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Photobilirubin: An early bilirubin photoproduct detected by absorbance difference spectroscopy

(*Z-E* configurational photoisomers/phototherapy/neonatal jaundice/bile pigments)

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ABSTRACT **Absorption of light converts bilirubin-IXα in solution to a mixture of what are probably *cis-trans* geometric isomers. This reaction is much faster than other photochemical reactions of bilirubin and reaches photoequilibrium before losses due to photooxidation are significant. At room temperature in the dark in the presence of trifluoroacetic acid or iodine or simply on standing, the photoproducts revert to the natural isomer. They also revert under visible light. Their formation and reversion can be followed by chromatography on polyamide and by absorbance difference spectroscopy.**

In 1970 Davies and Keohane (1) showed by absorbance difference (AD) spectrophotometry that, upon brief irradiation with visible light, bilirubin-IXα (BR) in chloroform or buffered serum albumin solution is converted rapidly to a photolabile product absorbing at about 490 nm. This transient early photoproduct, which we will call photobilirubin‡ (PBR), was not isolated or identified, but the suggestion has been made that it might play some part in phototherapy of neonatal jaundice (2). Direct evidence to substantiate this has not been published and most subsequent studies on the photochemistry and photometabolism of BR have ignored the reaction.

In this paper we wish to confirm and explain the observation of Davies and Keohane and show that PBR formation is an important general photochemical reaction of BR that accompanies or precedes other photochemical transformations of the pigment. Evidence that it is the key reaction in phototherapy of neonatal jaundice will be published elsewhere.

MATERIALS AND METHODS

Bilirubin [Sigma, Koch-Light (Colnbrook, U.K.), or Matheson] was purified as described (3) or by washing a chloroform solution of it three times with 5% aqueous $NaHCO_3$ and crystallization from chloroform/methanol, 1:1 (vol/vol). Purified BR contained less than 5% of IIIα and XIIIα isomer impurities (4) as determined by thin-layer chromatography (TLC) (4) or by high-performance liquid chromatography (HPLC) on a DuPont Zorbax-SIL column (25 cm × 4.6 mm; chloroform/0.75% ethanol/1% acetic acid) (5). Photoproduct formation was followed by TLC on Cheng Chin 50-μm polyamide sheets [(Gallard-Schlesinger, Carle Place, NY), methanol/1% conc. ammonium hydroxide] or HPLC on a DuPont Zorbax-ODS column (25 cm × 4.6 mm; 0.1 M methanolic ammonium acetate or methanol/water 95:5, containing 0.05 M ammonium formate). HPLC analyses were run on a Perkin–Elmer series 3 instrument or a DuPont model 848 liquid chromatograph equipped with a model 837 variable wavelength detector. Detectors were set at 450 nm.

Except where noted otherwise, photochemical experiments were carried out with 15–30 μM solutions of BR in stoppered 1-cm-pathlength quartz cuvettes. Three different light sources were used. 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 (generally at 440 nm and filtered through Lucite to remove shorter wavelength harmonics). Source B was a Duro-Test R57 400-W high-pressure Hg lamp filtered (Corning no. 3389) to transmit >391 nm. Source C was a 20-W Westinghouse F20T12/BB special blue fluorescent tube. In some experiments, solutions were deoxygenated with argon.

Absorbance and AD measurements were carried out on a Cary 219 or Cary 118 dual beam spectrophotometer. For AD measurements, aliquots of the same solution were placed in both sample and reference beams, and the offset baseline was balanced to zero absorbance. Then, changes produced in the sample solution by illumination were recorded.

Isolation of PBR was achieved by irradiation of a 0.1 mM solution of BR in 1% methanolic ammonia followed by HPLC separation and collection of the PBR peak. In this reverse-phase system, PBR is eluted before BR.

Analytical reagent grade solvents were used as supplied. Reagent grade solvents were purified and redistilled.

RESULTS AND DISCUSSION

Fig. 1 shows AD spectra generated by irradiation of bilirubin in chloroform and methanol (containing 0.2–1% concentrated ammonium hydroxide) with monochromatic (440 nm, 10-nm bandpass) light. These spectra represent changes in the irradiated sample relative to the unirradiated reference. As reported for chloroform and carbon tetrachloride solutions (1, 2), irradiation produced a "loss" peak near 460 nm and synthesis peaks near 500 and 350 nm. Essentially similar results were obtained with light source C and for solutions of BR in chloroform/1% *n*-hexane, chloroform/1% ethanol/5–50% triethylamine, chloroform/1% ethanol/40 mM 2,3-dimethyl-2-butene, and benzene. In benzene the 490-nm synthesis peak eventually merged with another broad synthesis peak near 670 nm that, presumably, is due to biliverdin (6). Irradiation of BR in chloroform/1% *n*-hexane (7) at 420 or 430 nm or in ammoniacal methanol at 400, 410, or 420 nm gave results similar to those obtained at 440 nm. However, some excitation wavelength dependence was observed. In chloroform/1% *n*-hexane no ≈500-nm synthesis peak was formed on irradiation at 340 or 280 nm.

The ≈500-nm AD band was formed in anaerobic or aerobic solutions; in the latter, its formation was not markedly in-

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Abbreviations: BR, bilirubin-IXα; PBR, photobilirubin; AD, absorbance difference; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

‡ The trivial name "photobilirubin" is introduced solely to facilitate discussion. It is not intended to imply chemical homogeneity.

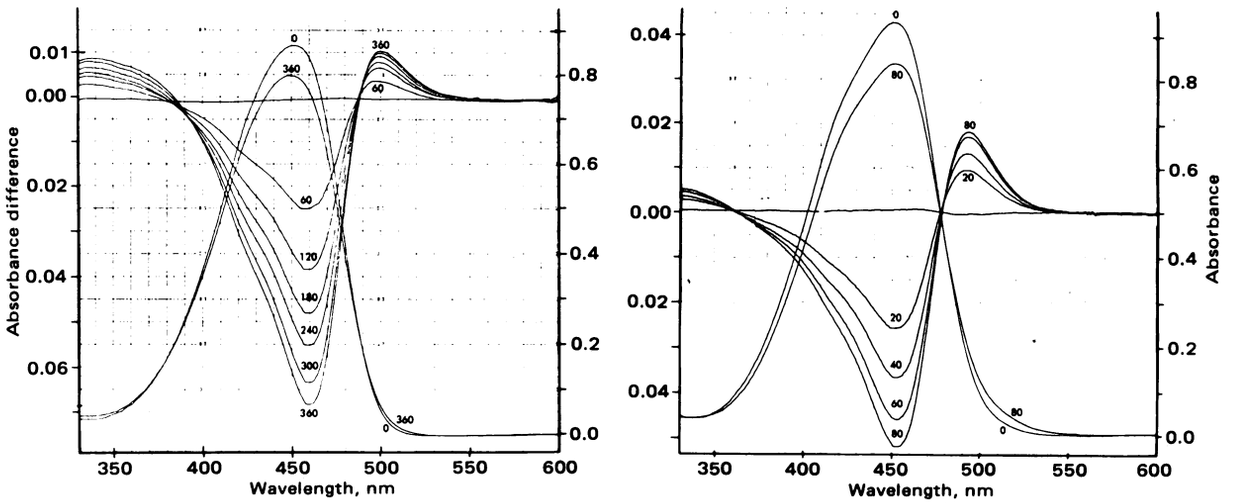


FIG. 1. AD spectra (sigmoid) obtained from irradiation of 15 μM BR solutions with source A. Cumulative irradiation time (sec) is indicated on each scan. Absorption curves of sample solutions before and after irradiation are superimposed on the AD spectra. (*Left*) In chloroform/1% ethanol; (*Right*) in methanol/1% conc. ammonium hydroxide.

fluenced by the presence of singlet oxygen scavengers such as dimethylbutene, diethylamine, and triethylamine. During irradiation, the ≈500-nm absorbance progressively increased to a maximum that was then maintained with further irradiation on the same time scale. This maximum was reached fairly rapidly (e.g., in 1–10 min with source A and in 1–3 min with source C) and more rapidly in basic solvents such as chloroform/1% ethanol/triethylamine and ammonia/methanol than in neutral solvents such as benzene, chloroform/1% ethanol, or chloroform/1% *n*-hexane. Further changes in the maximum occurred only with significantly longer irradiation times. Prolonged irradiation caused a decrease in the ≈500-nm synthesis peak and an increase in the ≈460-nm loss peak, presumably due to photooxidation (8) of BR and PBR. Apparently, a photoequilibrium is established rather rapidly before losses due to photooxidation become detectable.

When an irradiated solution of BR at photoequilibrium (as determined by AD spectra) was allowed to stand in the dark, slow but significant thermal (22°C) reversal occurred (Fig. 2) as shown by the decrease in the absolute values of the AD peaks at ≈460 and ≈500 nm and the tight isosbestic points. More rapid reversal occurred on addition of a trace of trifluoroacetic acid (essentially instantaneous upon mixing) or iodine (Fig. 3) or on irradiation of the sample with 510-nm (10-nm band pass) monochromatic light (Fig. 4). With the latter, a new photo-

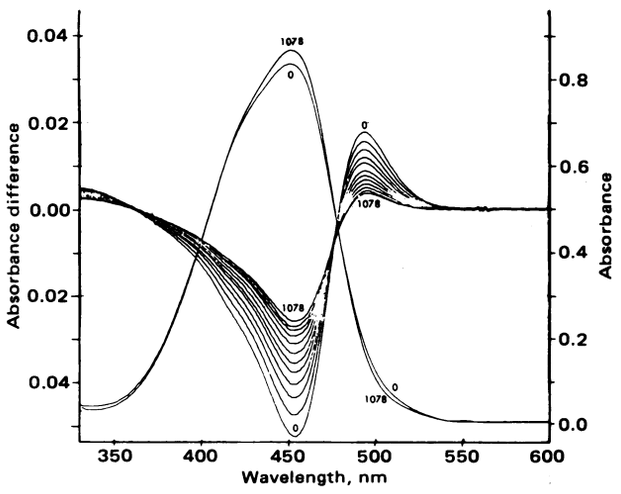


FIG. 2. Thermal reversion of photoisomerized BR, 15 μM in methanol/1% conc. ammonium hydroxide, as detected by AD spectroscopy. BR was irradiated to photoequilibrium [80 sec, source A (Fig. 1 *right*)] and then AD spectra were scanned every 97 min while the solution was kept in the dark at 22°C. Absorption curves of the sample solution at photoequilibrium (0) and late in the thermal reversion (1078 min = 18 hr) are superimposed on the AD spectra.

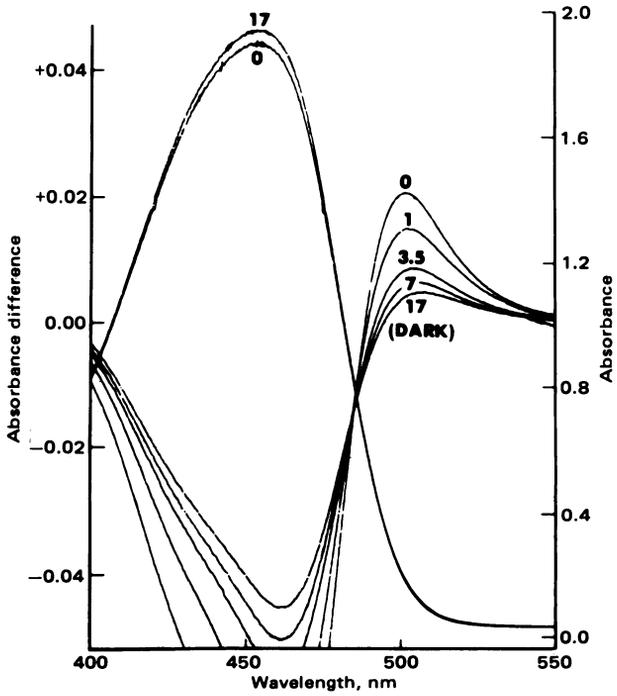


FIG. 3. Iodine-catalyzed reversion of photoisomerized BR in chloroform/1% ethanol as detected by AD spectroscopy. BR (30 μM) was irradiated (100 sec, source C) to photoequilibrium, equal amounts (1 μl) of 1.13 μM I_2 in chloroform/1% ethanol were added to each cuvette to a final concentration of 0.87 μM, and AD spectra were scanned with time (min) while the solution was kept in the dark. Absorption curves of the sample solution at photoequilibrium (0) and late in the I_2-catalyzed reversion (17 min) are superimposed on the AD spectra.

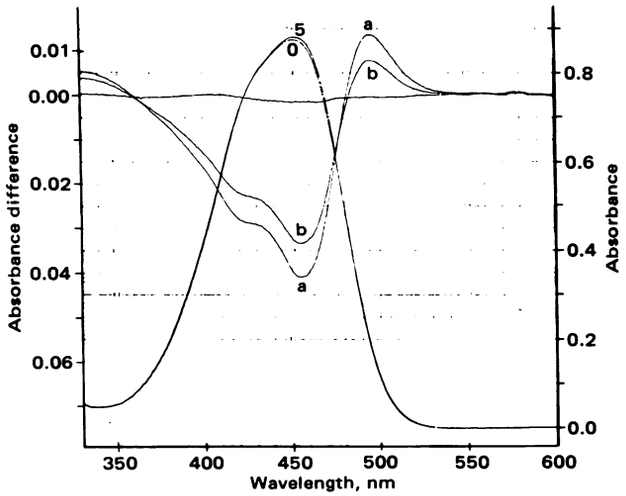


FIG. 4. Photoreversion of photoisomerized BR in chloroform/1% ethanol/10% triethylamine as detected by AD spectroscopy. BR was irradiated to photoequilibrium with 440-nm light (50 sec, source A, 10-nm bandwidth) to give AD spectrum a. Irradiation of the sample for 5 min with 510-nm light (10-nm bandwidth) from the same source gave AD spectrum b, which did not change on further brief irradiation (1–5 min). Absorption curves of the sample solution before and after 510-nm light irradiation are superimposed on the AD spectra.

stationary state, containing less PBR, was reached. Thus, irradiation leads to a photostationary state, and the photoproducts revert to starting material thermally, catalytically, or by photoirradiation in a region where they absorb more strongly than BR.

When irradiated solutions on the way toward and at photoequilibrium were sampled and analyzed by TLC, formation of a new yellow product, PBR ($R_F \approx 0.6$; BR has $R_F \approx 0.4$) could be seen (Fig. 5). Analysis by HPLC showed that the BR concentration decreased and the PBR concentration increased as photoequilibrium was approached. Once photoequilibrium

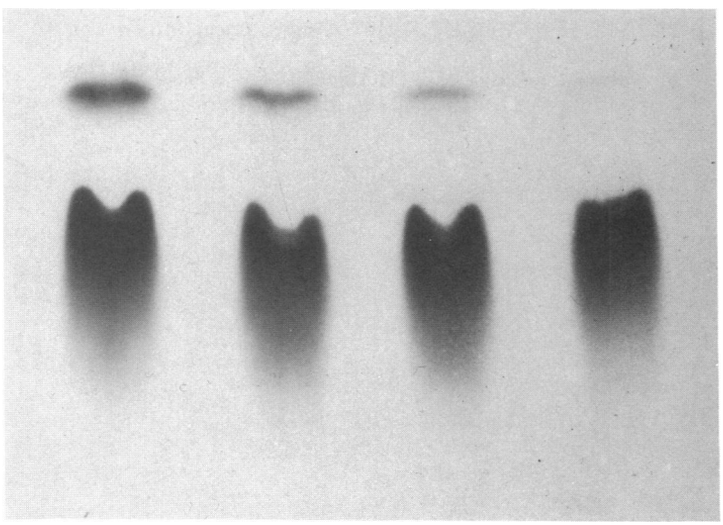


FIG. 5. Formation of PBR in chloroform. A solution (10 ml; 0.85 mM) of BR in chloroform in a 10-ml Pyrex erlenmeyer flask was purged with argon (10 min) and then irradiated, with continuous argon bubbling, with light source C placed horizontally beneath the flask. Aliquots of equal volume were removed at 2, 5, 10, and 15 min (right to left) from the start of irradiation and spotted on polyamide in dim light. The layer was kept in the dark until the last spot had been applied and then developed in the dark. The lower spot is BR. The photograph was taken through a blue filter (Kodak Wratten filter no. 47), and the plate was overloaded to increase visibility.

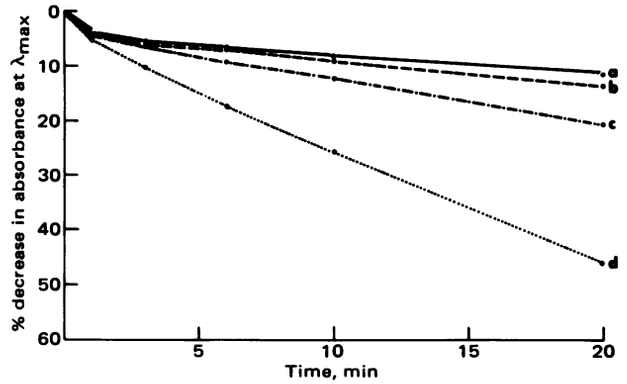
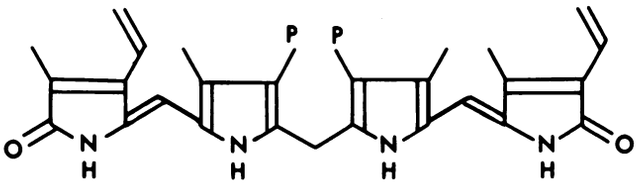
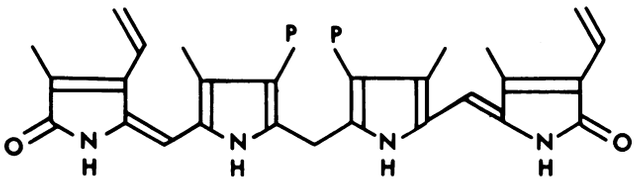


FIG. 6. Photooxidation of BR in chloroform. Solutions of BR (15 μM) in chloroform were irradiated with light source B, and loss of pigment was followed spectroscopically by the decrease in absorbance at λ_{max} (≈453 nm). Curves: a, argon-saturated; b, oxygen-saturated and containing 0.15 M 1,4-diazobicyclo[2.2.2]octane; c, oxygen-saturated and containing 1.5 mM dimethylbutene; d, oxygen-saturated.

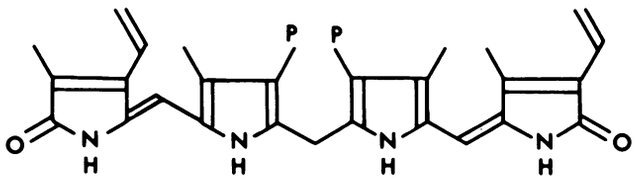
was reached, the BR and PBR concentrations did not change significantly with further short-term irradiation. Similarly, the photochemical and thermal reversion processes could be followed by TLC and HPLC; with the latter, a good 1:1 correspondence of PRB disappearance and BR appearance was ob-



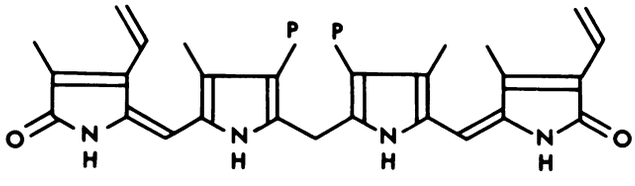
5Z, 15Z



5E, 15Z



5Z, 15E



5E, 15E

FIG. 7. Configurational isomers of BR. The top structure has the naturally occurring 5Z,15Z configuration. P, CH_2CH_2COOH.

served. PBR could not be detected by HPLC or TLC on silica because, being sensitive to acid, it reverts to BR.

Isolation of PBR by HPLC afforded yellow material with a single broad absorption band (λ_{max} 440 nm) and no peak near 500 nm. This material was always contaminated with varying amounts of BR due to thermal reversion after elution. Unreacted BR was also isolated and shown to be identical (UV-visible spectrum and HPLC) to starting BR. When isolated PBR in chloroform was placed in a sample cuvette with a chloroform solution of BR in the reference cuvette, AD spectra were obtained that mimicked those generated photochemically. Therefore, the peaks detected by AD spectroscopy on irradiation of BR are not the actual absorption maxima of BR or PBR. Rather, they result from the addition of positive and negative curves and actually represent differences in absorption between BR and PBR. An analogous effect is observed with oppositely signed overlapping circular dichroism spectra (9).

The rapid formation of PBR was also noticeable in kinetic studies of BR photooxidation. Fig. 6 shows the rate of self-sensitized photooxygenation of BR in chloroform in the presence of various inhibitory substances (10). Although the individual reaction rates were different after the first minute, the loss of BR during the first minute was similar in each case. This early oxygen-independent loss of absorbance is due to PBR formation. We have observed a similar phenomenon during photooxidation of BR in aqueous buffer (pH 7.4), in detergents (Triton X-100, cetyltrimethylammonium bromide, pH 7.4), in rat serum, in aqueous bovine serum albumin (pH 7.4), and in several organic solvents including dimethyl sulfoxide, ammoniacal methanol, benzene, and pyridine. This suggests that rapid formation of PBR occurs in all of these media. Early changes in the spectra of irradiated BR/albumin solutions also have been noted by others (1, 2, 11).

These findings show that when BR is irradiated in solution it is converted rapidly to an equilibrium mixture containing BR and a novel substance, PBR. The absorption spectra of BR and PBR are rather similar and almost congruent. But there are differences, and in several solvents formation or disappearance of PBR is manifested by complementary changes in the AD spectra near 460 and 500 nm. Formation of PBR does not require oxygen and is faster than the aerobic photooxidation of BR. The reaction occurs in a wide variety of polar and nonpolar media and under neutral or basic conditions, and it is reversible photochemically, thermally, and catalytically. The most plausible explanation for these findings is $Z \rightarrow E$ photoisomerization (12, 13) of BR at the exocyclic enamide carbon-carbon double bonds. In its most stable state, BR has the 5Z,15Z configuration (14). Photoisomerization should generate a pair of almost identical *Z,E* isomers and smaller amounts of an *E,E* isomer (8) (Fig. 7). We believe that PBR is a mixture of these geometric isomers containing predominantly the 5Z,15*E*/5*E*,15Z pair. Further evidence to support this conclusion and to show that PBR formation *in vivo* is crucial for the successful treatment of neonatal jaundice by phototherapy will be reported separately.

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