irradiated in toluene-ethanol or $CHCl_3$ with a photoflash (Source B) or monochromatic light (Source A) at 410 nm, changes in the ab-

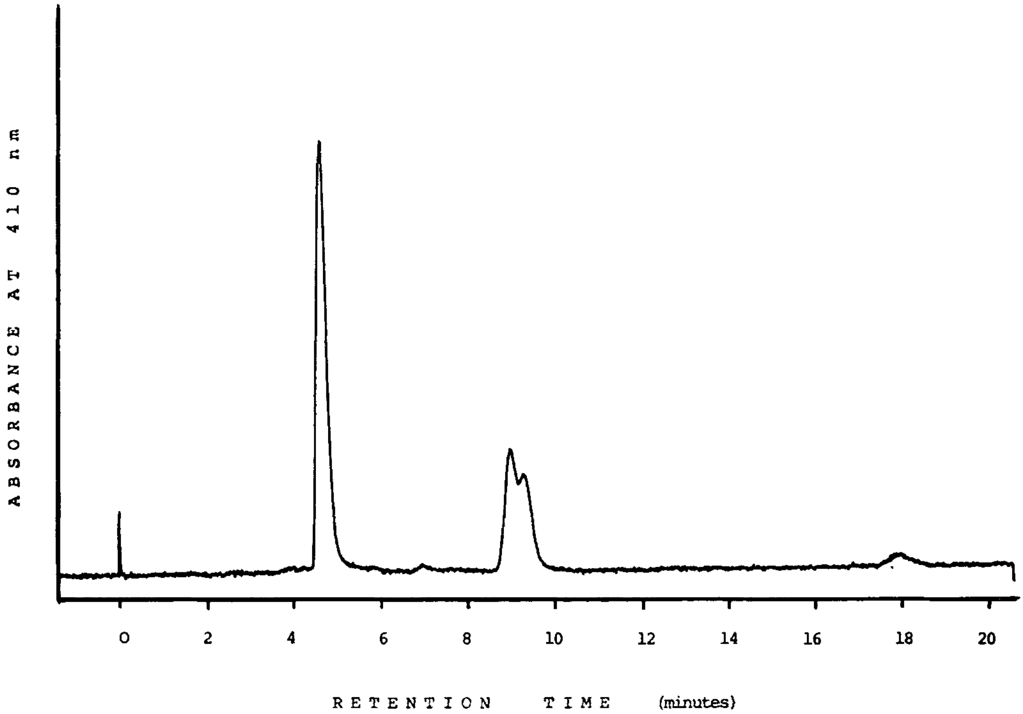

Figure 3 HPLC scan of BR-IXα dimethyl ester at photoequilibrium after irradiation in toluene-ethanol (95%) (95:5 v/v) with Source A at 410 nm (10 nm band-pass). The detector was set at 410 nm and the solvent flow rate was 1.0 ml/min. The retention times (min) of the isomers are: Z-Z (4.6), E-Z (8.9), Z-E (9.3) and E-E (17.9) using the same solvent system (14).

sorbance difference spectrum equivalent to those obtained with BR (Figure 2) were obtained, although shifted to shorter wavelengths. Just as with BR-IXα (11), the reaction proceeded to a stationary state which was maintained on further brief irradiation. Analysis of the photo-equilibrium mixture by HPLC revealed three new compounds as expected (Figure 3); two major components, with similar mobilities, and a more polar minor component. In contrast, the photo-equilibrium mixture obtained from BR-IIIα dimethyl ester or BR-XIIIα dimethyl ester (Figure 4) contained only two new compounds, a major one and a minor one. When the photo-equilibrium mixtures from BR-IXα dimethyl ester, BR-IIIα dimethyl ester or BR-XIIIα dimethyl ester were allowed to stand at room temperature in the dark, slow thermal reversion of the reaction occurred, as shown by difference spectroscopy and by HPLC. Recalling our previous observations on BR-IXα (11), we conclude that the three photoproducts from the IXα ester are E-Z, Z-E and E-E isomers and that the two photoproducts from each of the IIIα and XIIIα esters are E-Z and E-E isomers.

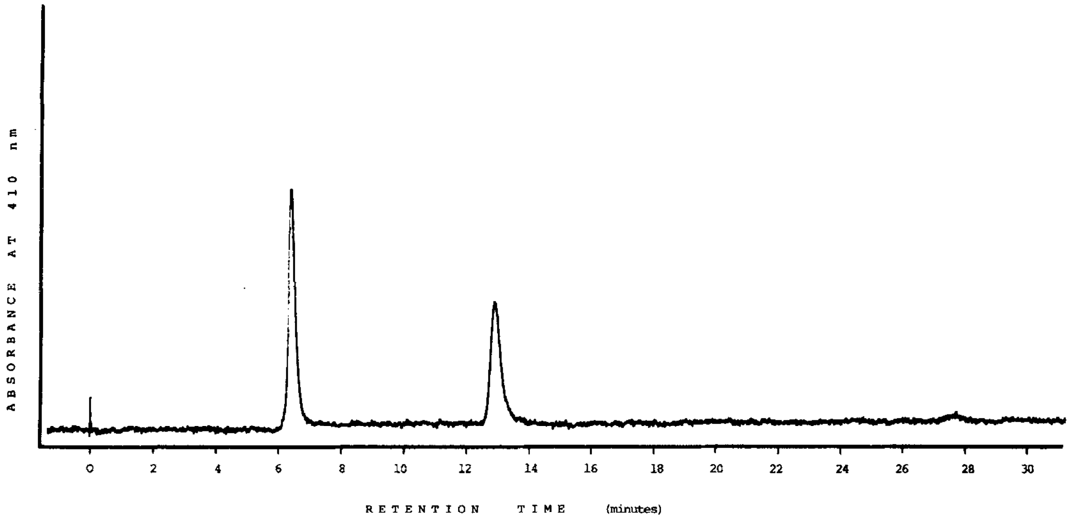

Figure 4 HPLC scan of Br-XIIIα dimethyl ester at photoequilibrium after irradiation in toluene-ethanol (95%) (95:5 v/v) with Source A at 410 nm (10 nm bandpass). The detector was set at 410 nm and the solvent flow rate was 1.0 ml/min. The retention times (min) of the isomers are: Z-Z (6.4), E-Z = Z-E (12.9) and E-E (27.6) using the same solvent system (14). For BR-IIIα dimethyl ester treated similarly, the retention times are: Z-Z (4.0), E-Z = Z-E (6.8) and E-E (12.6).

The two E-Z/Z-E isomers derived from the IXα ester did not revert to starting material at identical rates. The least polar of the pair reverted at a rate that was comparable to the E-Z → Z-Z reversion of BR-XIIIα dimethyl ester. The more polar member reverted to Z-Z BR-IXα dimethyl ester more slowly and at a rate that was similar to the E-Z → Z-Z reversion rate of BR-IIIα dimethyl ester. This suggests that the more mobile E-Z isomer of BR-IXα dimthyl ester is the one with an E double bond in the half of the molecule that contains an *endo* vinyl substituent (2, Figure 1). Conversely, the less mobile Z-E isomer is the one with an E double bond in the half of the molecule with an *exo* vinyl group (3, Figure 1).

Reversion of the E-E isomers of BR-IIIα, -IXα and -XIIIα dimethyl ester, measured by their rate of disappearance, was slower than the reversion of the corresponding Z-E or E-Z isomers. On the other hand, formation of the photostationary state mixture of configurational isomers was faster from BR-IXα dimethyl ester than from BR-IXα itself in the same solvent. This undoubtedly reflects the weaker intramolecular hydrogen bonding in the ester and the consequently lower activation energy for isomerization.

When considered together with our earlier observations on BR-IXα (11), the present observations provide compelling evidence that BR-IXα and its dimethyl ester readily undergo geometric photoisomerization in visible light, and that PBR contains E-Z and Z-E BR-IXα together with a small proportion of E-E BR-IXα. Other possible reactions (18) such as photooxidation, photoaddition, photodisproportionation to IIIα and XIIIα isomers, and formation of "430 pigment" are inconsistent with the experimental data and, and in any case, proceed more slowly than PBR formation.

The naturally occurring Z-Z isomer of BR-IXα has the extensively intramolecularly hydrogen-bonded structure depicted in Figure 1 (1,2). Consequently, it is lipophilic and very sparingly soluble in water at physiologic pH (3,4,5). Among the four configurational isomers of BR-IXα, the Z-Z is unique because, as molecular models show, none of the other three can assume such a compact and highly hydrogen-bonded conformation. The other three isomers, therefore, ought to be more polar, less lipophilic and possibly more acidic than the natural Z-Z pigment. This prognosis was confirmed by the following observations. 1. On polyamide TLC or reverse-phase HPLC (11) PBR migrated ahead of BR as expected for a more polar material. 2. When an irradiated solution of BR-IXα in $CHCl_3$ (4.3×10^{-4} M) on its way to or at photo-equilibrium was shaken briefly with an equal volume of 0.1 M $NaHCO_3$, 0.1 M Tris buffer (pH 8.5 or 9) or ammonium acetate buffer (pH 8.4), yellow pigment (PBR) was transferred to the aqueous phase. By contrast, no yellow coloration of the aqueous extracts was detected in control experiments with unirradiated $CHCl_3$ solutions of BR-IXα. 3. The residue obtained after evaporation of an irradiated solution of BR-IXα in $CHCl_3$ was partly soluble in CH_3OH; whereas, the residue from an unirradiated control solution was totally insoluble. The pigment that dissolved in the CH_3OH was identified as PBR by TLC and reverted to BR thermally and photochemically (Figure 5).

Although PBR partitioned from $CHCl_3$ into pH 8.5 - 9.0 Tris buffer, it was not extracted by 0.1 M phosphate buffer of pH 7.4. Aqueous extracts containing PBR were unstable. They became colloidal on standing, showing a Tyndall effect, and yielded only BR-IXα on acidification with acetic acid and back extraction into $CHCl_3$. Similarly, solutions containing PBR in methanol were unstable. On standing in CH_3OH at room temperature, or more rapidly on irradiation or warming, PBR reverted mainly to BR which precipitated from the solution (Figure 5). However, ammoniacal CH_3OH solutions containing PBR were considerably more stable, and thermal reversion occurred much more slowly (11).

CONCLUSION

The ease with which PBR is formed *in vitro*, even in the presence of oxygen (11), suggests that it may well be formed *in vivo* during phototherapy of jaundiced patients or on irradiation of congenitally hyperbilirubinemic rats (Gunn rats) (6). Indeed, the reaction kinetics make it a more likely reaction than

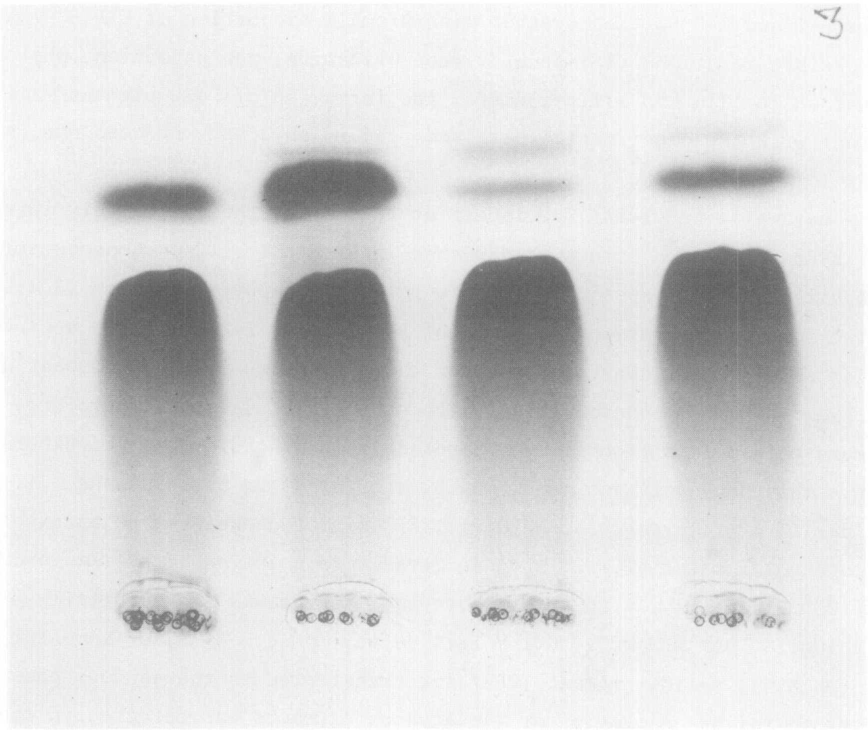

Figure 5 *Formation of PBR and its solubility and instability in* CH$_3$OH. The polyamide TLC plate (overloaded to facilitate photography and reproduction) shows, left to right: (1) Residue after irradiating BR in CHCl$_3$. (2) Product obtained after extracting (1) with CH$_3$OH. (3) Product obtained after irradiating (2) in CH$_3$OH. (4) Product obtained on warming (2) in CH$_3$OH. The main, lower R$_f$ band is BR; bands above this are PBR. The plate was photographed through a blue filter (Kodak Wratten 5A). For further details see Materials and Methods.

self-sensitized photooxidation of BR which has also been postulated to occur *in vivo* (6,18). Furthermore, the solubility properties of PBR, which resemble those of biliverdin-IXα, indicate that, like biliverdin (19), PBR would not require conjugation for its hepatic excretion. We suggest, therefore, that during phototherapy, PBR formed photochemically from BR near the surface of the skin is transported in plasma to the liver where it is taken up and excreted into bile. This would explain the almost instantaneous biliary pigment excretion that occurs when Gunn rats are exposed to light (20) and the presence of much unconjugated BR in extracts of pigmented bile obtained during phototherapy (7,21). Due to the facile thermal and photochemical reversion of PBR, solvent extraction of bile under the usual conditions would tend to yield BR rather than its isomers, even if they were present initially. Although the steady-state concentration of PBR in peripheral tissues would remain low during phototherapy, the efficiency of the overall process would be high because the PBR being continuously swept away and excreted would be replenished constantly by newly-formed PBR. Thus, PBR formation provides a particularly plausible rationale for the enhanced biliary excretion of unconjugated BR in jaundiced rats and humans exposed to light. Although excretion of unconjugated BR is not the only process that contributes to the effectiveness of phototherapy, the available data (6,18,21) indicate that it is quantitatively the most important. Therefore, if our hypothesis is correct, PBR formation is the most important and fundamental reaction in phototherapy of neonatal jaundice.

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