Phototherapy for Neonatal Jaundice. Configurational Isomers of Bilirubin

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Phototherapy is used routinely in hospitals for treating neonatal jaundice (hyperbilirubinemia).[1] Patients are irradiated with blue or white light to enhance elimination of the yellow neurotoxic metabolite (4*Z*,15*Z*)-bilirubin IXα (*Z*,*Z*-**1**, Figure 1). Enhanced elimination is due to formation of bilirubin photoproducts that are excreted rapidly in bile and, to a lesser extent, in urine.[2] There is abundant evidence that **1**,[3] like similar tetrapyrroles and model compounds,[4] undergoes rapid C=C configurational isomerization to unstable 4*E* and 15*E* isomers when irradiated in solution,[5] and we have previously presented compelling evidence that $Z \rightarrow E$ isomerization of *Z*,*Z*-**1** is crucially important in phototherapy.[6] In earlier work we separated and identified all four configurational isomers of the dimethyl ester of **1**.[3a,b,7] However, the corresponding biologically important free acids have not been completely separated and the identification of *E* isomers, particularly in biological tissues, has been uncertain and controversial.[8] We now report unambiguous structural assignments for the three *E* isomers of **1**. We also describe simple methods for their separation and detection in vitro and in vivo.

Photochemical studies were carried out on *Z*,*Z*-**1** and the symmetrical analogues (4*Z*,15*Z*)-bilirubin IIIα (*Z*,*Z*-**2**), (4*Z*,15*Z*)-bilirubin XIIIα (*Z*,*Z*-**3**), and (4*Z*,15*Z*)-mesobilirubin XIIIα (*Z*,*Z*-**4**) (Figure 1). Reactions were followed by absorbance difference spectroscopy and HPLC.[9] On photolysis of *Z*,*Z*-**2** in 50% $CHCl_3$–Et_3N the system rapidly reached a photostationary state with development of a characteristic sigmoidal difference

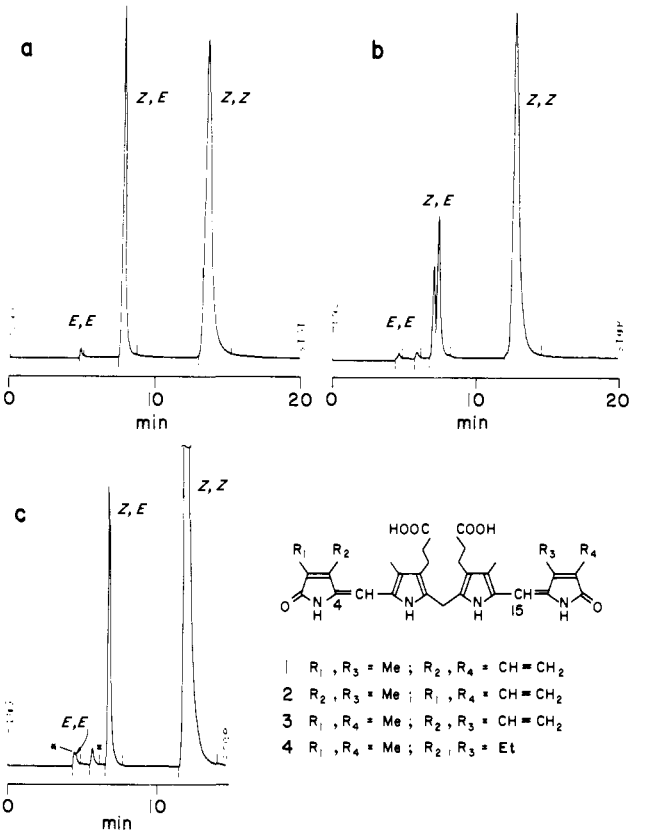

Figure 1. HPLC of samples at or near photoequilibrium from the photolyses of (a) bilirubin IIIα (*Z*,*Z*-**2**), (b) bilirubin IXα (*Z*,*Z*-**1**), and (c) bilirubin XIIIα (*Z*,*Z*-**3**) in $CHCl_3$-Et_3N. For photolysis and HPLC conditions see ref 9.

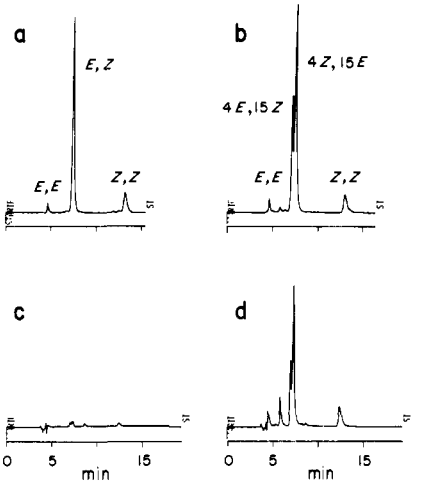

Figure 2. HPLC of (a) purified 4*Z*,15*E*-bilirubin IIIα, (b) purified (4*E*,15*Z*/4*Z*,15*E*)-bilirubin IXα, (c) hepatic bile from a jaundiced Gunn rat kept in the dark, (d) hepatic bile from the same rat after exposing the animal to blue light for 2 h.

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(3) (a) McDonagh, A. F.; Lightner, D. A.; Wooldridge, T. A. *J. Chem. Soc., Chem. Commun.* **1979**, 110–112. (b) Lightner, D. A.; Wooldridge, T. A.; McDonagh, A. F. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 235–243. (c) Lightner, D. A.; Wooldridge, T. A.; McDonagh, A. F. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 29–32. (d) Lamola, A. A.; Flores, J.; Doleiden, F. H. *Photochem. Photobiol.* **1982**, *35*, 649–654. (e) Lamola, A. A.; Flores, J. *J. Am. Chem. Soc.* **1982**, *104*, 2530–2534.

(4) (a) de Groot, J. A.; van der Steen, R.; Fokkens, R.; Lugtenburg, J. *Recl. Trav. Chim. Pays-Bas* **1982**, *101*, 219–223; (b) 35–40. (c) Hori, A.; Mangani, S.; Pepe, G.; Meyer, F.; Cullen, D. L.; Falk, H.; Grubmayr, K. *J. Chem. Soc., Perkin Trans. 2.* **1981**, 1525–1528. (d) Falk, H.; Grubmayr, K.; Höllbacher, G.; Hofer, O.; Leodolter, A.; Neufingerl, F.; Ribô, J. M. *Monatsh. Chem.* **1977**, *108*, 1113–1130. (e) Sheldrick, W. S.; Borkenstein, A.; Blacha-Puller, M.; Gossauer, A. *Acta Crystallogr., Sec. B* **1977**, *B33*, 3625–3635. (f) Lightner, D. A.; Park, Y.-T. *J. Heterocycl. Chem.* **1977**, *14*, 415–422. (g) Falk, H.; Grubmayr, K.; Herzig, V.; Hofer, O. *Tetrahedron Lett.* **1975**, 559–562.

(5) Rapid configurational photoisomerization of *Z*,*Z*-**1** occurs in organic and aqueous albumin solutions. In water, disproportionation to IIIα and XIIIα structural isomers occurs (see: McDonagh, A. F., *Ann. N.Y. Acad. Sci.* **1975**, *244*, 553–569), and $Z \rightarrow E$ isomerization is undetectable.

(6) McDonagh, A. F.; Palma, L. A.; Lightner, D. A. *Science (Washington, D.C.)* **1980** *208*, 145–151. See also: (a) Onishi, S.; Isobe, K.; Itoh, S.; Kawade, N.; Sugiyama, S. *Biochem. J.* **1980**, *190*, 533–536. (b) Lamola, A. A.; Blumberg, W. E.; McClead, R.; Fanaroff, A. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 1882–1886.

(7) The four configurational isomers are the 4*Z*,15*Z* isomer and the three photochemically accessible 4*Z*,15*E*, 4*E*,15*Z*, and 4*E*,15*E* isomers. For the symmetrically substituted compounds bilirubin IIIα (**2**), mesobilirubin XIIIα (**4**), and bilirubin XIIIα (**3**) only three configurational isomers are possible, 4*Z*,15*Z*, 4*Z*,15*E* (≡4*E*,15*Z*), and 4*E*,15*E*.

(8) (a) McDonagh, A. F.; Lightner, D. A. *Pediatrics* **1981**, *67*, 929–930. (b) Ostrow, J. D.; Cohen, A. N. *Ibid.* **1981**, *67*, 930–931. (c) Stoll, M. S.; Vicker, N.; Gray, C. H.; Bonnett, R. *Biochem. J.* **1982**, *201*, 179–188.

(9) Solutions ($\simeq 10^{-3}$–10^{-4} M) were degassed with argon and photolyzed with blue light (Westinghouse special blue 20-W fluorescent tube, λ_{max} 436, 446 nm) or for wavelength-dependence studies, with 20-nm band-pass light from a variable-wavelength monochromator. Products were analyzed by reverse-phase HPLC (Beckman-Altex Ultrasphere-IP or -ODS columns, 5 μm, C-18, 25 × 0.46 cm; with a Beckman-Altex ODS precolumn, 4.5 × 0.46 cm) by using 0.1 M di-*n*-octylamine acetate in MeOH as eluant (0.75 mL/min, detector 435 or 450 nm). Retention times may vary with age and origin of column. Retention volume = 0.75 × R_t. Details of the HPLC analyses will be published separately. Solutions were considered to have reached a photostationary state if there was no further marked change in composition on doubling the irradiation time.

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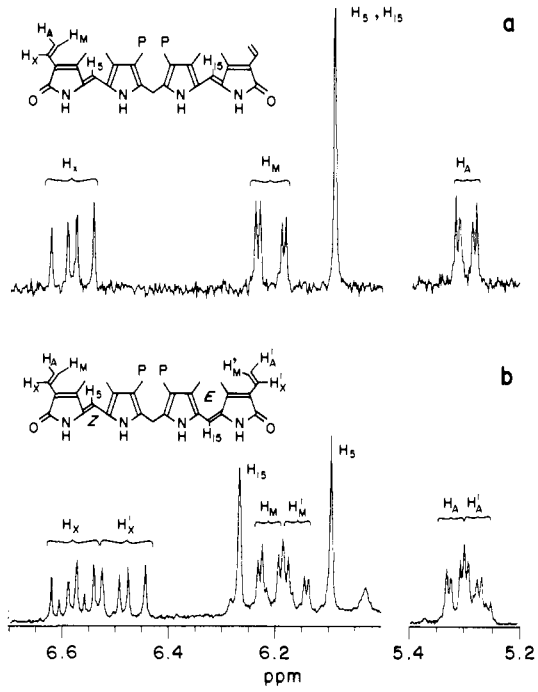

Figure 3. Olefinic region of the 360-MHz ^{1}H NMR (Me_2SO-d_6) of (a) bilirubin IIIα, *Z,Z*-**2**, and (b) purified *Z,E*-**2** (P = $CH_2CH_2CO_2H$). Proton resonances are in ppm downfield from Me_4Si.

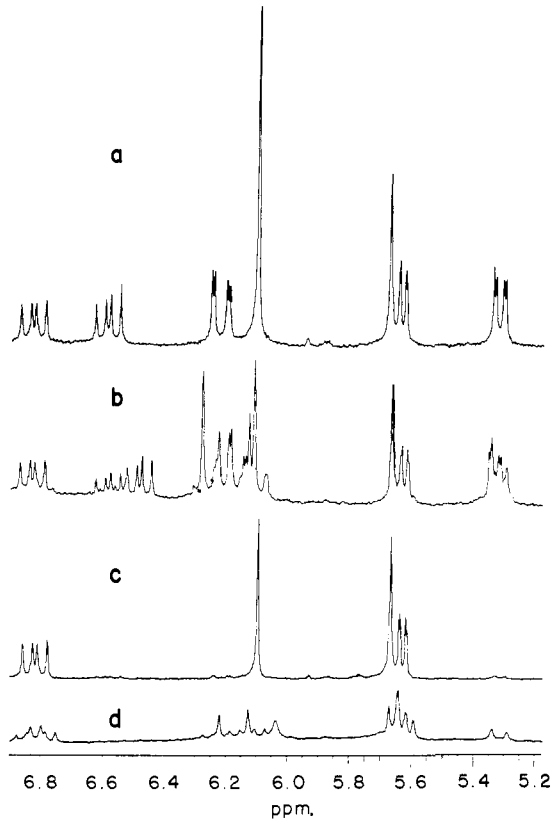

Figure 4. Olefinic region of the 360-MHz ^{1}H NMR spectra (Me_2SO-d_6) of (a) bilirubin IXα, *Z,Z*-**1**, (b) purified (4*E*,15*Z*/4*Z*,15*E*)-bilirubin IXα with the 4*Z*,15*E* diasteromer predominating, (c) bilirubin XIIIα, *Z,Z*-**3**, and (d) purified (4*Z*,15*E*)-bilirubin XIIIα. The sample used in d contained 52% of the *Z,E* diastereomer and 34% of the *Z,Z* diastereomer and was contaminated with ~10% of the corresponding IXα isomer.

spectrum[3a-c] showing a positive peak (487 nm), a negative peak (456 nm), and *tight* isosbestic points (360 and 476 nm). HPLC at photoequilibrium (Figure 1a) revealed, in addition to *Z,Z*-**2**, two rather more polar compounds that reverted to *Z,Z*-**2** slowly on warming and instantly on treatment with a trace of CF_3CO_2H.[3a,c,10] No new peaks appeared on further prolonged irradiation. *Z,Z*-**4** behaved almost identically with *Z,Z*-**2**. *Z,Z*-**3** behaved somewhat similarly to *Z,Z*-**2** in that brief irradiation led to a pronounced difference spectrum and the appearance of two new acid-labile peaks on HPLC (Figure 1c). However, a true photostationary state was not reached, and in contrast to **2** and **4**, continued irradiation led to further changes in the difference spectrum with loss of isosbestic points and the slow emergence of additional peaks on HPLC (marked by asterisks in Figure 1c). (These additional products are discussed in the following communication.) The behavior of the naturally occurring IXα isomer, *Z,Z*-**1**, was intermediate between that of *Z,Z*-**2** and *Z,Z*-**3**, and HPLC near photoequilibrium showed three new acid-labile products (Figure 1b), as expected, each more polar than the parent isomer.[11]

These observations, taken alone or in conjunction with previous work,[3,4] are consistent with the structural assignments shown in Figure 1, and they provide strong evidence that **1** undergoes configurational photoisomerization as follows:[12]

$$4Z,15Z \rightleftarrows 4E,15Z\ (+4Z,15E) \rightleftarrows 4E,15E.$$

To confirm these assignments and distinguish the 4*E*,15*Z* and 4*Z*,15*E* isomers of **1**, we examined the 360-MHz ^{1}H NMR spectra of purified photoisomers derived from **1**–**4**. Preparations highly enriched in the polar *E* photoisomers were prepared by solvent extraction of crude photolysis products at 0 °C.[3b,13] Typical enrichments are shown in Figure 2. Comparison of the ^{1}H NMR spectra of photoisomerized **2** and its structurally symmetric parent *Z,Z* isomer reveals quite strikingly the anticipated doubling of most of the H resonances due to desymmetrization of the molecule. Thus, there appear two meso-bridge olefinic proton signals, two vinyl group multiplets (Figure 3b), four CH_3 singlets, and four NH proton signals. One set of signals in the photoisomer preparation corresponds closely to the spectrum of the *Z,Z* isomer; the second set, equal in intensity to the first, exhibits chemical shifts characteristic of *E* configuration dipyrrylmethenones.[4a,b,d] In particular, the approximately 0.1 ppm deshielding of the meso-*E* H at C-15 is similar to that observed in numerous model compounds.[4a,b,d,f] Analogous features (i.e., signal doubling and characteristic chemical shifts) were observed in the spectra of

(10) The composition at photoequilibrium was weakly and reversibly wavelength dependent. For example, photolysis at 450 nm produced slightly more of the major photoproduct (*Z,E*-**2**) than photolysis at 490 nm.

(11) Base-line HPLC separation of the two *Z,E* isomers of **1** was obtained with 0.1 M di-*n*-dodecylamine acetate in MeOH as eluant (1 mL/min).

(12) Reversibility also was demonstrated by irradiating purified *Z,E* isomers obtained from *Z,Z*-**1**, *Z,Z*-**2**, and *Z,Z*-**4**. In each case the photostationary mixture of isomers obtained was nearly the same as that obtained on irradiation of the corresponding parent *Z,Z* isomer. The possibility of phototautomerism (lactam ⇄ lactim) rather than geometrical configurational photoisomerization may be rejected as follows: (i) The 0.2 ppm deshielding of H_{15} to δ 6.07 in the 4*Z*,15*E* diastereomer of **2** falls in the range found for meso-H deshieldings of *E* isomers in model dipyrromethenones and benzalpyrrolinones, not in the region of the more strongly deshielded (δ 6.4–6.5) H_5/H_{15} signals of the bis-lactim methyl ether of **1** dimethyl ester. (ii) The N–H signals of the *E* half of *Z,E*-**4** (δ 9.88 (H_{21}), 10.45 (H_{22}), 9.68 (H_{23}), 10.06 (H_{24})) are shifted upfield from the parent *Z,Z*-**4** (δ 9.83 (H_{21} = H_{24}) 10.34 (H_{22} = H_{23})), whereas the N–H signals (δ 10.95 (H_{22} = H_{23})) of the bis-lactim methyl ether of **1** dimethyl ester appear farther downfield.

(13) Solutions of *Z,Z* isomers in purified Et_3N–$CHCl_3$ (1:1) were irradiated to near photoequilibrium. The odorless residue obtained after evaporation of solvent in vacuo was mixed with MeOH at 0 °C, and the mixture was filtered rapidly into cold Et_3N–$CHCl_3$. Removal of solvent at <25 °C gave the enriched isomer preparation (e.g., see Figure 2). These preparations contained traces of solvent residues that inhibited the autocatalytic thermal *E* → *Z* reversion of *E* isomers in Me_2SO and facilitated the NMR studies. Nonphotochemical manipulations were done under a safelight.

purified photoisomers from bilirubin XIIIα (3) (Figure 4d) and mesobilirubin XIIIα (4). The photoisomer preparation derived from bilirubin IXα (1) contains two major components in the ratio of about 1:2 (Figure 2b). The NMR spectrum of this material (Figure 4b) was essentially a composite of the spectra of the corresponding IIIα and XIIIα photoisomers. Comparison of all of these spectra (Figures 3 and 4) showed unambiguously that the predominant photoisomer in the preparation derived from *Z,Z*-**1** is the 4*Z*,15*E* diastereomer, as designated in Figure 2b.

These data provide conclusive evidence that *E,Z* isomers are the main photoproducts formed on short-term irradiation of bilirubin in vitro,[14] and they confirm our previous structural assignments for "photobilirubin".[15] Clear evidence that they are the predominant yellow photoproducts excreted by the liver in vivo during blue-light irradiation of jaundiced rats is shown in Figure 2.[20]

Acknowledgment. These studies were supported by Grants AM-26307, AM-11275, and AM-26743 (A.F.M.) and HD-09026 (D.A.L.) from the National Institutes of Health, Grant CHE 79-10133 (D.A.L.) from the National Science Foundation, and a research grant (MSC Springer No. 34) from the University of California, San Francisco (A.F.M.). F.R.T. thanks the Caixa de Barcelona for a fellowship. The 360-MHz spectra were run at the University of California, Davis, NMR Facility with the assistance of Drs. J. L. Dallas and G. B. Matson. We are grateful to Diana Fedorchak and Michael Karasik for fast editorial aid.

(14) Assignment of the *E,E* configuration to the minor, most polar photoproducts from **1**–**4** follows from their thermal and acid lability, comparison with analogous *E,E* isomers,[3b,4a,b] and the absence of a similar product from the photolysate of (*Z*)-xanthobilirubinic acid.

(15) Previously we used the term "photobilirubin" to refer to the mixture of photoisomers obtained on irradiating *Z,Z*-**1** to photoequilibrium. Now that the individual isomers have been characterized, we recommend that the photobilirubin nomenclature be discontinued for the individual isomers. Several other "photobilirubins" have been reported and designated as configurational isomers of bilirubin. Many of these structural assignments are inconsistent with our data. Stoll et al.[16] isolated four substances by preparative TLC on silica and referred to them as photobilirubins IA, IB, IIA, and IIB. IA and IB were designated as 4*Z*,15*E*-**1** and 4*E*,15*Z*-**1**, respectively. IIA and IIB were considered to be conformational isomers of 4*E*,15*E*-**1**. We have been unable to separate or purify authentic *E,Z* isomers of **1** by TLC as described (see also: Sloper, R. W.; Truscott, T. G. *Photochem. Photobiol.* **1982**, *35*, 743–745). HPLC of IA and IB[9,11] revealed that both are complex mixtures containing only trace amounts of the designated *E,Z*/*Z,E* isomers and that neither IIA nor IIB is the 4*E*,15*E* diastereomer of **1**. Our data show unequivocally that none of the structural assignments in ref 16 is correct. These incorrect assignments have been perpetuated in subsequent papers.[8b,c,17] Onishi et al. detected more than 24 components after irradiating *Z,Z*-**1** anaerobically in $CHCl_3$.[18] At least seven of these were stated to be geometric isomers of **1**, but structures and adequate supporting data were not presented. Many of the observed products were probably secondary products and artifacts unrelated to the primary photochemistry of **1** and resulting from overirradiation and radical reactions. Isobe and Onishi have isolated three substances, designated as peaks 1, 2, and 3, from photolysis of *Z,Z*-**1** in aqueous serum albumin.[19] Peak 1 was not identified, peak 2 was attributed to *E,E*-**1**, and peak 3 was attributed to a mixture of the two *E,Z* isomers of **1**. On the basis of data described in this and the following communication, it is clear that peak 2 is not *E,E*-**1** and probable that peak 3 is almost exclusively 4*Z*,15*E*-**1**.

(16) Stoll, M. S.; Zenone, E. A.; Ostrow, J. D.; Zarembo, J. E. *Biochem. J.* **1979**, *183*, 139–146.

(17) Cohen, A. H.; Ostrow, J. D. *Pediatrics* **1980**, *65*, 740–750. Stoll, M. S.; Zenone, E. A.; Ostrow, J. D. *J. Clin. Invest.* **1981**, *68*, 134–141.

(18) Onishi, S.; Itoh, S.; Kawade, N.; Isobe, K.; Sugiyama, S. *Biochem. Biophys. Res. Commun.* **1979**, *90*, 890–896.

(19) Isobe, K.; Onishi, S. *Biochem. J.* **1981**, *193*, 1029–1031.

(20) Details of the animal experiments will be published separately. The structure of the minor photometabolite at R_f 6.1 min in Figure 2d is discussed in the following communication.

(21) Note **Added in Proof**: After this communication had been accepted, we were made aware of work in press on the ^1H NMR spectral analysis of the mixture of 4*Z*,15*Z*-**1** and 4*E*,15*E*-**1** obtained by anaerobic irradiation of 4*Z*,15*Z*-**1** (Falk, H.; Müller, N.; Ratzenhofer, M.; Winsauer, K. *Monatsh. Chem.*). In this work the authors also converted the mixture of *E* isomers of **1** to the corresponding, previously characterized, 4*Z*,15*E* and 4*E*,15*Z* biliverdins IXα, thus providing independent confirmation of the structural assignments made in the present communication.

Phototherapy for Neonatal Jaundice. Stereospecific and Regioselective Photoisomerization of Bilirubin Bound to Human Serum Albumin and NMR Characterization of Intramolecularly Cyclized Photoproducts

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The photochemistry of (*Z,Z*)-bilirubin IXα (**1**) is important because of the clinical use of phototherapy for treating neonatal hyperbilirubinemia (neonatal jaundice). It is now established that **1** undergoes rapid reversible configurational photoisomerization to *E* isomers in organic solvents, in serum, and in jaundiced mammals.[1] The *E* isomers of **1** are unstable and revert to the parent *Z,Z* isomer photochemically or thermally. At room temperature thermal reversal is highly solvent dependent, being slow in basic organic solvents, serum, or aqueous albumin and rapid in acidic or polar solvents. Previously, we described the formation of additional yellow products, more stable than the *E* isomers, on prolonged photolysis of **1**.[1,2] Small amounts of these products are formed in jaundiced rats exposed to blue light and in humans during phototherapy.[1,3] We now show that these compounds are structural isomers of **1**. We also show that complexation of **1** with serum albumin (SA) has a marked species-dependent influence on bilirubin photoisomerization and, in particular, on the regioselectivity of the configurational isomerization.

On photolysis of **1** in $CHCl_3$–Et_3N (1:1) (Figure 1a–d) the system reached a photostationary state in ca. 10 min.[4] HPLC revealed the expected *E,E* and *E,Z* isomers (R_f 4.8 and 7.4 min, respectively) and a minor peak at R_f 6.1 min.[4] On continued photolysis the 6.1-min peak grew slowly, without affecting the *E,Z*:*Z,Z* ratio, along with a smaller peak (R_f 4.7 min) that ran close to *E,E*-**1**. All peaks except the R_f 6.1-min peak and a minor unidentified peak at R_f 6.7 min disappeared on treatment of the photolysate with CF_3CO_2H[1] (Figure 1e). Similar rapid configurational isomerization accompanied by slow growth of the 4.7- and 6.1-min peaks was observed when complexes of **1** with SA were irradiated at pH 7.4.[5] However, the identity of the albumin

(1) McDonagh, A. F.; Palma, L. A.; Trull, F.; Lightner, D. A. *J. Am. Chem. Soc.*, preceding communication in this issue, and references cited therein. See the preceding communication for the structure of **1**.

(2) McDonagh, A. F. "Phototherapy in the Newborn: An Overview"; Odell, G. B., Schaffer, R., Simopoulos, A. P., Eds.; National Academy of Sciences, Washington, DC, 1974; pp 56–73. McDonagh, A. F. "Bilirubin Metabolism in the Newborn (II)"; Bergsma, D., Blondheim, S. H., Eds.; Excepta Medica: Amsterdam, 1976; pp 30–38.

(3) McDonagh, A. F.; Ennever, J. F.; Palma, L. A., manuscript in preparation.

(4) Solutions containing 1.25 mg of pigment/10 mL were photolyzed under argon with a 20-W blue fluorescent tube.[1] Albumin solutions were made up in 0.1 M phosphate buffer, and the albumin:pigment ratio was 1.1:1. The solvent for reverse-phase HPLC (Beckman-Altex Ultrasphere-IP or Ultrasphere-ODS column, 5 μm, C-18, 25 × 0.46 cm; with a Beckman-Altex ODS precolumn, 4.5 × 0.46 cm) was 0.1 M di-*n*-octylamine acetate in MeOH (0.75 mL/min, detector 450 nm). Retention times may vary with age and origin of column. Retention volume = $0.75R_t$. For HPLC, albumin solutions were diluted 1:4 or 1:9 with ice-cold mobile phase, and 20 μL of the supernatant was injected. Lumirubins were isolated by solvent extraction[2] and TLC on silica (solvent, 1% AcOH in 10% MeOH–$CHCl_3$). Nonphotochemical work was done under safelights. Mass spectra were field desorption (FD) spectra.

(5) (a) **1** forms strong association complexes ($K_a \simeq 10^7$–10^8 M^{-1}) with mammalian serum albumins (See: McDonagh, A. F. "The Porphyrins"; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. 6, Chapter 6, pp 392–491). (b) Reactions were studied by using Cohn Fraction V serum albumins from horse, human, guinea pig, ox, and rat and also by using adult rat and human serum. (c) For photolysis of **1** in anaerobic conditions, see: McDonagh, A. F. *Ann. N.Y. Acad. Sci.* **1975**, *244*, 553–569.

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