Preparation and Properties of Bilirubin Photoisomers

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Polar photoisomers of bilirubin were formed by irradiation of bilirubin in chloroform solution in the absence of O₂. Two pairs of compounds were isolated with molecular weights identical with bilirubin. One pair reverted to bilirubin in polar media and gave chemical reactions similar to bilirubin; the other pair were not reconverted into bilirubin by chemical means and gave reactions distinct from those of bilirubin. However, both groups were reconverted into bilirubin by irradiation in chloroform solution in the absence of O₂. The probable role of these photoisomers in the catabolism of bilirubin during phototherapy of neonatal jaundice is discussed.

Phototherapy is the standard technique for decreasing serum concentrations of unconjugated bilirubin in neonatal jaundice (Seligman, 1977). Many studies of the effects of light on bilirubin have emphasized the formation of oxidation products, principally through attack of singlet oxygen (Bönnett, 1976), in turn formed by the reaction between excited bilirubin and ground-state triplet oxygen (McDonagh, 1971; Berry *et al.*, 1972; Lightner, 1977). Since these oxidation products of bilirubin are more polar than bilirubin and are readily excreted in the bile, or urine, it has been postulated that photo-oxidation is the major mechanism of increased bilirubin catabolism during phototherapy (Ostrow, 1971; Ostrow *et al.*, 1974; McDonagh & Palma, 1977).

Two observations from studies of phototherapy of the Gunn rat (Ostrow, 1971; Ostrow *et al.*, 1974), used as a model for neonatal jaundice, are not consonant with the postulated principal role of photo-oxidation reactions *in vivo*. (1) The major bilirubin photoderivative excreted in bile is a yellow labile polar compound that has not been found among the photo-oxidative derivatives of bilirubin formed *in vitro*. (2) A much increased output of unconjugated bilirubin in the bile occurs during phototherapy; something not easily explicable by an

oxidative mechanism. Excretion of the major photoderivative and the unconjugated bilirubin in the bile together account for 70% of the increased bilirubin turnover during phototherapy of the Gunn rat (Ostrow *et al.*, 1974). Therefore the mechanisms of their formation, and not oxidative processes, presumably represent the primary means of elimination of bilirubin during phototherapy.

These considerations prompted the present study of the anaerobic photoisomerization of bilirubin in chloroform solution with visible light. Two pairs of compounds, designated IA and IB and IIA and IIB respectively, have been isolated. They are shown to be isomers of bilirubin that are more polar than, and readily revert to, the parent pigment. Their properties can account for both the major photoproduct *in vivo*, and for the increased output of unconjugated bilirubin in bile, during phototherapy of the Gunn rat.

Experimental

Materials

Bilirubin was from BDH Chemicals, Broom Road, Poole, Dorset BH12 4NY, U.K. Chloroform, methanol, dimethyl sulphoxide, methyl acetate and acetone (J. T. Baker Co., Phillipsburg, NJ, U.S.A.) were analytical reagent grade. An unfiltered 100W high-pressure mercury spot lamp (model H-100, PSP44-4; General Electric Co.) was used as the source of radiation. Otherwise all operations were performed under subdued light. Silica gel G was from Analtech Inc., Newark, DE, U.S.A., and silica gel D-O was from Camag Inc., New Berlin, WI, U.S.A.

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Preparation of photobilirubins IA and IB

To bilirubin (100mg/litre) in chloroform was added a trace of EDTA. The solution was purged for 10min with 99.997% N₂ and then irradiated with the mercury lamp for 2min/mg of bilirubin. The solution was contained in a vessel, in turn placed in a beaker of water that was stirred magnetically and maintained at $23 \pm 0.5^\circ\text{C}$ by addition of ice as necessary. At the site of irradiation the inner vessel was placed a few millimetres from the inner wall of the beaker so that the bilirubin solution would be 2cm from the u.v. lamp at its closest point. Mixing of the bilirubin solution within the light beam was maintained by bubbling of N₂ through the solution. After irradiation, chloroform was removed by flash evaporation at 50°C *in vacuo* and photoproducts were extracted twice into acetone (1ml/3mg of bilirubin). The acetone extract was evaporated to dryness and the residue dissolved in chloroform for application to thin layers of silica gel. Chromatography was performed by using system I (composition given in Table 1), until the solvent front had ascended 12cm from the origin. Pigments were eluted individually with the development solvent, and evaporated to dryness *in vacuo* at 50°C .

Preparation of photobilirubins IIA and IIB

A trace of EDTA was added to bilirubin (150mg/litre) in dimethyl sulphoxide, and the solution purged with N₂ and irradiated as described for the preparation of photobilirubins IA and IB. After irradiation, chloroform (10vol.) was added, and the mixture washed four times with an equal volume of water to remove all dimethyl sulphoxide. The chloroform phase was evaporated to dryness, the polar photoproducts extracted twice into methanol (1ml/2mg of bilirubin) and the extract was evaporated to dryness *in vacuo* at 50°C . The residue was dissolved in chloroform, leaving a small residue of biliverdin, and subjected to t.l.c. in system II (composition given in Table 1). The pigments were eluted with the developing solvent, the eluate washed once with 0.1 M-NaHCO₃ (2vol.) and then twice with water to remove all traces of formic acid, and the solution evaporated to dryness *in vacuo* at 50°C .

Preparation of ^{14}C -labelled photobilirubins

[^{14}C]Bilirubin was prepared biosynthetically from δ -amino[4- ^{14}C]laevulinic acid (New England Nuclear Corp., Boston, MA, U.S.A.) injected into rats with a bile fistula. The labelled bilirubin was isolated and crystallized and its specific radioactivity determined (Ostrow *et al.*, 1961). From this [^{14}C]bilirubin, labelled photobilirubins were prepared as described above, in order to determine their molar absorption coefficients, their yields from bilirubin and the quantities of bilirubin and photo-

bilirubins extracted from t.l.c. plates during decay experiments.

Radioassay

^{14}C -labelled pigments were transferred to scintillation vials in a suitable solvent, which was evaporated to dryness. The pigment residue was dissolved in 0.2ml of 1 M-Hyaminate in methanol (New England Nuclear Corp.) and then 3.0ml of ethanol, 10ml of 2,5-diphenyloxazole in toluene (8g/litre) and 1 drop of acetic acid were added sequentially. Counting was performed to 10K in a Packard Tri-Carb model 2650 liquid-scintillation spectrometer at 87% efficiency, with an external standard.

Physical properties of the photobilirubins

Electronic spectroscopy. Spectra were recorded in spectroscopic-grade chloroform or methanol on a Coleman-Hitachi model 124 double-beam spectrophotometer. Molar absorption coefficients were determined by using ^{14}C -labelled pigments derived from [^{14}C]bilirubin of known specific radioactivity, with the concentration of pigment used for spectroscopy calculated from the radioactivity in the solution.

I.r. spectroscopy. Approx. 0.5mg of pigment was ground with 100mg of spectral-grade KBr, by using an agate mortar and pestle. A wafer was formed by compression at 138 MPa, and spectra were obtained with a Perkin-Elmer model 727 i.r. spectrophotometer between 600 and 4000cm^{-1} .

Mass spectrometry. Mass spectra were obtained by field desorption on a Varian CH5 mass spectrometer at a wire current of 24mA. Electron-impact spectra of the dimethyl esters were obtained on the same instrument, by using a different probe (Berry *et al.*, 1972). The dimethyl esters were prepared by addition of excess ethereal diazomethane to a solution of the pigment in chloroform. After standing at room temperature for 10min, the solution was evaporated to dryness *in vacuo* at 30°C . The esters were freed from small amounts of impurities by t.l.c. on Analtech silica gel G, with benzene/ethanol (10:1, v/v) as the solvent system.

Chemical and photochemical reactions of the photobilirubins

Diazo reaction. Pigments were dissolved in chloroform and reacted with diazotized sulphanilic acid by the method of Malloy & Evelyn (1937).

Dehydrogenation with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone. Pigment was dissolved in dimethyl sulphoxide, and a freshly prepared solution of 2,3-dichloro-5,6-dicyano-*p*-benzoquinone in dimethyl sulphoxide (1mg/ml) was added dropwise with mixing until no further colour change was observed (Stoll & Gray, 1977).

Anaerobic chemical reversion to bilirubin in the dark

In aqueous buffer solutions. The photobilirubin (100–250 µg) was dissolved in 10ml of each selected buffer (see Table 3) that had been previously deoxygenated by N₂ diffusion. The solution was then filtered rapidly through cotton wool to remove undissolved pigment (reverted bilirubin IX-α). While the solution was maintained in the dark at 23°C, serial 1ml portions were removed to a stoppered tube containing chloroform (2ml) and acetic acid (3 drops). After immediate vigorous shaking, the colourless aqueous upper layer was removed, and the chloroform phase was washed twice with 10ml of water. The pigments, recovered by evaporation of the chloroform under N₂, were then separated by t.l.c. in solvent system I until the front had ascended to 12cm. The t.l.c. plate was then removed and covered with a plain-glass plate to protect the pigments from air. Photobilirubin and bilirubin bands were individually removed and eluted with the development solvent into 1 or 2ml volumetric flasks. To minimize unequal decomposition on the t.l.c. plate, the two bands derived from each portion were removed sequentially and eluted before the components from the next portion were uncovered. The photobilirubin and bilirubin in each portion were quantified by measurement of their absorbance at 450nm on an Hitachi model 100-40 single-beam spectrophotometer, with ¹⁴C-labelled compounds as standards. Total recoveries of pigments from the eluted bands always exceeded 90% of the applied pigments. Decay constants were determined by linear regression analysis of the semi-logarithmic first-order decay curves of the disappearance of photobilirubin.

In Gunn-rat bile. The photobilirubin was dissolved in 5ml of Gunn-rat bile, obtained during the first 10h

after cannulation of the bile duct under pentobarbital anaesthesia. Dissolution of the pigment was aided by sonication for 1min before filtration. Serial 0.5ml portions were taken, and the extractant consisted of chloroform (2ml), water (0.5ml), 6mM-disodium EDTA (150 µl) and acetic acid (3 drops). Extraction, washing, t.l.c. analysis and calculation of decay constants were performed as above.

Anaerobic photochemical reversion of photobilirubin to bilirubin

The photobilirubin (10 µg) was dissolved in 1ml of chloroform, purged with N₂ and then irradiated for 1min as described above for bilirubin. The pigments were recovered by evaporation of the chloroform and identified by t.l.c. in two systems: chloroform/methanol/formic acid (30:2:1, by vol.) (Berry *et al.*, 1972) on Analtech silica gel G, and chloroform/acetic acid (100:1, v/v) on Analtech silica gel H (McDonagh & Assisi, 1971).

Results*Physical properties of the photobilirubins*

The major products consisted of two pairs of photobilirubins. All four had lower mobilities than bilirubin on silica gel adsorption chromatography, indicating that the photobilirubins were more polar than bilirubin. The slower moving pair (photobilirubins IIA and IIB) was cleanly resolved into two components only in t.l.c. system III (for composition, see Table 1). In the acidic systems II and III, steady conversion into bilirubin during chromatography led to tailing of the less mobile photobilirubins IA and IB. Yields of the photobilirubins calculated from ¹⁴C-radioassay data were: IA, 6.4%; IB, 1.4%; and II, 2.8%. Some minor green and violet

Table 1. *Mobilities of bilirubin and photobilirubins in various t.l.c. systems*

Gel layers were 0.5mm thick, and were activated for 1h at 120°C before use. Tanks were lined and pre-equilibrated with the developing solvent for at least 2h before use.

System number	Gel	Developing solvent	<i>R_F</i> values				
			Bilirubin	Photobilirubins			
				IA	IB	IIA	IIB
I	Silica gel D-O containing 0.17% (w/w) disodium EDTA	Chloroform/methanol/water (40:9:1, by vol.)	0.61	0.52	0.42	0.27	0.27
II	Silica gel G	Chloroform/methanol/formic acid (30:3:1, by vol.)	0.90	Decomposition and tailing		0.20–0.40	0.20–0.40
III	Silica gel G	Chloroform/methyl acetate/methanol/formic acid (14:26:3:1, by vol.)	—	Decomposition and tailing		Separate on double development	

Table 2. *Electronic-spectral properties of bilirubin and photobilirubins in chloroform and methanol*

Compound	Solvent			
	Chloroform		Methanol	
	$\lambda_{\max.}$ (nm)	$\epsilon_{\max.}$ (litre·mol ⁻¹ ·cm ⁻¹)	$\lambda_{\max.}$ (nm)	$\epsilon_{\max.}$ (litre·mol ⁻¹ ·cm ⁻¹)
Bilirubin	453	60.0×10^3	Insoluble	
Photobilirubin IA	446	56.6×10^3	439	53.4×10^3
Photobilirubin IB	444	38.4×10^3	433	42.8×10^3
Photobilirubins IIA and IIB	441	40.9×10^3	429	45.0×10^3

Fig. 1. *I.r. spectra in KBr pellets of bilirubin (a), photobilirubins IA and IB (b) and photobilirubins IIA and IIB (c)*

bands were observed also, but were not identified further.

Electronic spectroscopy (Table 2). The photobilirubins exhibited visible-absorption maxima at shorter wavelengths than that of bilirubin, and the maxima were at shorter wavelengths in methanol than in chloroform. In all cases the molar absorption coefficients were lower than that of bilirubin (60.0×10^3 litre·mol⁻¹·cm⁻¹ in chloroform at 453 nm).

I.r. spectroscopy (Figs. 1a–1c). The i.r. spectrum of photobilirubins IA and IB (Fig. 1b) and II (Fig. 1c) showed prominent peaks at 2850, 2920 and 1380 cm⁻¹, absent or much decreased in the spectrum of bilirubin (Fig. 1a). The spectrum of photobilirubin II

differed from those of both bilirubin and photobilirubin I over the range 600–1800 cm⁻¹, showing enhanced peaks at 1740, 1670, 1570, 1450 and 1190 cm⁻¹. The peak at 3440 cm⁻¹ was progressively less prominent in photobilirubins I and II, compared with bilirubin, whereas the peak at 1250 cm⁻¹ was similar in all three pigments.

Mass spectrometry. On field-desorption mass spectrometry photobilirubin I showed a weak molecular ion at *m/e* 584 and no other ions. Photobilirubin II showed a molecular ion at *m/e* 584, a prominent *M*+1 ion at *m/e* 585, and no other ions. Its dimethyl ester showed a molecular ion at *m/e* 612 only.

All attempts to obtain electron-impact spectra of the free photobilirubins were unsuccessful. The electron-impact spectrum of the dimethyl ester of photobilirubin II confirmed the molecular ion at *m/e* 612 and gave a fragmentation pattern similar to that of bilirubin dimethyl ester. However, the ions at *m/e* 298, 300, 312 and 314, due to fission at the central methylene bridge, were much less prominent for the photobilirubin ester than for bilirubin.

Chemical properties of the photobilirubins

The Malloy and Evelyn diazo reaction. Photobilirubins IA and IB yielded a normal violet colour indistinguishable from the reaction given by bilirubin. Photobilirubin II, however, slowly turned an atypical pale orange, similar to that seen in Gunn-rat bile after phototherapy (Ostrow, 1971).

Reaction with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone. Photobilirubins IA and IB rapidly yielded a green colour, indistinguishable from that given by bilirubin, indicating the formation of biliverdin. Photobilirubin II, however, gave a plum red colour that, on extraction and t.l.c., revealed the presence of a large number of compounds that were not characterized further.

Anaerobic photoreversion to bilirubin. Brief illumination of each of the photobilirubins in chloroform solution under N₂ gave, as the major product,

Table 3. Reaction-rate data for the anaerobic conversion of photobilirubin IA into bilirubin in the dark in various polar media at 23°C

Initial concentrations of photobilirubins ranged from 16 to 36 μM . $t_{\frac{1}{2}}$ values were calculated from semi-logarithmic regression lines of the disappearance of photobilirubin. Correlation coefficients ranged from 0.90 to 0.99.

Solution in which photobilirubin was dissolved	pH	1 mM-Disodium EDTA present	$t_{\frac{1}{2}}$ (min)
Sodium phosphate buffer (0.1 M)	7.36	+	70.2
	8.27	+	19.0
	8.50	+	16.9
Phosphate/borate (Koltoff) buffer ($I/2 = 0.1$)	8.49	+	20.0
		—	8.4
	8.99	+	20.0
Gunn-rat bile (bile acids, 6.7 mM)		—	4.7
	8.07	—	12.0

bilirubin IX- α , as characterized by t.l.c. (McDonagh & Assisi, 1971).

Behaviour in various solutions in the absence of light and O_2 . Photobilirubin IA. This reverted rapidly to bilirubin IX- α in a variety of aqueous media. In all cases, the decay followed first-order kinetics, with a half-life that varied from 70.2 min at pH 7.36 in the presence of disodium EDTA, to 4.7 min at pH 8.99 in the absence of disodium EDTA (Table 3). The half-life decreased as the pH increased from 7.36 to 8.50, and disodium EDTA retarded the decay process markedly. In Gunn-rat bile, at the physiological pH of 8.07, the half-life of photobilirubin IA was 12.0 min.

Photobilirubin II. In contrast with photobilirubin I, all attempts to revert the more polar photobilirubin into bilirubin by chemical means were unsuccessful. As long as light and O_2 were excluded, photobilirubin II was stable in chloroform, methanol, dimethyl sulphoxide and 0.1 M-NaOH. Exposure to acetic acid led to formation of insoluble brown products, which remained at the origin during t.l.c. in system II, but no bilirubin. In toluene-*p*-sulphonic acid in benzene (1 mg/ml), at temperatures from 23 to 80°C, extensive degradation occurred, with multiple products found on t.l.c., but no bilirubin. Exposure to air in chloroform solution, even in the dark at 4°C, slowly yielded multiple violet compounds that could be separated by t.l.c. in system II. These decomposed to ill-defined grey products during elution from the gel, precluding their identification.

Discussion

Recent X-ray-diffraction studies (Bonnett *et al.*, 1976) have demonstrated that crystalline bilirubin

IX- α is normally in the *Z,Z*-configuration, containing six internal hydrogen-bonds, all of which involve the carboxy groups (Fig. 2a). As a result, the ionization of the two carboxy groups is suppressed, accounting for the high pK_a (7.95) and water insolubility of bilirubin (Overbeek *et al.*, 1955). N.m.r. studies by Falk *et al.* (1975) have shown that a model dipyrromethanone, which serves as a model for half of the bilirubin molecule, is likewise normally in the *Z*-configuration. It is partially converted by irradiation into a more polar *E*-isomer, which in turn readily reverts to the more stable *Z*-form in non-polar solvents. These observations suggested to us that *Z,Z*-bilirubin might likewise undergo *cis-trans* isomerization to *E*-isomers during irradiation. Molecular models indicated that the carboxy groups in such *E*-isomers would be incapable of the same degree of internal hydrogen-bonding as the parent *Z,Z*-compound. In the *E*-isomers, the carboxy groups would therefore be free to ionize, so that these isomers should be more polar than *Z,Z*-bilirubin (Figs. 2b and 2c). Moreover, since the *E*-form would be thermodynamically less stable, it should revert to the *Z*-form under a variety of conditions, among which should be irradiation.

To test this hypothesis, we attempted to produce such *E*-isomers by irradiation of bilirubin under anaerobic conditions. O_2 was excluded because it was considered likely that the photoisomers might otherwise rapidly react with singlet oxygen, and thus deteriorate before they were detected and isolated. As predicted by these hypotheses, four major new photoproducts were obtained in the absence of O_2 , all of which were isomers of bilirubin. This was documented by the molecular ions at *m/e* 584 and *m/e* 612, obtained by mass spectrometry of the free photoproducts and their dimethyl esters respectively.

The physical and chemical properties of these photobilirubins are compatible with their being *E*-isomers of bilirubin, but, so far, there is no direct evidence to support this contention. Indirect evidence in favour of this proposal includes: (1) the high polarity of the photobilirubins, consistent with freedom of the carboxy groups to ionize, arising from disruption of the normal internal hydrogen-bonding of bilirubin (models have shown that this would be the case in *E*-bilirubins; Figs. 2b, 2c, 2d and 2e); (2) the shorter wavelengths of the absorption maxima of the photobilirubins compared with that of bilirubin, compatible with non-coplanarity of the two rings in the dipyrrolic chromophore, thus allowing less π -electron overlap (this too is found in models of the *E*-bilirubins); (3) the large absorption bands at 2850 and 2920 cm^{-1} in the i.r. spectra of the photobilirubins, which are absent in that of bilirubin (this is indicative of modification of the carboxy -OH group, again compatible with disruption of the normal hydrogen-bonding in bilirubin); (4) the

Fig. 2. Structures of bilirubins

(a) *Z,Z*-Bilirubin IX- α (normal form in crystalline state elucidated from X-ray diffraction data); (b) *E,E*-bilirubin IX- α ('open' rotamer elucidated from model data); (c) *E,E*-bilirubin IX- α ('closed' rotamer elucidated from model data); (d) *E,Z*-bilirubin IX- α ('open' rotamer elucidated from model data); (e) *Z,E*-bilirubin IX- α ('open' rotamer elucidated from model data).

rapid reversion to bilirubin IX- α of all the photobilirubins on exposure to light, and of the less stable photobilirubins IA and IB on exposure to polar media (this indicates that they are either isomers of bilirubin, or are addition compounds that eliminate the added species on reversion to bilirubin). However, the mass-spectral data, and the formation of the same photobilirubins on illumination of bilirubin in different solvents, argues against addition products. Some recent work (Lightner *et al.*, 1979) dealing with irradiation of bilirubin dimethyl ester supports the structural interpretation proposed in the present paper.

If the photobilirubins are indeed geometrical isomers of bilirubin, it seems most likely that the less stable photobilirubins IA and IB are the two isomeric forms of *E,Z*-bilirubin (Figs. 2d and 2e), and that the more stable photobilirubin II is the *E,E*-isomer (Figs. 2b and 2c). In keeping with the less extensive structural alteration of an *E,Z*-isomer, photobilirubins IA and IB have chemical and spectral properties more akin to bilirubin, and are more readily reverted to bilirubin in the absence of light, than is photobilirubin II. Moreover, there was a higher yield of photobilirubin II than IA and IB when bilirubin was irradiated in dimethyl sulphoxide, whereas only traces of photobilirubin II were generated on irradiation of bilirubin in chloroform. This is compatible with the greater hydrogen-bond-breaking power of dimethyl sulphoxide, which should thermodynamically favour formation of the *E,E*-isomer (II), which has fewer hydrogen bonds than the *E,Z*-bilirubins.

Of the two *E,Z*-isomers, the one with the altered *E*-configuration about the 4,5-double bond would throw the more tightly conjugated endovinyl group out of alignment with the conjugated diene system in the B-ring, giving its absorption maximum a shorter wavelength and lesser intensity than that of the 15*E*,16*E*-isomer. This would suggest that the photobilirubin IB is the 4*E*,5*E*-isomer (Fig. 2d), and that photobilirubin IA, spectrally more akin to bilirubin, is the 15*E*,16*E*-isomer (Fig. 2e).

On high-resolution t.l.c., photobilirubin II was separated into two components. However, individual elution and rechromatography of either component always yielded approximately equal quantities of both components. It is therefore postulated that these two components represent two readily interconvertible forms of *E,E*-bilirubin, e.g. the 'closed' and 'open' rotamers represented in Figs. 2(b) and 2(c). Molecular models indicate that both could exist, and that they would be separated by a steric energy barrier. The easy interconversion of photobilirubins IIA and IIB renders it unlikely that one component is the cyclized furan isomer of the D-ring of bilirubin produced by irradiation of bilirubin in dimethylformamide in the presence of reducing agents (Roos *et al.*, 1977).

Several puzzling features remain. (1) Photobilirubin II differs chemically in many ways from photobilirubins IA and IB, and is much more stable in solution. This might be explained by the formation of a new stable hydrogen-bonded configuration, requiring interactions between the two free carboxy groups. (2) Brief irradiation of photobilirubin II gave some bilirubin, but yielded no photobilirubin I, an anticipated intermediate if the proposed structures are correct. However, the excited states of *Z,E*- and *E,Z*-bilirubins may be so unstable that they do not accumulate in detectable amounts during photoconversion of photobilirubin II into bilirubin. (3) Rechromatography of the isolated photobilirubins IA and IB yielded both photobilirubin I and bilirubin. Subsequent isolation of the photobilirubin component and rechromatography showed the same phenomenon. This suggested that photobilirubins IA and IB exist in solution as complexes with bilirubin, but that on chromatography the components separate, the photobilirubin component presumably being stabilized by binding to the silica gel. On elution from the gel the photobilirubin immediately undergoes disproportionation, with formation of sufficient bilirubin to reform the relatively stable equimolar complex. It therefore appears that all the studies reported in the present paper on photobilirubins IA and IB might actually be studies of photobilirubin I-bilirubin complexes.

Further studies of these compounds will be required to establish definitive structures. Unfortunately their relative instability has thus far precluded satisfactory n.m.r. and X-ray diffraction studies. However, whatever their structures, the properties of these isomers indicate that they might play an important role in the phototransformation of bilirubin *in vivo*.

Thus, the more stable photobilirubin II has spectral and chemical properties that strongly resemble the major photoproduct (called C-9) isolated by Berry *et al.* (1972) and Ostrow *et al.* (1974) from the bile of the Gunn rat during phototherapy. It also resembles the '430 pigment' described by Kapitulnik, which was found in the excreta of an infant with the Crigler-Najjar syndrome (Kapitulnik *et al.*, 1974), and can be ultrafiltered from bilirubin-albumin solutions after illumination (Kapitulnik *et al.*, 1973). The common properties include the R_F values in t.l.c. system I, the absorption maximum near 430 nm, the atypical diazo-reactivity and the tendency to form brown insoluble (presumably polymeric) residues in acid solutions.

The unstable photobilirubins IA and IB revert within a few minutes to *Z,Z*-bilirubin in alkaline aqueous solutions in the dark, a degradation retarded by disodium EDTA. This suggests that bivalent cations may catalyse this reversion, akin to their role in the oxidation of bilirubin in alkaline solutions.

The more rapid reversion of photobilirubins IA and IB to bilirubin with increasing pH suggests that ionization of the Z,Z-bilirubin product, which becomes significant above pH 7.4 and is nearly complete at pH 8.5 (Overbeek *et al.*, 1955), stabilizes the product and favours the reversion reaction. This reversion also occurs rapidly in Gunn-rat bile at the physiological pH of 8.07 ($t_{1/2}$ = 12 min), a rate that would render difficult the detection of unstable photobilirubin I in fistula bile collected from the Gunn rat during phototherapy. However, excretion of the polar unstable photoisomers IA and IB, with subsequent reversion to bilirubin during passage down the biliary tree, could readily account for the dramatic increase in unconjugated bilirubin observed in common-duct bile during phototherapy of the Gunn rat (Ostrow, 1971; Ostrow *et al.*, 1974). Presumably, the lower pH values in plasma (7.4), and within the hepatocyte (approx. 6.8–7.0), would slow the reversion of the photobilirubins IA and IB. Their configuration would thus be preserved during passage from their presumed site of formation in the skin, through the plasma and liver, until excreted in the bile.

Thus, the bilirubin photoisomers described here could account both for the major photoproduct *in vivo*, and for the increased appearance of unconjugated bilirubin in Gunn-rat bile, during phototherapy. These possibilities have been confirmed by studies of the excretion of labelled products in bile after intravenous administration of pure [^{14}C]photobilirubins to Gunn rats (M. S. Stoll, E. A. Zenone & J. D. Ostrow, unpublished work). Therefore the major mechanism for increased bilirubin turnover during phototherapy may be anaerobic photoisomerization, rather than photo-oxidation, as had been suggested previously (McDonagh, 1976; Lightner, 1977).

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