Molecular Mechanisms of Phototherapy for Neonatal Jaundice

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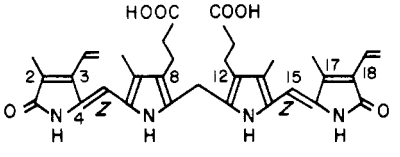
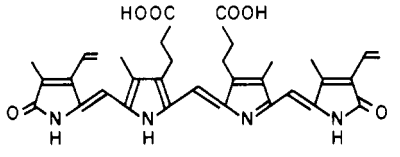
Go into any intensive-care nursery and the chances are that one or two of the tiny infants will be lying, almost naked but securely blindfolded, in a pool of bright blue or white light. The light is not illuminational but therapeutic. The infants, like tens of thousands of others per year, are receiving phototherapy—a treatment to prevent accumulation of the potentially toxic yellow metabolite bilirubin (1). Light has been used prophylactically in this way for nearly a quarter of a century without any firm idea of why it works. In this Account we describe some of our studies on the mechanism of phototherapy. The underlying photochemistry has turned out to be unexpectedly subtle and simple. It demonstrates that quantum-efficient photochemical reactions observed in the test tube can also be carried out in as complex a vessel as the human body. And it provides a textbook example of interdependence among structure, conformation, H bonding, and biological transport. The studies have also revealed a system where chemical intuition can be misleading, where a carboxylic acid may be less polar than its methyl ester, and where compounds with similar structures exhibit profound differences in their chemical and biological properties. For example, of **1** and **2**, who would guess that only **2** is soluble in methanol?



BILIRUBIN (1)



BILIVERDIN (2)

David Lightner was born in Los Angeles, CA, in 1939, obtained his A.B. in 1960 from the University of California, Berkeley and his Ph.D. from Stanford University in 1963. After postdoctoral studies at Stanford and the University of Minnesota, he served on the faculty at UCLA and Texas Tech. Presently he is R. C. Fuson Professor of Chemistry at the University of Nevada, Reno, a Fellow of the Center for Advanced Study, and a member of the Cellular and Molecular Biology faculty. His research interests include synthesis and stereochemistry, photochemistry, photobiology, and phototherapy.

Tony McDonagh was born in England in 1938, carried out his undergraduate education at the Royal College of Advanced Technology, Salford, U.K. and the University of London, and received his Ph.D. in 1967 from Vanderbilt University. After postdoctoral studies at Queen Mary College, University of London, he joined the Gastrointestinal Unit of the Department of Medicine, UCSF in 1970, where he is a Research Chemist. Dr. McDonagh's scientific interests include tetrapyrrole chemistry, photobiology and phototherapy, hepatic metabolism and transport processes, and organic stereochemistry.

Bilirubin and Jaundice. Bilirubin (BR) is produced in nature by enzymic ring opening of heme (Figure 1).[1] Most heme in the body is contained in red cells, and these are the major source of BR. They are synthesized and destroyed at about 3×10^6 cells/s, thereby generating ~300 mg of BR/day. BR formation requires two enzymes, one membrane bound, the other soluble. The membrane-bound enzyme, heme oxygenase, clips open the heme ring regiospecifically at its most lipophilic edge, extruding the bridging α-carbon as CO.[2] Elision of CO and loss of iron from the resulting complex affords the blue-green pigment biliverdin (**2**), better known, perhaps, as the blue of birds eggs and the green of the praying mantis.[3a] Although compounds similar to **2**, e.g., phytochrome[3b] and phycocyanobilin,[3c] are vital in plants and algae, **2** has no known function in mammals.[3] Once formed, it is reduced by the abundant enzyme biliverdin reductase to another metabolically useless substance, BR (**1**).[1] As explained below, the chemical properties of BR are anomalous, and in contrast to **2**, it is lipophilic. It also is poorly soluble in water at physiologic pH and binds avidly to certain proteins, particularly serum albumin and ligandin, a protein found in liver cells.[1a,b,3a] These properties make BR essentially unexcretable, i.e., too lipophilic and protein bound to pass efficiently out of the circulation across either of the selective barriers, kidney and liver, into urine or bile (Figure 2). Nature circumvents this problem with a device that is also used to detoxify and excrete many of substances of exogenous origin: "conjugation" with glucuronic acid. The COOH groups of BR are esterified by a glucuronyl transferase enzyme to give mono- and di-1-*O*-acyl-β-glucuronides (Figure 1). The glucuronides, synthesized mainly in the liver, are more polar than BR, so polar that they are excreted readily in bile.[1a,b,4]

Normally, BR and its metabolites cycle through us silent and unseen. Get a bruise or liver disease, and they may appear. The yellow of a healing bruise is due

(1) (a) Schmid, R.; McDonagh, A. F. In "The Metabolic Basis of Inherited Disease"; Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Eds.; McGraw-Hill: New York, 1978; Chapter 51. (b) Chowdhury, J. R.; Wolkoff, A. W.; Arias, I. M. In "The Liver"; Arias, I. M.; Popper, H.; Schachter, D.; Shafritz, D. A., Eds.; Raven Press: New York, 1982; Chapter 18. (c) Kikuchi, G.; Yoshida, T. *Mol. Cell. Biochem.* **1983**, *53/54*, 163.

(2) Humans, even nonsmokers, continuously exhale endogenous CO, and expired CO can be used to measure the rate of red-cell destruction.

(3) (a) McDonagh, A. F. In "The Porphyrins"; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. 6, Chapter 6. (b) Smith, W. O. *Photochem. Photobiol.* **1981**, *33*, 961. (c) Lagarias, J. C.; Glazer, A. N.; Rapoport, H. *J. Am. Chem. Soc.* **1979**, *101*, 5030.

(4) It is unknown why some animals reduce biliverdin, which is excretable, to BR, which is toxic and unexcretable. If the heme ring were opened at any other bridge but the α, reduction would be inconsequential because the corresponding biliverdin *and* BR isomers would all be polar and excretable.

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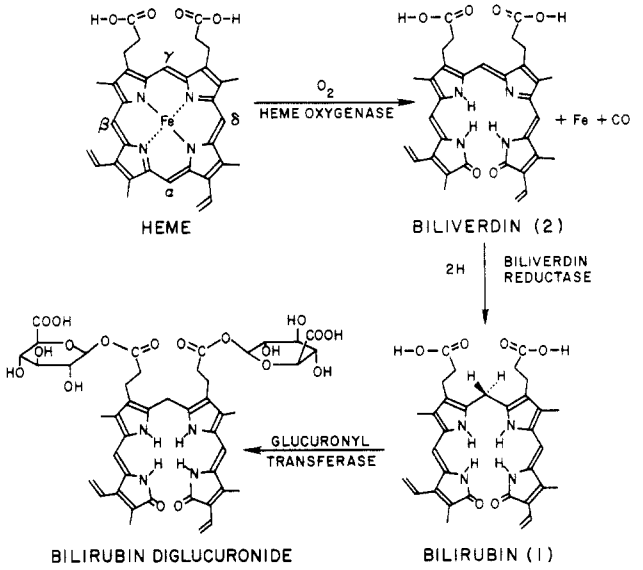


Figure 1. Biosynthesis of BR. In the first step, heme is oxidized at the α-bridge and this carbon extruded as CO. Nonenzymic ring opening in protein-free solvents occurs randomly at all four bridges, yielding three additional isomers. See: Bonnett, R.; McDonagh, A. F. *J. Chem. Soc., Perkin Trans. 1* **1973**, 881. Although only one end product is shown, the glucuronyl transferase catalyzes formation of both mono- and diglucuronides of BR. If these accumulate in vivo, they undergo isomerization by migration of the BR acyl group to vicinal OH groups of the sugar (Jansen, P. L. M. *Clin. Chim. Acta* **1981**, *110*, 309. Compernolle, F.; Van Hees, G. P.; Blanckaert, N.; Heirwegh, K. P. M. *Biochem. J.* **1978**, *171*, 185.).

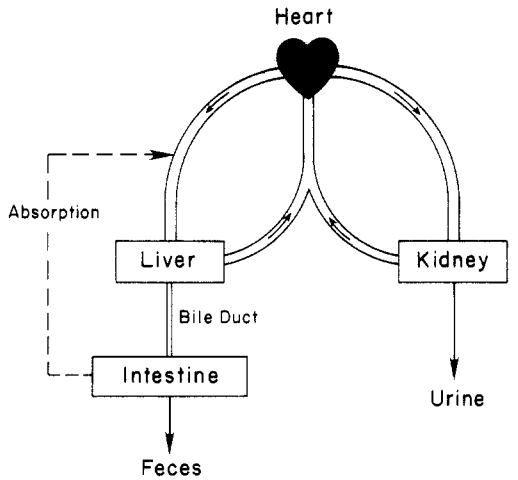


Figure 2. Chemist's eye view of organs involved in hepatic and renal excretion.

to BR formed from red cells squashed out of the capillary bed. In several diseases, especially disorders of the hepatobiliary system, formation and/or excretion of BR glucuronides become impaired. BR and its glucuronides then accumulate in the circulation, producing *hyperbilirubinemia*. Measuring the concentrations of BR species in blood plasma or serum often helps in diagnosing the underlying disorder.[1a,b] In moderate to severe hyperbilirubinemia, the yellow color of the accumulated pigments becomes visible in the whites of the eyes or the skin. This symptom is known as *jaundice* or icterus. Jaundice and hyperbilirubinemia, therefore, are not diseases but symptoms, usually of liver disease.

To this general scheme, there are two exceptions: the human fetus and newborn. BR glucuronyl transferase activity in fetal and newborn liver is very low.[5] This presents no problem for the fetus, because BR, being lipophilic, can pass readily across the placenta from the fetal to maternal circulation to become glucuronidated and excreted by the mother's liver.[1a,b] The newborn, however, does not have this option. So after birth BR begins to accumulate. Since BR forms a high-affinity association complex with albumin,[3a] much of the accumulated pigment is sequestered, bound to albumin, in interstitial fluid or blood.[3] Consequently, newborn infants invariably develop hyperbilirubinemia. Often they become perceptibly yellow and are said to have *neonatal jaundice* or physiologic jaundice of the newborn.[6] Neonatal jaundice is, of course, only transient. During the first week of life, BR glucuronyl transferase activity in the liver increases, and within about 7–14 days the baby is usually able to synthesize and excrete BR glucuronides as efficiently as a grown-up.[5,6] Any jaundice gradually dwindles. Unfortunately, *bilirubin is toxic*[3a,b] and in severe hyperbilirubinemia sufficient pigment may partition into the brain to cause irreversible damage, even death. This becomes more likely as the amount of BR in the blood increases and as the capacity of albumin to bind and sequester it decreases. A plasma BR concentration of $\gtrsim$0.25 mM, or roughly one-half the albumin concentration ($\sim$0.5 mM), is generally considered to be potentially hazardous.[6-8] Such severe hyperbilirubinemia occurs in only a small percentage of babies and is most common in premature low-birthweight infants and those with excessive BR production. For babies considered to be at risk, three things can be done: nothing, just wait and hope that the liver matures soon enough to clear the BR unaided; exchange transfusion, in which blood—and, along with it, the threatening pigment—is drained from the child's body and replaced with "clean" blood; or phototherapy, irradiate the baby with visible light.

Phototherapy. The discovery of phototherapy stems from the observations of J. Ward, a nurse who supervised a premature baby unit in Essex, U.K.[9] Apparently an advocate of the fresh-air-and-sunshine school of medicine, Sister Ward would sometimes take her wee patients outdoors when the weather was fine. One summer, she noticed that the skin of a jaundiced infant had become bleached where it had been exposed to sunlight. When the doctors at the unit purposely exposed jaundiced babies to brief periods of sunlight, they observed not only blanching of the skin but also a distinct drop in serum BR with each exposure (Figure 3a).[9] Thus began phototherapy. These seminal observations have been confirmed many times, but using artificial light instead of sunlight (Figure 3b).[7a] The treatment is now routine. It has no known serious harmful side effects and has been used on countless

(5) Kawade, N. Onishi, S. *Biochem. J.* **1981**, *196*, 257.

(6) (a) Valman, H. B. *Br. Med. J.* **1980**, 543. (b) Paxson, C. L. In "Van Leeuwen's Newborn Medicine"; Paxson, C. L., Ed.; Yearbook Medical Publishers, Inc.: Chicago, 1979. (c) Brown, R. J. K.; Valman, H. B. "Practical Neonatal Pediatrics"; Blackwell: Oxford, 1979.

(7) (a) Brown, A. K.; McDonagh, A. F. *Adv. Pediatr.* **1980**, *27*, 341. (b) Karp, W. B. *Pediatrics* **1979**, *64*, 361.

(8) Although jaundice can occur at any age, BR toxicity (BR encephalopathy) is rare after the neonatal period. Jaundice in later life is generally due to liver disease, in which case the yellow pigments in the circulation are a complex mixture containing BR, BR glucuronides, BR glucuronide isomers, and a biliprotein.

(9) (a) Dobbs, R. H.; Cremer, R. J. *Arch. Dis. Child.* **1975**, *50*, 833. (b) Cremer, R. J.; Perryman, P. W.; Richards, D. H. *Lancet* **1958**, *1*, 1094.

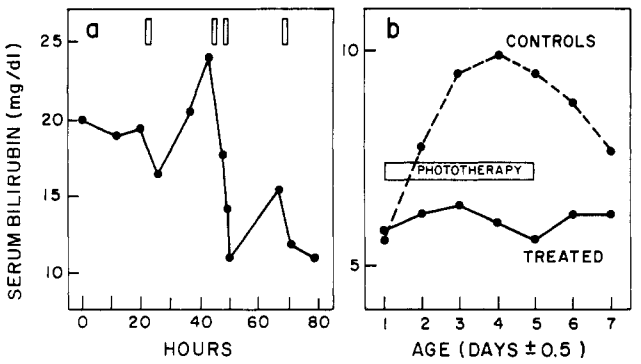
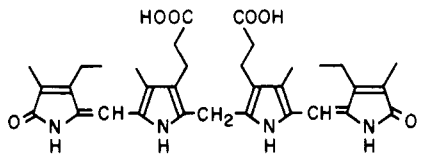


Figure 3. Effect of phototherapy with sunlight and artificial light on serum BR concentrations in jaundiced babies. (a) Single infant exposed for 2-h periods (bars) to sunshine.[9b] (b) Data from ~500 control and ~500 treated low-birthweight infants.[7a] The treatment group received phototherapy for 96 h starting at 24 ± 12 h after birth. Infants in both groups received exchange transfusions if serum BR levels exceeded 10–15 mg %; 24% of the control infants and 4% of the irradiated infants required transfusions. (Redrawn from published graphs.)

thousands of children. Generally blue or white fluorescent tubes are used, and the patient is irradiated for hours to days. This successfully reduces the level of BR in the blood or retards its rate of increase and presumably also lowers the risk of brain damage.[10] To understand how it works requires a closer look at BR structure and chemistry.

Bilirubin: Structure and Properties. Some properties of BR are obvious from structure **1**. Like biliverdin (**2**), it is soluble in dilute alkali, insoluble in water, and readily forms esters and amides.[3a,11] Other properties are less predictable. Thus, it readily disproportionates to symmetrical isomers (Figure 4)[3a] and, though soluble in 0.1 M NH_4OH, is insoluble in 0.1 M ND_4OD.[11] But its most surprising property is its lipophilicity. This property makes BR anomalous compared to most natural "linear" tetrapyrroles and dominates its biological transport, making it essentially unexcretable yet readily transportable across membrane barriers such as the placenta, the blood–brain barrier, and the liver cell plasma membrane.[1a] In contrast to **2**, BR is insoluble in MeOH, soluble in $CHCl_3$ (Figure 5), and not extractable from $CHCl_3$ into 0.1 M $NaHCO_3$.[3a] These properties, which seem paradoxical on the basis of linear representations such as **1** and **2**, become less puzzling when three-dimensional structure[12] is considered.

BR contains three features that together have a dominating effect on its shape: an sp^3 carbon at C-10, which constrains the molecule to bend in the middle and allows the twin pyrromethenone chromophores to rotate independently about the C-9,10 and C-10,11 single bonds; two *Z*-configuration C=C bonds at C-4 and C-15, each within a syn-periplanar chromophore; and two propionic acid groups at C-8 and C-12, which can form *intra*molecular H bonds with the pyrrole and lactam functions in the opposite half of the molecule. These features permit, almost force, BR to adopt either of the two enantiomeric conformations **1A** and **1B** (Figure 6), which are each stabilized by six H bonds. Both conformers occur in crystalline BR[13] and appear to be equally preferred in achiral organic solvents, where they interconvert rapidly.[14] Ionization of the carboxyl groups reduces the number of H bonds but probably increases the strength of the remainder so that conformers like **1A** and **1B** prevail also in the dianion.[13c] The preference for intramolecularly H-bonded conformers (Figure 6), in which polar functions are neutralized internally, explains why BR, though amphiphilic, is predominantly lipophilic and requires addition of a polar function, such as glucuronic acid, for excretion. The same properties are shared by analogues that also have an sp^3 carbon at C-10, *Z* double bonds at C-4 and C-15, and propionic acid groups at C-8 and C-12, e.g., **3**, **4**, and **5**. However, congeners that lack any one



(5)

of these features are less lipophilic than BR and do not require glucuronidation for hepatic excretion, e.g., **2**, which has an sp^2 carbon at C-10, BR isomers with *E* double bonds at C-4 or C-15, or the three BR isomers that correspond to cleavage of heme (Figure 1) at any other position (β, γ, or δ) than the α.[11,15] A curious corollary of these structural considerations is that the mono- and dimethyl esters of BR are *more polar* on silica TLC or reversed-phase HPLC than the free acid itself, presumably because they are not zipped up so tightly into nonpolar conformers by intramolecular H bonding.

Mechanisms of Phototherapy: Models

Normally, rats do not get neonatal jaundice. However, in 1934, the geneticist Charles Gunn noticed that three rat pups in a litter of 13 were decidedly yellowish.[16] He found that they had an hereditary disease, the yellow being BR. Later workers showed that these "Gunn rats" were unable to glucuronidate BR.[1a,b] Consequently, they develop life-long jaundice—provided, of course, that they survive the toxic effects of BR. Gunn rats are useful for studying phototherapy, and they respond to visible light much as jaundiced babies, with a slow decline in serum BR levels.[17] Experiments on Gunn rats provided the first key clues to the chemistry of phototherapy. First, wavelength-de-

(10) Decreased serum BR does not necessarily reflect increased BR excretion. It can also reflect a shift of BR from blood to other tissues. Aspirin and several other common drugs, for example, compete with BR for albumin binding (see, for example: Brodersen, R.; Ebbesen, F. *J. Pharm. Sci.* **1983**, *72*, 248). Some of these, when given to jaundiced infants, can lower serum BR levels by displacement. Use of an antibacterial sulfonamide drug in jaundiced infants in the 1950s led to a small "epidemic" of deaths from BR encephalopathy due to this displacement mechanism (Silverman, W. A.; Andersen, D. H.; Blanc, W. A.; Crozier, D. N. *Pediatrics* **1956**, *18*, 614).

(11) Lightner, D. A.; McDonagh, A. F., unpublished observations.

(12) For leading references, see: Lightner, D. A. In "Bilirubin"; Heirwegh, K. P. M., Brown, S. B., Eds.; CRC Press: Boca Raton, FL; 1982; Vol. 1, Chapter 1.

(13) (a) Bonnett, R.; Davies, J. E.; Hursthouse, M. B.; Sheldrick, G. M. *Proc. R. Soc. London, Ser. B* **1978**, *202*, 249. (b) LeBas, G.; Allegret, A.; Manguen, Y.; DeRango, C.; Bailly, M. *Acta Crystallogr., Sect. B* **1980**, *B36*, 3007. (c) Mugnoli, A.; Manitto, P.; Monti, D. *Acta Crystallogr., Sect. C* **1983**, *C39*, 1287.

(14) (a) Manitto, P.; Monti, D. *J. Chem. Soc., Chem. Commun.* **1976**, 122. (b) Kaplan, D.; Navon, G. *Isr. J. Chem.* **1983**, *23*, 177. (c) Navon, G.; Frank, S.; Kaplan, D. *J. Chem. Soc., Perkin Trans. 2* **1984**, 1145.

(15) Blanckaert, N.; Heirwegh, K. P. M.; Zaman, Z. *Biochem. J.* **1977**, *164*, 229.

(16) Gunn, C. H. *J. Hered.* **1938**, *29*, 137.

(17) Ostrow, J. D. *J. Clin. Invest.* **1971**, *50*, 707.

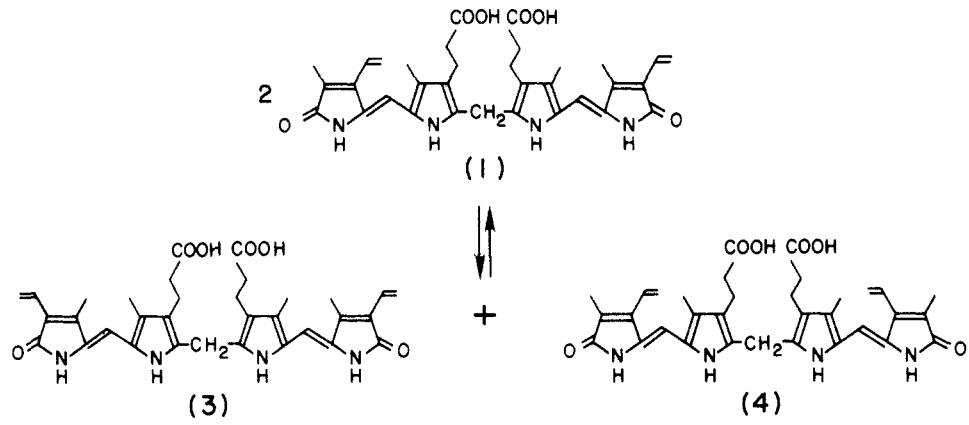


Figure 4. Disproportionation of BR (1) to symmetrical isomers 3 and 4.

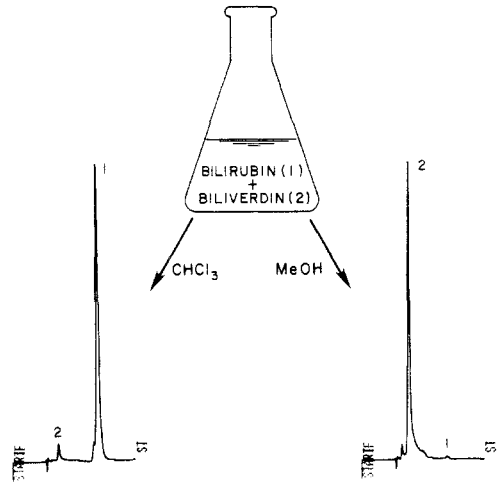


Figure 5. Solubility of BR (1) and biliverdin (2) in $CHCl_3$ and MeOH. Mixtures containing equal weights, ~50 μg, of crystalline 1 and 2 were stirred for 1 h in the dark at 25 °C in MeOH and $CHCl_3$ (1 mL). The solutions were filtered and evaporated and the residues analyzed by HPLC with detection at an isosbestic point (502 nm).

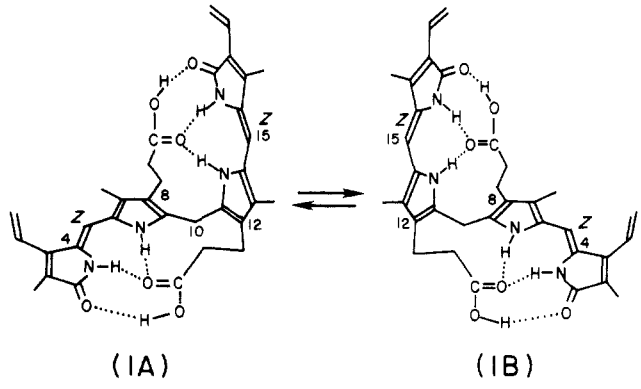


Figure 6. Enantiomeric H-bonded conformers of BR. The stereochemical inversion **1A** ⇄ **1B** requires rupture and reformation of all six H bonds. The rate of inversion in $CDCl_3$ has been estimated independently by two different NMR methods as 7.2 ± 0.4 s^{-1} (~53 °C) and 3–95 s^{-1} (50–95 °C) with an activation barrier of ~18 kcal/mol.[14]

pendence studies indicated that the photoreceptor is BR itself.[18] Second, excretion studies showed that the slow decline in serum BR during phototherapy is preceded by a much faster, almost instantaneous, excretion of yellow pigment in bile.[17,19] When extracted, this

(18) Ballowitz, L.; Geutler, G.; Krochmann, J.; Pannitschke, R.; Roemer, G.; Roemer, I. *Biol. Neonate* **1977**, *31*, 229.

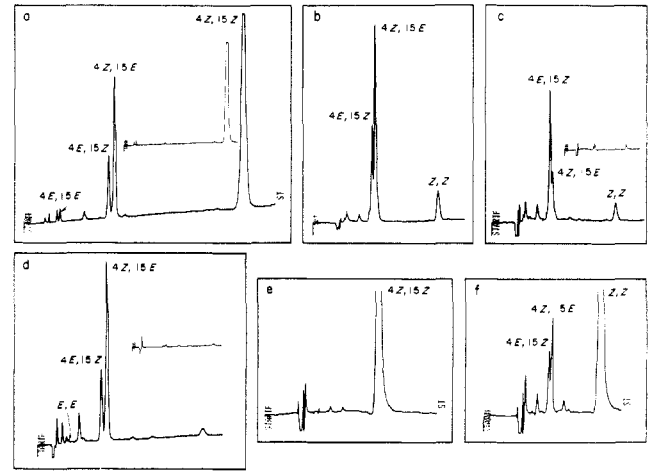


Figure 7. HPLC of BR photoisomers.[11,23] (a) BR before (inset) and after irradiation to near photoequilibrium in $CHCl_3/Et_3N$. (b) Pigments isolated from (a) by MeOH extraction and dissolved in serum. (c) Gunn rat bile before (inset) and 12 min after intravenous injection of the mixture shown in (b). (d) Bile from a Gunn rat before (inset) and 3 h after starting phototherapy. (e, f) Serum from Gunn rats undergoing phototherapy without (e) and with (f) the bile duct tied. (Chromatograms not run under identical conditions, but order of elution is constant.)

pigment seemed to contain mainly BR.[17,20,21] Since BR normally has to be made more polar to be excreted, these observations suggested that the light was perhaps converting BR to a less H-bonded isomer, more polar than the biosynthetic isomer, that did not require conjugation for excretion.[19b] Such isomers, however, were unknown and in vitro studies had to be carried out to find them.

Photolysis of BR or its dimethyl ester in anaerobic organic solvents led rapidly to a photoequilibrium mixture containing two new major products, more polar than the parent compound (Figure 7a).[21-23] These were thermally unstable and reverted quantitatively to BR on acid or radical catalysis or even on standing. ^1H

(19) (a) McDonagh, A. F.; Ramonas, L. M. *Science* **1978**, *201*, 829. (b) McDonagh, A. F.; Palma, L. A. *J. Clin. Invest.* **1980**, *66*, 1182.

(20) McDonagh, A. F. *Lancet* **1975**, *1*, 339.

(21) McDonagh, A. F.; Palma, L. A.; Lightner, D. A. *Science* **1980**, *208*, 145.

(22) (a) Lightner, D. A.; Wooldridge, T. A.; McDonagh, A. F. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 235. (b) Lightner, D. A.; Wooldridge, T. A.; McDonagh, A. F. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 29. (c) McDonagh, A. F.; Lightner, D. A.; Wooldridge, T. A. *J. Chem. Soc., Chem. Commun.* **1979**, 110.

(23) (a) McDonagh, A. F.; Palma, L. A.; Trull, F. R.; Lightner, D. A. *J. Am. Chem. Soc.* **1982**, *104*, 6865. (b) McDonagh, A. F.; Palma, L. A.; Lightner, D. A. *Ibid.* **1982**, *104*, 6867.

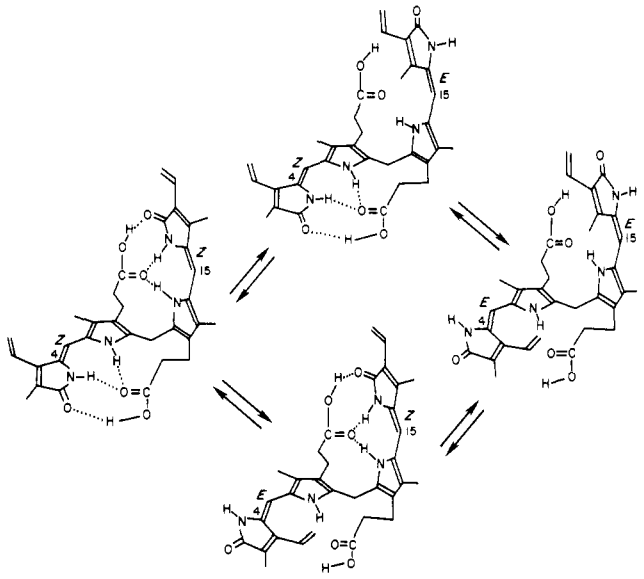


Figure 8. The four BR configurational isomers.

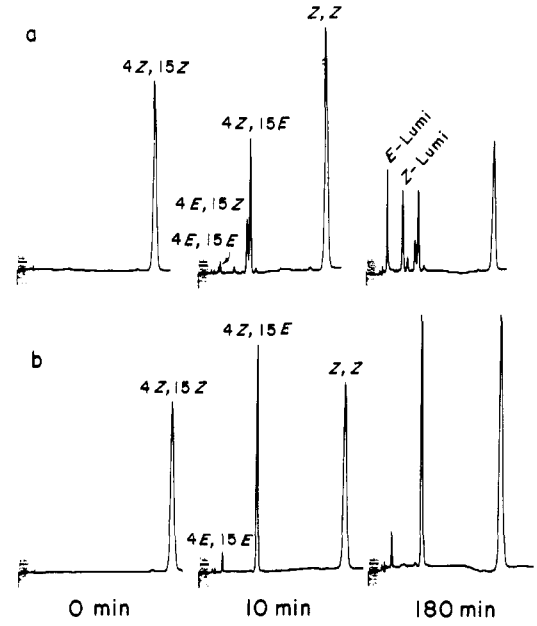


Figure 9. Photolysis of (a) BR (1) and (b) the symmetrical isomer 3 (Figure 4), which has no endo vinyl substituent.[11,23] Samples were photolyzed in $CHCl_3/Et_3N$ under Ar and analyzed by HPLC after 10 min and 180 min.

NMR established their structures as 4*E*,15*Z* and 4*Z*,15*E* isomers of BR (Figure 8), and a minor, more polar component of the photoequilibrium mixture was assigned to the corresponding 4*E*,15*E* isomer.[23a] Symmetrically substituted analogues such as **3**, **4**, and **5** behaved similarly but yielded only a single *E*,*Z* isomer along with a minor amount of *E*,*E* isomer.[11,23a] Experiments with pyrromethenones and benzalpyrrolinones showed the reaction to be general.[12,24]

$Z \rightleftarrows E$ isomerization of BR is very efficient with a quantum yield for the forward ($Z \rightarrow E$) reaction of about 0.2 and even higher for the reverse ($E \rightarrow Z$) reaction.[25] With blue fluorescent lights of the sort used in phototherapy, the isomer mixture at photoequilibrium in solvents such as $NH_4OH/MeOH$ and $CHCl_3/Et_3N$ contains ~80% 4*Z*,15*Z*, 14% 4*Z*,15*E*, 6% 4*E*,15*Z*, and 1% 4*E*,15*E*.[11] Surprisingly, protein binding does not inhibit photoisomerization, though it does influence its regioselectivity (see below). However, protein binding has a marked effect on the thermal reversion of *E* isomers in the dark. In rat bile, which contains little protein, the 4*E*,15*Z* and 4*Z*,15*E* isomers revert completely to the 4*Z*,15*Z* isomer within ~45 min at 37 °C, but in serum or aqueous albumin, they are stable for hours.[11,21] Their stability depends on the solvent. They are very unstable in H_2O or MeOH, more stable in less polar solvents such as $CHCl_3$ and even more stable in basic solvents such as $CHCl_3/Et_3N$ or $NH_4OH/MeOH$. In contrast to the parent 4*Z*,15*Z* isomer, the more polar *E* isomers are soluble in MeOH and, despite their thermal instability in this solvent, MeOH extraction provides a useful practical method for separating them from unisomerized BR (cf. Figure 7b).[11,22]

Having synthesized and characterized the geometric isomers of BR and developed sensitive HPLC methods for detecting them, we were able to study their metabolism and formation in the Gunn rat. When synthetic 4*Z*,15*E*/4*E*,15*Z* isomers were injected intravenously in the dark, they were excreted rapidly in bile without metabolic alteration (Figure 7c),[11] unlike the 4*Z*,15*Z* isomer, which is not excreted to any significant extent. Interestingly, the 4*E*,15*Z* isomer, which elutes just before the 4*Z*,15*E* isomer on reversed-phase HPLC, is also excreted slightly faster than the 4*Z*,15*E* isomer in vivo.[11] Thus, the liver can distinguish these remarkably similar diastereomers (Figure 8), acting in effect as a low-resolution reversed-phase chromatography system.

When shaved Gunn rats were irradiated with blue light, there was immediate excretion of pigment in bile.[19] The pigment concentration gradually increased and reached a steady state by 60–90 min. When the light was switched off, the pigment concentration decreased abruptly, eventually returning to the initial preirradiation level.[11,19] HPLC of bile collected during the steady-state period showed mainly (4*Z*,15*E* + 4*E*,15*Z*)-BR (Figure 7d) along with smaller amounts of other pigments including the 4*Z*,15*Z* isomer and a trace of the 4*E*,15*E* isomer.[23a] This composition was seen only when extreme precautions were taken to avoid thermal isomerization of pigments in bile after their excretion from the liver. When these were omitted, the apparent proportion of 4*Z*,15*Z* isomer increased.

Since blood is the vehicle that transports *E* isomers from their site of formation (skin) to their exit point (liver), we expected that they would be detectable in blood during phototherapy. They were, but barely. Despite copious excretion in bile, their concentration in serum in the rat remained very low (Figure 7e). However, when their egress from the liver was blocked by tying the bile duct, the isomers accumulated in blood and were detectable quite readily (Figure 7f).[11,19b,21] Their removal from blood by the liver is therefore efficient and concentrative.

Although geometric isomers were the major products detected in Gunn rat bile during phototherapy or

(24) See, for example: (a) Lightner, D. A.; Park, Y.-T. *J. Heterocycl. Chem.* **1977**, *14*, 415. (b) Falk, H.; Grubmayr, K.; Neufingerl, F. *Monatsh. Chem.* **1979**, *110*, 1127. (c) deGroot, J. A.; Jansen, H.; Fokkens, R.; Lugtenburg, *J. Recl. Trav. Chim. Pays-Bas* **1983**, *102*, 114. (d) Lamola, A. A.; Braslavsky, S. E.; Schaffner, K.; Lightner, D. A. *Photochem. Photobiol.* **1983**, *37*, 263.

(25) (a) Greene, B. I.; Lamola, A. A.; Shank, C. V. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 2008. (b) Lamola, A. A.; Flores, J.; Doleiden, F. H. *Photochem. Photobiol.* **1982**, *35*, 649.

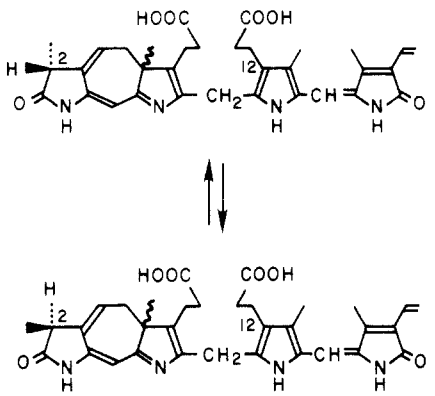


Figure 10. Lumirubin diastereomers. Interconversion by epimerization at C-2 is catalyzed by base.[23b] Intramolecular H bonding between the C-12 propionic acid group and the opposing erstwhile pyrromethenone group is sterically impossible, making each epimer more polar than BR.

short-term photolysis of BR in vitro, other minor pigments were also present. These became major products in vitro on longer term photolysis (Figure 9a). Similar products are formed from BR dimethyl ester and analogues containing a vinyl group at the C-3 or C-17 (endo) position, e.g., **4**, but never from compounds lacking an endo vinyl group, e.g., **3** and **5** (Figure 9b).[11,23b] The major longer term photolysis product of BR separated into two virtually identical components on silica TLC. These interconverted in base. Though their structures have not been established unambiguously,[26] they appear to be diastereomeric structural isomers formed by cycloaddition of the endo vinyl group to the adjacent pyrrole ring (Figure 10).[23b] These structural isomers (lumirubins) still contain an exocyclic 15*Z* double bond, about which rapid, reversible $Z \rightleftarrows E$ isomerization occurs on exposure to light.

So anaerobic photolysis of BR in vitro results in two types of isomerization: configurational and structural. The former is rapid and reversible and accounts for the initial products seen on short-term photolysis. The latter is irreversible and is responsible for the additional products formed as irradiation continues.[27] These different isomers account for all of the enhanced pigment excretion in Gunn rat bile during phototherapy, with configurational (4*E*,15*Z*, 4*Z*,15*E*, and 4*E*,15*E*) isomers contributing ~90% and structural isomers ((*Z*)- and (*E*)-lumirubins) ~10%.[11] The photochemistry of BR in the rat is therefore remarkably similar to that in organic solvents, a point that eluded earlier workers because they often restricted in vitro studies to aqueous solutions or overirradiated at light fluxes unattainable in vivo. Mammals may be mostly water, but it is not necessarily very wet where the action is.

Mechanisms of Phototherapy: Babies

Phototherapy is obviously more difficult to study in babies than rats, and the complete mechanism in humans is not yet known. It is nonetheless already clear that the same basic photochemistry occurs in both species. As in the Gunn rat, phototherapy leads to increased pigment output in bile,[28] which seems to be

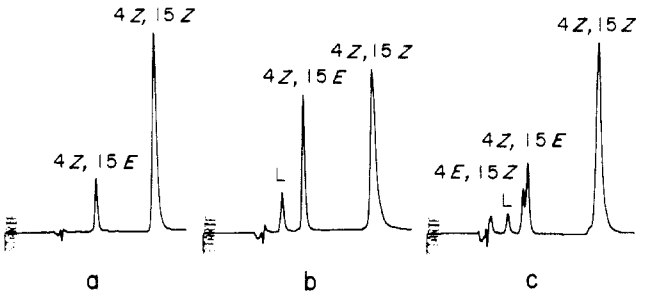


Figure 11. HPLC of serum from a jaundiced baby undergoing phototherapy (a) and photolysis products from in vitro (30 min) irradiation of 0.2 mM BR in 0.2 mM aqueous human albumin (b) and guinea pig albumin (c).[11] L stands for (*Z*)-lumirubin.

due to isomers.[11,28b] Using HPLC we have detected both structural and configurational isomers of BR in serum and bile from babies undergoing irradiation. In fact, small amounts of (4*Z*,15*E*)-BR are sometimes present in the serum of jaundiced babies *before* phototherapy, presumably due to exposure of the infant to ambient light. There are, however, important differences between the two species. Whereas *E* isomers do not accumulate in the blood of Gunn rats undergoing phototherapy, they do accumulate in humans, and they may comprise up to ~15% of the total rubinoid pigment in the blood of jaundiced babies undergoing treatment.[11,28b] This suggests that infants are unable to excrete *E* isomers of BR as readily as the Gunn rat. Intriguingly, only one of the two possible *Z*,*E* isomers, the 4*Z*,15*E*, appears in the infant's blood. The 4*E*,15*Z* isomer is barely detectable (Figure 11a).[23b] Although this could be caused by selective excretion of one particular diastereomer, our in vitro studies indicate that it is more likely due to species-specific regioselective photochemistry.

As noted above, binding of BR to albumin does not inhibit photoisomerization. With some albumins, e.g., human albumin, the reverse is true and the rates of both structural and configurational isomerization are actually higher than in, say, $CHCl_3$. Furthermore, the configurational isomer composition at photoequilibrium in vitro is markedly dependent on the species of albumin with which the BR is complexed.[23b] Photolysis of aqueous solutions of BR containing equimolar concentrations of albumin from ox, rat, guinea pig, horse, chicken, or rabbit gives both 4*E*,15*Z* and 4*Z*,15*E* isomers in ratios of 2:1 to 1:2 (cf. Figure 11c). In contrast, with human serum albumin the reaction becomes almost completely regioselective for the 4*E*,15*Z* isomer (Figure 11b). The explanation of this is unclear. However, it is not simply a question of only one end of the molecule binding to the protein leaving the other exposed and free to isomerize, because the rate of isomerization is independent of the viscosity of the aqueous medium.[29]

Thus far we have described two fundamental reactions that, by disruption of intramolecular H bonds, give products that are sufficiently polar to be excreted without needing the normal pathway of glucuronidation. Although these are the most quantum-efficient photoreactions of BR, they are by no means

(26) Stoll, M. S.; Vicker, N.; Gray, C. H.; Bonnett, R. *Biochem. J.* **1982**, *201*, 179.

(27) Anaerobic photolysis in some solvents also causes constitutional isomerization to symmetrical isomers via free-radical-initiated disproportionation (Figure 5).[3a] There is no evidence that this reaction occurs in vivo during phototherapy.

(28) (a) Lund, H. T.; Jacobsen, J. *J. Pediatr.* **1974**, *85*, 262. (b) Onishi, S.; Isobe, K.; Itoh, S.; Kawade, N.; Sugiyama, S. *Biochem. J.* **1980**, *190*, 533.

(29) Lamola, A. A.; Flores, J. *J. Am. Chem. Soc.* **1982**, *104*, 2530.

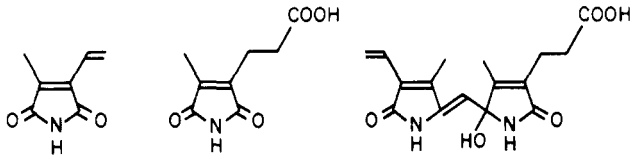


Figure 12. In vitro photooxidation products of BR.[12] Other isomers of the dicyclic compound are also formed.

the only ones.[3a,30] The originators of phototherapy assumed that the treatment depended on photooxidation of BR to biliverdin or other excretable compounds of lower molecular weight.[9] It is well-known that BR undergoes photooxidation, with eventual destruction of the chromophore, especially in the presence of O_2. The products of aerobic photooxidation are time, solvent, and concentration dependent. Typically they include colorless water-soluble oxidized mono- and dipyrroles (Figure 12).[3a,12,30] At physiologic O_2 concentrations and the sort of light intensities that might be encountered in vivo during phototherapy, BR photooxidation is slow—too slow to account for the rather prompt response of jaundiced rats and babies to light. Nevertheless, the quantum yield for photooxidation is not zero, and irradiation times in phototherapy extend from hours to days. Therefore, some photooxidation is expected. Consistent with this, compounds of the type shown in Figure 12 and their hydrolysis products have been identified unambiguously in urine of infants undergoing treatment.[31] Without a complete material balance, it is impossible to estimate accurately the contribution of this pathway to the overall process. However, the known photochemistry of BR, the seemingly low concentration of photooxidation products in urine, and recent animal studies[32] make it unlikely that photooxidation is a major contributor. Were this the sole reaction, phototherapy would probably be ineffective.

Concluding Remarks

So in Sister Ward's sunshine baby the main photoreactions of BR were probably configurational and structural isomerization along with some oxidation. Certainly there is no doubt that the rapid isomerization pathways that occur in vitro also occur in vivo. However, dynamic processes of transport and excretion are superimposed on the photochemistry in vivo. These can influence the product distribution and also prevent $Z \rightleftarrows E$ isomerization from reaching photoequilibrium. Since the relative rates of production, hepatic uptake, and excretion of the individual BR isomers are probably species dependent, the contribution of each isomerization pathway to the overall effect of phototherapy is likely to be different in humans and rats. How different is unknown. In rats $Z \rightarrow E$ isomerization is unquestionably the predominant process leading to enhanced pigment excretion from the liver. Therefore, light does not really stimulate the hepatic excretion of (4*Z*,15*Z*)-BR itself, as once thought—only isomers of it. Since most of these isomers gradually revert to (4*Z*,15*Z*)-BR in the bile or intestine, the major net effect in the rat is translocation of BR from the illuminated region to the intestine.[33] Unisomerized BR can then

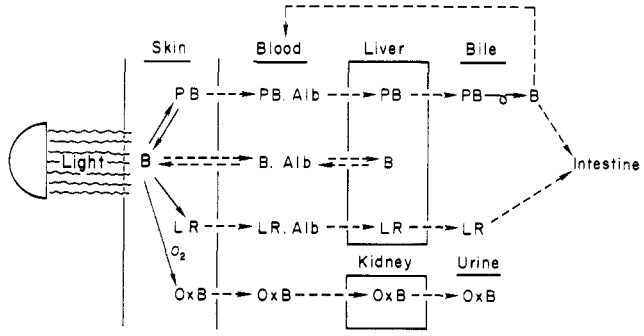


Figure 13. General mechanism of phototherapy. Solid arrows represent chemical reactions; dashed arrows, transport processes. Pigments may be protein bound in compartments other than blood. Although not indicated, small amounts of photoisomers are also excreted in urine. B = (4*Z*,15*Z*)-bilirubin, PB = *E* photoisomers of bilirubin, LR = *E* and *Z* isomers of lumirubin, OxB = oxidation products of bilirubin, Alb = albumin.

migrate to vacated sites in the skin or circulation and undergo the same photometabolic cycle (Figure 13).

In terms of the overall expulsion of BR during phototherapy, there is one other complicating factor to be considered. (4*Z*,15*Z*)-BR, the stable isomer, can be absorbed across the intestinal wall into the circulation (Figure 2).[34] This diminishes the net contribution of the $Z \rightarrow E$ pathway and effectively increases the relative contribution of the photooxidation and structural isomerization pathways. The extent of intestinal reabsorption of BR in patients under phototherapy is unknown. Also unknown are the specific microscopic sites where photoisomerization occurs in vivo and the contribution of protein binding to the stereoselectivity of the photochemistry.

Despite these uncertainties, most data indicate that photoisomerization is far more important quantitatively than photooxidation in human infants. However, the exact relative contribution of each type of isomerization pathway to the eventual decrease in serum BR concentration in humans is still uncertain. From the photochemical point of view, then, the mechanism of phototherapy is similar to those of vision and vitamin D synthesis, which both involve light-driven isomerization reactions.

To many, BR is an obscure waste product. To us, this small yellow compound and its isomers have revealed a rich lode of fascinating biological chemistry. They provide a handy paradigm of the importance of shape and conformation in biological function and the ability of H bonding to control these, they demonstrate the delicate sensitivity of hepatic transport to lipophilicity and polarity, and they show how seemingly small stereochemical changes can have a large effect on biological properties. The pronounced species-dependent effect of protein binding on the photochemistry of BR shows how proteins can be used for selective control of photochemical reactions and suggests that BR may turn out to be a useful photochemotaxonomic probe for serum albumins. Lastly, the photoisomers of BR, which are chiral internal amphiphiles, promise to be useful for probing the mechanism of BR diglucuronide formation in the liver and the topology of BR glucuronyl trans-

(30) (a) Lightner, D. A. *Photochem. Photobiol.* **1977**, *26*, 427. (b) Landen, G. L.; Park, Y.-T.; Lightner, D. A. *Tetrahedron* **1983**, *39*, 1893.
(31) Lightner, D. A.; Linnane, W. P.; Ahlfors, C. E. *Pediatr. Res.* **1984**, *18*, 696–700.
(32) Davis, D. R.; Yeary, R. A. *J. Pediatr.* **1981**, *99*, 956.
(33) Falk et al. (Eichinger, D.; Falk, H.; Sobczak, R. *Photochem. Photobiol.* **1983**, *38*, 193) described recently an in vitro model of this overall process in which light is used to "pump" BR from one side of a fluid hydrophilic barrier to the other.
(34) Lester, R.; Schmid, R. *N. Engl. J. Med.* **1963**, *269*, 178.

ferase in the endoplasmic reticulum membrane.

Phototherapy was spawned by the chance effect of sunlight on a jaundiced baby and an icteric serum sample.[9a] Curiously, long before phototherapy was invented, an American physician named Edward Bliss Foote wrote a bestseller called "Plain Home Talk About the Human System" in which he wrote: "When people allow the sun to paint their faces brown, torpid livers are less liable to paint them yellow."[35] As it turned out, for all the little people with neonatal jaundice, he was not far wrong.

(35) Foote, E. B. "Dr. Foote's New Book on Health and Disease, With Recipes, Including Sexology"; Murray Hill Publishing Co.: New York, 1903; p 305. (Later edition of the title cited in text.)

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