Biochem. J. (1982) **201**, 179–188
Printed in Great Britain

Concerning the structure of photobilirubin II

Mark S. STOLL, Nigel VICKER and Charles H. GRAY
Division of Clinical Chemistry, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, U.K.

and Raymond BONNETT*
Department of Chemistry, Queen Mary College, Mile End Road, London E1 4NS, U.K.

(*Received 30 April 1981/Accepted 10 September 1981*)

Evidence is presented which supports the postulate that the photobilirubins IIA and IIB are diastereoisomers in which the C-3 vinyl group has cyclized intramolecularly. The evidence comes principally from proton n.m.r. spectroscopy at 400MHz and from chemical considerations. The cyclic structures require the *E*-configuration at the C-4 double bond in the precursor; this is the first structural evidence for the $Z \rightarrow E$ isomerization in bilirubin and supports the view that the precursor (photobilirubin IA or IB) is (4*E*,15*Z*)-bilirubin. Brief irradiation of photobilirubin II gives bilirubin, a new compound (photobilirubin III) and unchanged starting material. The various photoisomers are discussed in terms of their inter-relationships and biological fates.

Since its discovery by Cremer *et al.* (1958) the phototherapy of neonatal hyperbilirubinaemia has gained increasing acceptance in clinical medicine (Seligman, 1977). The lowering of serum bilirubin concentrations appears to occur along at least two distinct pathways. The reported increase in propentdyopent adducts in the urine after phototherapy (Porto, 1970) suggests a photodegradation pathway. This has attracted considerable interest, as a result of which bilirubin is now known to be readily photo-oxidized in hydroxylic solvents to various water-soluble products, including propentdyopent adducts (Bonnett & Stewart, 1975). There is much evidence which suggests that singlet oxygen is involved in this reaction (McDonagh, 1971; for review; see Lightner, 1977).

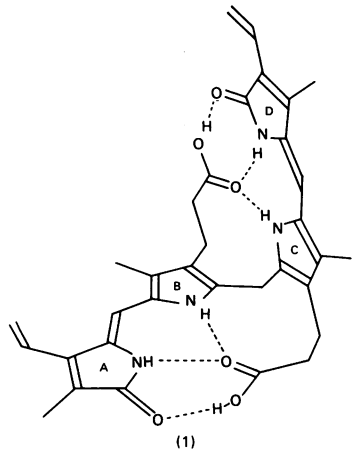
A second mechanism, which is at present generally regarded as the major pathway, involves photosolubilization. Early physiological experiments on human infants (Lund & Jacobsen, 1972) and on Gunn rats (Ostrow, 1971) showed that irradiation of the animal led to the increase of bilirubin secretion in the bile. The light had solubilized the bilirubin (1), so that it could be excreted by the liver without hydrophilic conjugation. The cause of the insolubility of bilirubin was evident from X-ray crystal analysis, which established the 4*Z*,15*Z* structure (1) with a closely-knit system of six hydrogen bonds (Bonnett *et al.*, 1976). Clearly, a photoisomerization at the C-4–C-5 and/or C-15–C-16 bond would generate a system (e.g. structure 2) in which the hydrogen bonding propensities of the various polar groups could no longer be satisfied intramolecularly and the molecule would be expected to hydrogen-bond to molecules of solvent water, and thus to become more soluble in that solvent (Bonnett, 1976; McDonagh & Palma, 1977; Stoll *et al.*, 1977; Bonnett *et al.*, 1978). This idea required as a basis the secure knowledge, which the X-ray work afforded, of the configuration of ordinary bilirubin.

Evidence for a photoequilibrium with small amounts of photobilirubins which are more soluble in aqueous media, and which revert, more or less readily, to bilirubin, has come from irradiation of bilirubin solutions, preferably in the absence of oxygen. Three research groups have been active, and have used different nomenclatures for the photobilirubins. Although reasonable doubt has been expressed about the identity of the various photoproducts (McDonagh *et al.*, 1980) the relationship between the photoproducts is probably as shown in Table 1.

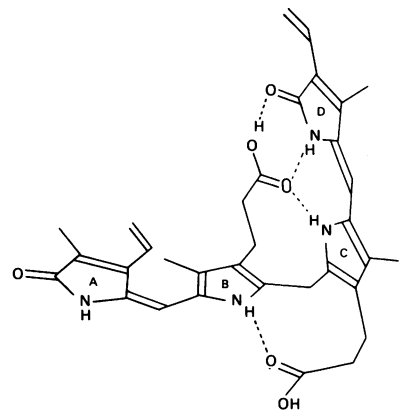
The major photoproduct (*EZ*/*ZE*, peak 3, photobilirubin IA/IB) is probably a mixture of (4*E*,15*Z*)-bilirubin (2) and (4*Z*,15*E*)-bilirubin, as proposed by Stoll *et al.* (1977, 1979), Lightner *et al.* (1979*a*,*b*) and McDonagh *et al.* (1979), although because these substances revert so readily to (4*Z*,15*Z*)-bilirubin (1), chemical proof of this is still lacking. These photoproducts have been detected in the blood

* To whom reprint requests should be sent.

0306-3275/82/010179-10$01.50/1



(1)



(2)

serum of jaundiced infants (Onishi *et al.*, 1979, 1980*a*) and of Gunn rats (McDonagh *et al.*, 1980) undergoing irradiation.

Another photoproduct (peak 2, 'unknown pigment', photobilirubin IIA/IIB) has been detected in the bile of irradiated jaundiced infants (Onishi *et al.*, 1980*a*) and of irradiated Gunn rats (Ostrow *et al.*, 1974; Zenone *et al.*, 1977; Stoll *et al.*, 1981). It is rapidly excreted in bile when injected into Gunn rats (Zenone *et al.*, 1977; Stoll *et al.*, 1981). Here it will be referred to as photobilirubin II. Its structure is unknown, and is the subject of the present paper.

Experimental

Materials and methods

Bilirubin was from Koch–Light Laboratories (Colnbrook, Bucks., U.K.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chloroform (stabilized with 1% ethanol), acetone, methanol and formic acid were analytical reagent grade, dimethyl sulphoxide and EDTA (disodium salt) were reagent grade (identical results were obtained with AR grade dimethyl sulphoxide). Thin-layer plates (20cm × 20cm × 0.2mm) were silica type K6 (Whatman).

^{1}H n.m.r. spectra were recorded on a Bruker WH400 instrument in [$^2H_6$]dimethyl sulphoxide (Goss, Ingatestone, Essex, U.K.) dried over molecular sieve type 4A. I.r. spectra were recorded on a model 225 grating spectrophotometer (Perkin–Elmer, Beaconsfield, Bucks., U.K.) in a KBr disc.

Elemental analysis was performed by Butterworths Microanalytical Laboratory, Teddington, Middx., U.K.

Photobilirubin II

The method employed was that of Stoll *et al.* (1979) with modification. Bilirubin (200mg) in dimethyl sulphoxide (200ml) containing disodium EDTA (5mg) was contained in a 220ml centrifuge glass bottle, which in turn was placed in a beaker (1 litre) of stirred water at 25°C. The solution was purged with argon for 10min before, and during, irradiation. The solution was irradiated for 60min with light from a 100W medium pressure mercury lamp (Blak-Ray model B-100A, Ultraviolet-Products, Cambridge, U.K.) filtered through a Wratten type 2A filter (Kodak), cut off 405nm, transmission 80% at 438nm. The filter was held between glass plates and cooled by an air blower to prevent damage by overheating. The lamp face was 7cm from the filter and 10cm from the front of the irradiation vessel.

After irradiation, chloroform (400ml) and water (1 litre) were added to the reaction mixture, and, after shaking, the chloroform layer was separated. The aqueous phase was extracted with chloroform (4 × 50ml). The combined chloroform solution was washed with water (3 × 1 litre), each washing being back-extracted with chloroform (2 × 50ml) to minimize loss of pigment. Each chloroform washing was recombined with the main chloroform solution. The resulting chloroform solution was filtered through a chloroform-moistened cottonwool plug and taken to dryness under reduced pressure at <40°C. The residue, containing mainly bilirubin with some photobilirubins, was leached with methanol (3 × 10ml). The methanol extracts were decanted from the insoluble bilirubin, filtered through methanol-moistened cottonwool (previously washed with methanol) and evaporated to dryness. The residue was again treated with methanol (2 × 10ml). If at this stage there was an appreciable residue, the procedure was repeated using methanol (2 × 5ml). This process reduces the amount of bilirubin and

Table 1. *Photobilirubins formed by irradiating bilirubin*

Authors	Solvents used for irradiation	Designations of photoproducts (increasing polarity, left to right)			
		EZ/ZE	—	*EE*	—
Lightner *et al.* (1979*a*,*b*); McDonagh *et al.* (1979)	Chloroform/ethanol/triethylamine				
Onishi *et al.* (1980*a*,*b*)	Phosphate buffer (pH 7.4) containing human serum albumin	Peak 3 (photobilirubin)	Peak 2 ('unknown pigment')	—	Peak 1
Stoll *et al.* (1979); the present authors	Chloroform/ethanol/EDTA or dimethyl sulphoxide/EDTA	Photobilirubins IA/IB	Photobilirubins IIA/IIB	—	Photobilirubin III
Evidence for equivalences		Onishi *et al.* (1980*b*); Stoll *et al.* (1981); Isobe & Onishi (1981)	Onishi *et al.* (1980*b*); Stoll *et al.* (1981); Isobe & Onishi (1981)		

photobilirubin I reaching the chromatography stage. The insoluble residue from the methanol extractions is bilirubin of adequate purity for re-use in this preparation.

The final methanol extract was taken to dryness under reduced pressure and applied in chloroform to a thin-layer plate and developed with chloroform/methanol/water (40:9:1, by vol.). Photobilirubin II appeared as a major yellow band at R_F about 0.41. A minor band just resolved from and below this band is regarded as the XIIIα isomer of photobiliburin II; the proportion of this material increased as the bilirubin was recycled. The evidence for the identity of this lower band was that it gave bilirubin XIIIα on irradiation. The major bands of high R_F were bilirubin (R_F about 0.87) and photobilirubins IA (R_F about 0.80) and IB (R_F about 0.65). Photobilirubin II was eluted from the silica with the development solvent slightly enriched in methanol, and the solution was evaporated under reduced pressure at <40°C. To remove traces of water the residue was dissolved in acetone, and again taken to dryness as before. Such a procedure typically gave about 2mg (1%) of photobilirubin II as an amorphous yellow–brown solid.

Photobilirubins IIA and IIB

As reported previously (Stoll *et al.*, 1979), photobilirubin II is separated into two components by t.l.c. [Whatman K6; chloroform/butanone/methanol/formic acid (20:20:3:1, by vol.) as solvent], but on isolation and rechromatography each component is partially converted into the other, thus frustrating attempts to study the individual compounds.

For n.m.r. examination, the photobilirubin II sample was prepared as follows. The pigment was suspended/dissolved in [^{2}H]chloroform (about 0.1ml), and the solvent was evaporated in a stream of argon. The procedure, designed to remove any proton-containing residual solvent, was repeated once. The residue was dissolved in [$^2H_6$]dimethyl sulphoxide (3mM) with tetramethylsilane as internal reference. The comparison spectrum of bilirubin was measured on a 9mM solution.

Irradiation of photobilirubin II

Photobilirubin II (1mg) in chloroform (20ml) was purged with argon and irradiated with the filtered light source for 5min. The solvent was removed under reduced pressure and the residue was applied in chloroform/methanol (2:1, v/v) to a silica-gel plate (Whatman K6) and developed as before. The three major yellow bands were bilirubin (R_F about 0.9, about 3%), photobilirubin II (R_F about 0.4, about 62%) and a new pigment, termed photobilirubin III (R_F about 0.1, about 8%, assuming

$\varepsilon_{\lambda(max.)} = 40000\,M \cdot cm^{-1}$). On repeating the t.l.c., photobilirubin III was converted mainly into photobiliburin II.

Results and discussion

The spectrum of bilirubin has been recorded previously at lower field (McDonagh, 1979) and the chemical-shift assignments are given in Fig. 1. Expanded portions of the ^1H n.m.r. spectra of bilirubin and photobilirubin II are shown in Figs. 2, 3, 4 and 5.

The spectrum of photobilirubin II shows a number of high-field signals, some of which are interpreted as impurities. The sample of photobilirubin II examined is a mixture of two components (IIA/IIB, separable by analytical t.l.c., but so far resistant to purification by preparative-scale t.l.c.). Moreover the manner of preparation of photobilirubin II, which involves purification by differential extraction and not by crystallization, is more likely to be associated with the presence of minor contaminants. Initial spectra taken at lower field could not be interpreted, but the spectrum at 400 MHz permits structural conclusions.

The key differences between the two spectra are as follows.

(*a*) *Olefinic protons* (*Fig. 2*)

The olefinic region of the spectrum of bilirubin shows an ABX system attributed to the vinyl group at C-3, and an AMX system attributed to the vinyl group at C-18, together with a singlet for the two *meso* protons. A comparison with this region in photobilirubin II (Table 2) reveals that one vinyl group has changed and that, provided the original assignments in Fig. 1 are correct, then the vinyl group at C-3 is the one that is affected. It is also evident that one *meso* proton (δ6.11 p.p.m.) has been altered in some way, and that two new olefinic protons (δ5.78 and 5.89) are revealed.

(*b*) *C-10 protons* (*Fig. 3*)

The protons of the C-10 methylene group are

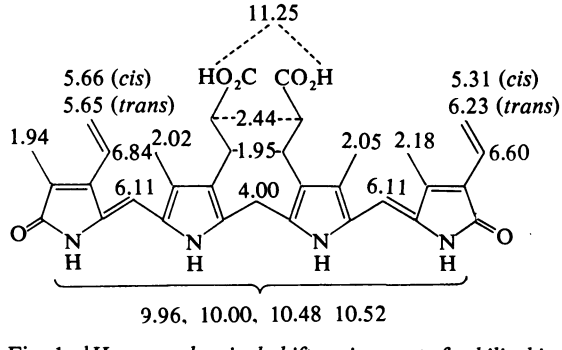


Fig. 1. *^1H n.m.r. chemical-shift assignments for bilirubin IXα*
The numbers refer to δ (p.p.m.) based on tetramethylsilane $\delta = 0$.

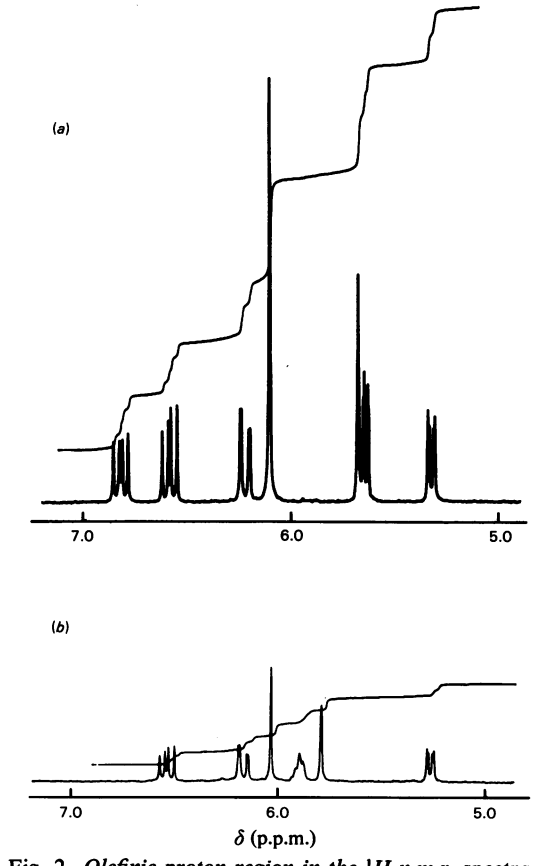


Fig. 2. *Olefinic proton region in the ^1H n.m.r. spectra of bilirubin IXα (a) and of photobilirubin II (b)*

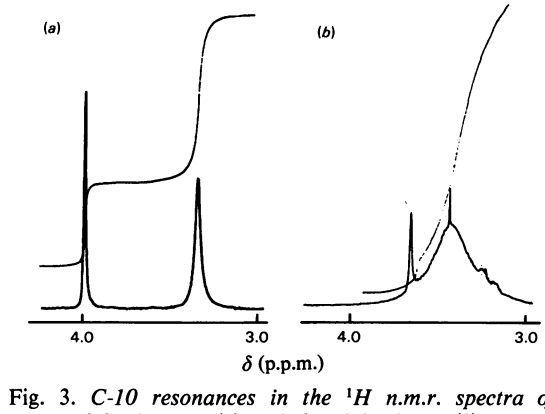


Fig. 3. *C-10 resonances in the ^1H n.m.r. spectra of bilirubin IXα (a) and photobilirubin II (b)*

Table 2. *Comparison of chemical shifts in [$^2H_6$]dimethyl sulphoxide of olefinic protons in bilirubin and in photobilirubin II*

Bilirubin				Photobilirubin II		
δ (p.p.m.)	Multiplicity [coupling constants (Hz)]	Number of protons	Assignment (ring, position)	δ (p.p.m.)	Multiplicity [coupling constants (Hz)]	Number of protons
6.84	Double doublet (J = 11.8, 17.8)	1	A (3^1)			
6.60	Double doublet (J = 11.8, 17.0)	1	D (18^1)			
				6.55	Double doublet (J = 11.6, 17.6)	1
6.23	Double doublet (J = 2.6, 17.4)	1	D ($18^{2\text{-}trans}$)			
				6.17	Double doublet (J = 3.0, 17.4)	1
				6.03	Singlet	1
6.11	Singlet	2	*meso* (5,15)			
				5.89	Multiplet	1
				5.78	Singlet (see the text)	1
5.66	Double doublet (J = 1.6, 10.4)	1	A ($3^{2\text{-}cis}$)			
5.65	Double doublet (J = 1.2, 18.6)	1	A ($3^{2\text{-}trans}$)			
5.31	Double doublet (J = 2.8, 11.2)	1	D ($18^{2\text{-}cis}$)			
				5.24	Double doublet (J = 3.0, 11.4)	1
	Total =	8			Total =	6

more shielded in photobilirubin II (δ3.62) than they are in bilirubin (δ4.00). The signal at about δ3.4 in both spectra, particularly broad in the case of photobilirubin II, is attributed to water.

(*c*) *Methyl groups attached to unsaturated centres* (*Fig. 4*)

In bilirubin the four *C*-methyl groups are clearly distinguished and are assigned as shown in Table 3. These assignments are based on comparisons with the spectra of bilirubins IIIα and XIIIα, which were also recorded but are not shown, and those of biliverdin IIIα, IXα and XIIIα dimethyl esters and dihydrobiliverdin IIIα, IXα and XIIIα dimethyl esters reported previously (Bonnett & McDonagh, 1970; Stoll & Gray, 1977). In photobilirubin II, two *C*-methyl resonances have disappeared from this region; again it is the A/B rings that are affected, i.e. the signals due to the methyl groups at C-2 and C-8 are missing.

Changes are also apparent in the propionic acid side chains. In bilirubin the signals occur as multiplets at δ1.95 (α-CH_2) and δ2.44 (β-CH_2), whereas in photobilirubin II this region is much more complex and broad, and structured signals are seen at about δ2.3 and δ2.6. This region is complicated by the appearance of the signal due to [$^2H_6$]dimethyl sulphoxide and by a peak at about δ1.9.

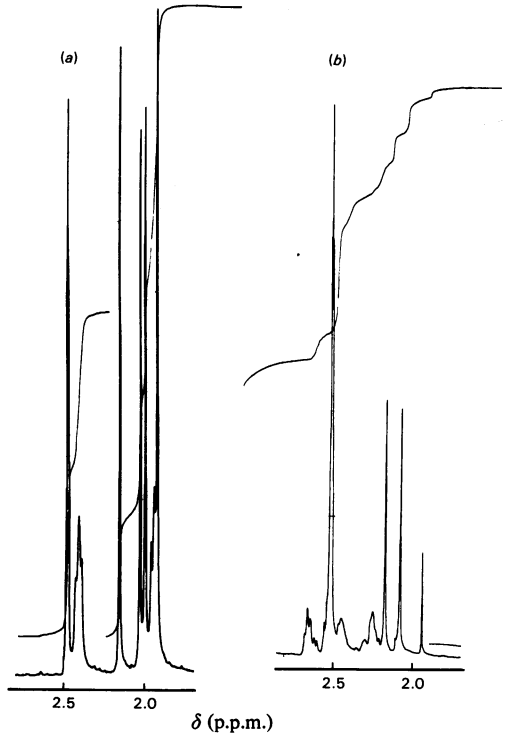


Fig. 4. *Resonances due to methyl groups attached to unsaturated centres in the ^1H n.m.r. spectra of bilirubin IXα (a) and photobilirubin II (b)*

Table 3. *Comparison of chemical shifts in [$^2H_6$]dimethyl sulphoxide of methyl groups attached to unsaturated centres in bilirubin and in photobilirubin II*

Bilirubin				Photobilirubin II		
δ (p.p.m.)	Multiplicity	Number of protons	Assignment (ring, position)	δ (p.p.m.)	Multiplicity	Number of protons
1.94	Singlet	3	A (2^1)			
2.02	Singlet	3	B (7^1)			
2.05	Singlet	3	C (13^1)	2.05	Singlet	3
				2.15	Singlet	3
2.18	Singlet	3	D (17^1)			

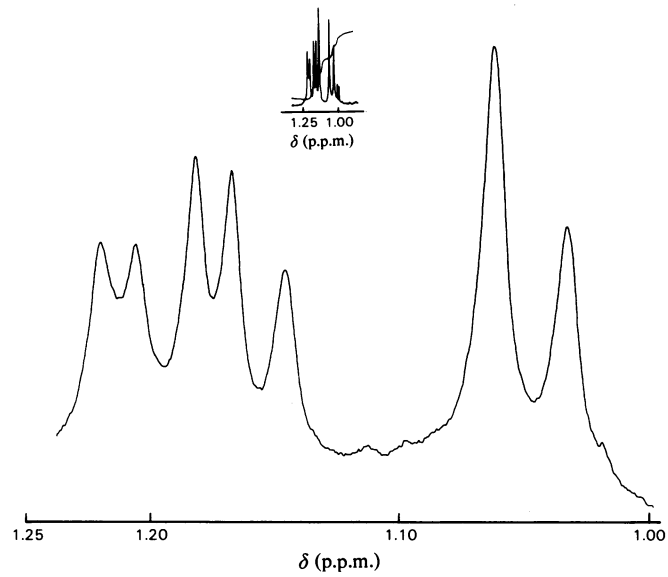


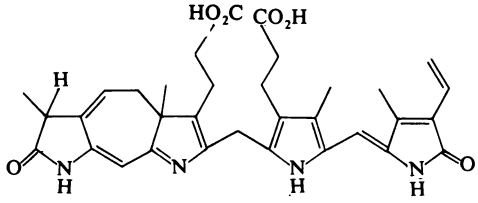
Fig. 5. *High-field region in the ^1H n.m.r. spectrum (expanded and non-expanded) of photobilirubin II (see the text)*

(*d*) *Methyl groups attached to saturated centres* (*Fig. 5*)

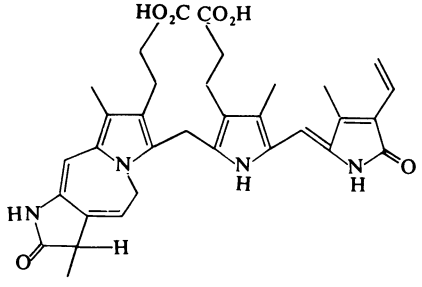
The spectrum of bilirubin shows no signals in this region, apart from an unassigned peak attributed to an impurity at approx. δ1.15, which is also found in the spectrum of photobilirubin II. The latter shows a set of signals (Fig. 5), which are crucial to the present interpretation, and which represent methyl groups in saturated environments. This region sometimes reveals impurities in samples prepared by evaporation, but in the present study these peaks, and their relative areas, were reproducible from one preparation to another, whereas the signals attributed to impurities were variable. This is illustrated in Fig. 5, since the expanded spectrum was from a sample different from that which gave the non-expanded spectrum. The signal at δ1.15 has a different intensity relative to the remaining signals in the two spectra.

At high field there are two signals (δ1.07 and 1.03); irradiation at these positions caused no discernible alteration elsewhere in the spectrum, and the signals were not affected by irradiation elsewhere. It is concluded that they are genuinely singlets and represent the system Me—C(—)—. The total integration corresponds to three protons; the ratio of the two areas is about 3:2 respectively.

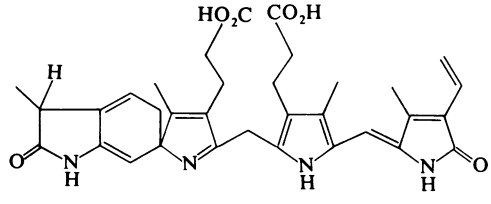
The remaining four peaks comprise two doublets, with chemical shifts and coupling constants of δ1.23 ($J = 8.0$ Hz) and δ1.18 ($J = 7.2$ Hz). Again the total integration corresponds to approximately three protons and this time the area ratio is about 2:3 respectively. Double-irradiation experiments showed that irradiation of these doublets resulted in the sharpening of signals at δ3.15 and 3.25. Conversely irradiation at δ3.15 caused the doublets at δ1.23 and δ1.18 to collapse.



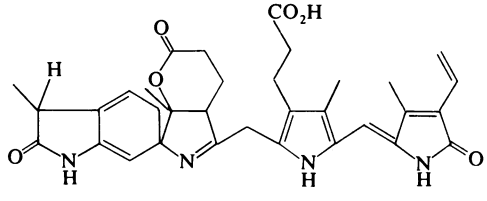
(3) Six olefinic protons



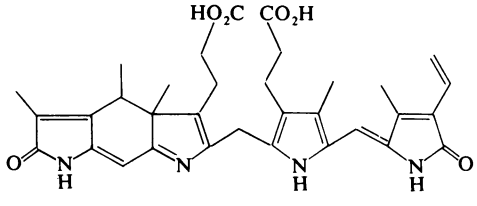
(4) Six olefinic protons



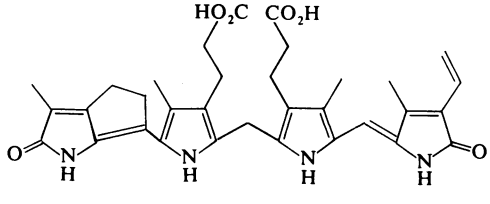
(5) Six olefinic protons



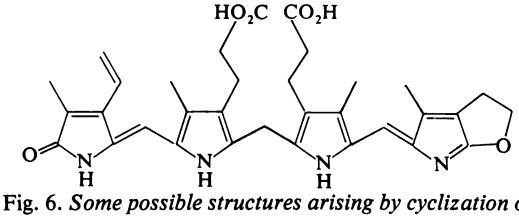
(6) (i) Six olefinic protons
(ii) $\nu_{C=O}$ expected at approx. 1735 cm^{-1}



(7) (i) Five olefinic protons
(ii) Alternative cyclizations (3^1–22 and 3^1–6) of this type can be drawn. Both have five olefinic protons.



(8) (i) Five olefinic protons
(ii) Normal diazo reaction expected



(9) (i) Wrong vinyl group
(ii) Five olefinic protons

Fig. 6. *Some possible structures arising by cyclization of β-vinyl substituents in bilirubin IXα*

The recognition of the signals at δ3.15 and δ3.25 is hampered by a broad impurity signal in this region. This signal was removed by irradiating its low-field edge, which revealed the two multiplets clearly.

It is concluded that the doublets arise from systems of the type Me–CH, the coupled methine protons being at δ3.15 and δ3.25. Evidently there are two similar systems present, and the integration values point to two compounds in the ratio 2 (δ1.23 and 1.03) to 3 (δ1.18 and 1.07).

Proposed structure for photobilirubin II

The n.m.r. results indicate that the C-3 vinyl group disappears when photobilirubin II is formed. The possibility that solvent has added to the vinyl group has been considered, since this is a known reaction (Manitto, 1971; Manitto & Monti, 1974). However, the product would be expected to give a normal diazo reaction, which photobilirubin II does not (Stoll *et al.*, 1979). Moreover there is some evidence that photobilirubin II is isomeric with bilirubin (Stoll *et al.*, 1979). Attempts to crystallize photobilirubin II have so far proved unsuccessful. Elemental analysis on the amorphous material shows that it contains no sulphur, so that dimethyl sulphoxide has not added to the molecule. This is further supported by our isolation of photobilirubin II after irradiation of bilirubin in 8M-urea and in chloroform. An isomeric structure is suggested by the mass-spectral observations on photobilirubin II and its dimethyl ester (Stoll *et al.*, 1979) and by the photochemical reversion of photobilirubin II into bilirubin.

If photobilirubin II is an isomer of bilirubin, then the only way that the vinyl group at C-3 can disappear with overall loss of olefinic protons (8→6, Table 2) is by an intramolecular cycloaddition reaction. Several possible modes of cyclization are considered in Fig. 6; no attempt is being made to present conformation or hydrogen bonding. The commentary in Fig. 6 argues against structures (7), (8) and (9).

Structures (3), (4), (5) and (6) represent the products of alternative pathways for cyclization between C-3[2] and ring B; in each case the C-4–C-5 bond must have *E*-configuration. The examination of models does not convincingly indicate a preference for any one of these cyclizations. However, the ^{1}H n.m.r. spectrum clearly favours (3) or (6), since these are the only structures that have two methyl groups attached to saturated centres, one of the type Me–C– and one of the type Me–C–H.

Moreover, the former 3-vinyl group, now present as a $=CH-CH_2-$ unit in all four structures (3–6), is detected in the n.m.r. spectrum by double-resonance experiments. The multiplet at δ5.89, assigned to the olefinic component of this unit, became two unequal singlets on irradiation at δ2.5. Conversely irradiation at δ5.89 caused modification in the δ2.5 region; this region is less easy to interpret because it is partly obscured by a β-methylene resonance of the propionic acid side chains, but there was clear evidence for the collapse of two doublets at δ2.64 and 2.60 to singlets (Fig. 7). This chemical shift, δ2.6, would be appropriate for structures (3), (5) or (6); it rules out structure (4), where the methylene resonance would be much lower [cf. *N*-methylpyrrole, *N*-CH_3, δ3.42 in tetrachloromethane (Anderson, 1965); 8α-protons in phorcabilin, δ4.0 in [^{2}H]chloroform (Bois-Choussy & Barbier, 1977).

The following evidence accords with either of the working proposals (3) and (6). (i) The signal due to the C-10 protons (δ3.62) has moved upfield relative

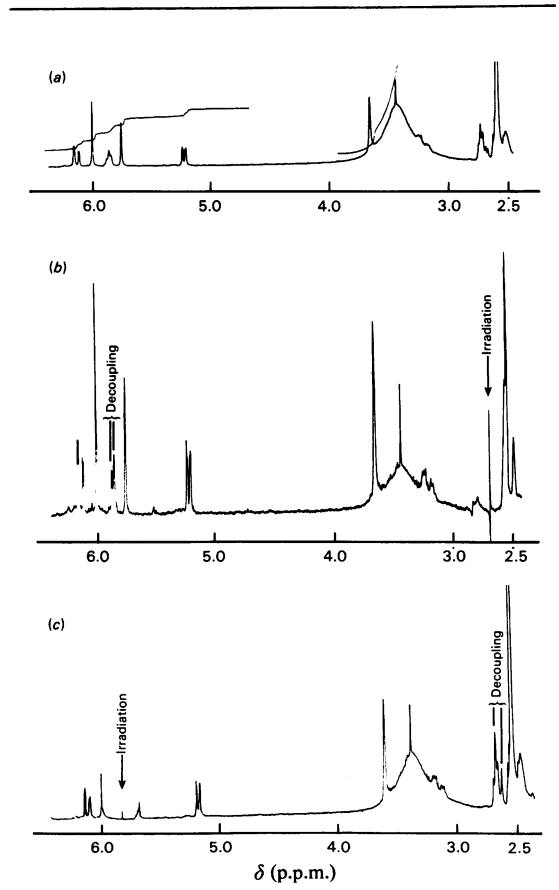
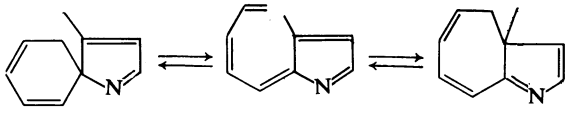
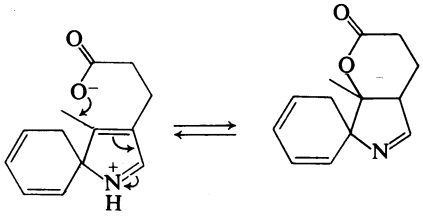


Fig. 7. *Double-resonance experiment showing (a) signals in the δ6.5–2.5 region in the ^{1}H n.m.r. spectrum of photobilirubin II, (b) collapse of the multiplet at δ5.89 on irradiation at δ2.5 and (c) collapse of two doublets to singlets at δ2.64 and δ2.60 on irradiation at δ5.89*

to the corresponding signal in bilirubin (δ4.00) because ring B is no longer aromatic. The signal due to the C-5 olefinic proton shifts in the same direction (δ6.11→5.78) for the same reason. On expansion this signal shows two components, differing by about 0.003 p.p.m., attributed to the two components IIA and IIB. (ii) The visible spectrum shows a pyrromethenone band [$\lambda_{max.}$ (chloroform) 441 nm, ε 40500 M·cm^{-1}], showing a lower intensity and a bathochromic shift with respect to bilirubin [$\lambda_{max.}$ (chloroform) 453 nm, ε 60000 M·cm^{-1}] (Stoll *et al.*, 1979). This accords with the presence of one pyrromethenone unit, together with the less conjugated system now present in the A/B rings. (iii) On irradiation bilirubin XIIIα gives a product analogous to photobilirubin II, whereas bilirubin IIIα does not. (iv) Photobilirubin II does not give biliverdin on attempted dehydrogenation (Stoll *et al.*, 1979). (v) The photocyclization is reversible; this might be explained by electrocyclic reactions of the types



We know at present of no exact analogies for these postulated reactions, which require an initial tautomerization in the A/B pyrromethenone system. The forward reaction to structure (3) has some parallel in the photocyclization of phorcabilin to sarpedobilin (Bois-Choussy & Barbier, 1977). The route to structure (6) requires lactonization by conjugate addition to an α,β-unsaturated azomethine.



Because the C-8 hydrogen in structure (6) is acidic, this addition would be expected to be readily reversible.

Only one piece of evidence from the n.m.r. spectrum appears not to support structures (3) and (6); this is in the NH/OH region, where the signals are broader than they are in bilirubin, and where they integrate to only 3–4 protons. However, since such signals are susceptible to broadening and to change of chemical shift we prefer to place more reliance on the preponderance of evidence from signals due to carbon-bound protons.

The i.r. spectrum should clearly differentiate structures (3) and (6) and indeed the published spectrum (Stoll *et al.*, 1979) shows a sharp peak at 1740 cm^{-1}, which might be attributed to a δ-lactone. Moreover, structure (6), with an electron-withdrawing group (C=N) substituted at C-10, would account well for the failure of photobilirubin II to give a normal diazo reaction (Stoll *et al.*, 1979). However, our present measurements of the i.r. spectrum of photobilirubin II do not reveal a clear peak at 1740 cm^{-1}, although there is extra absorption in this region. On balance we prefer at this preliminary stage to leave the choice between structures (3) and (6) an open question. Initial attempts to measure ^{13}C n.m.r. spectra of photobilirubin II have been unsuccessful due to its instability.

However, on the evidence presented here the intramolecular cyclization of the 3-vinyl group is clear. The product and its precursor must for steric reasons have the 4*E*-configuration. This constitutes the first structural evidence for the occurrence of a *Z*→*E* change at a bridge double bond on irradiation of bilirubin. Thus it is postulated that photobilirubins IA/IB are (4*E*,15*Z*)-bilirubin and (4*Z*,15*E*)-bilirubin, the former of which gives rise to the diastereoisomeric photobilirubins IIA and IIB possessing the cyclo-4*E*,15*Z*-configuration. This view is supported by the formation of a new photobilirubin, photobilirubin III, on irradiating

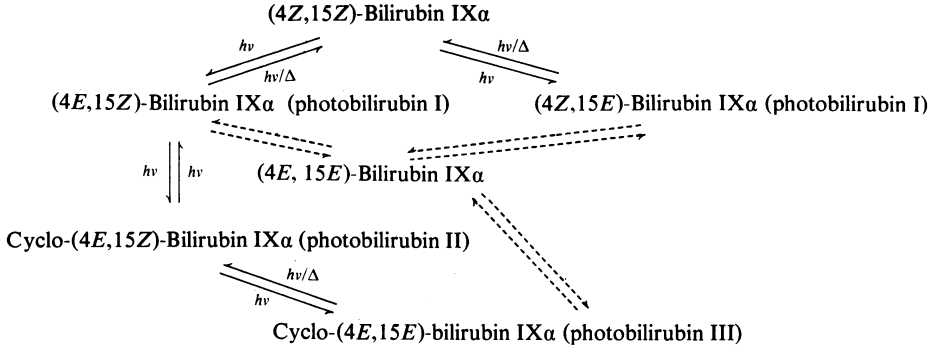


Fig. 8. *Proposed interrelationship of bilirubin IXα and its photoisomers*

photobilirubin II. Photobilirubin III appears to bear the same structural relationship to photobilirubin II as photobilirubin I bears to bilirubin. Photobilirubin III presumably has the cyclo-4*E*,15*E*-configuration. The proposed relationships between the photoproducts, including those not yet detected but which would be expected to occur in low concentrations in photoreaction mixtures, are given in Fig. 8.

Physiological significance of the photobilirubins

Recent work has indicated that photobilirubin I reaches a significant concentration in serum during neonatal phototherapy (Onishi *et al.*, 1980*a*), but is excreted by the liver without conjugation and reverts spontaneously to bilirubin in the bile. Photobilirubin II is excreted more efficiently so that it is not detected in the serum during phototherapy but is found in relatively high concentration in the bile (Onishi *et al.*, 1980*a*). The roles of photobilirubin III and (4*E*,15*E*)-bilirubin IXα in phototherapy have not been demonstrated but they may be important as rapidly excreted substances, never reaching significant concentrations in the serum, but reverting in the bile to photobilirubin II and bilirubin respectively and contributing significantly to the overall bilirubin turnover.

The ^{1}H n.m.r. spectra were recorded at Queen Mary College on the ULIRS WH400 service. We are grateful to Dr. G. E. Hawkes and Mr. M. S. Buckingham for their painstaking efforts in measuring the spectra. We are grateful for the support of the Medical Research Council (to R. B. and C. H. G.).

References

Bois-Choussy, M. & Barbier, M. (1977) *Experientia* **33**, 1407–1410

Bonnett, R. (1976) Biochem. Soc. Meeting, Leeds, noted in *J. Chem. Soc. Chem. Commun.* (1977) 830

Bonnett, R. & McDonagh, A. F. (1970) *J. Chem. Soc. Chem. Commun.* 238–239

Bonnett, R. & Stewart, J. C. M. (1975) *J. Chem. Soc. Perkins Trans. I*, 224–231

Bonnett, R., Davies, J. E. & Hursthouse, M. B. (1976) *Nature (London)* **262**, 326–328

Bonnett, R., Davies, J. E., Hursthouse, M. B. & Sheldrick, G. M. (1978) *Proc. R. Soc. London Ser. B* **202**, 249–268

Cremer, R. J., Perryman, P. W. & Richards, D. H. (1958) *Lancet* **i**, 1094–1097

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

Lightner, D. A. (1977) *Photochem. Photobiol.* **26**, 427–436

Lightner, D. A., Wooldridge, T. A. & McDonagh, A. F. (1979*a*) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 29–32

Lightner, D. A., Wooldridge, T. A. & McDonagh, A. F. (1976*b*) *Biochim. Biophys. Res. Commun.* **86**, 235–243

Lund, H. T. & Jacobsen, J. (1972) *Acta Paediatr. Scand.* **61**, 693–696

Manitto, P. (1971) *Experientia* **27**, 1147–1149

Manitto, P. & Monti, D. (1974) *Gazz. Chim. Ital.* **104**, 513–521

McDonagh, A. F. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1306–1311

McDonagh, A. F. (1979) in *The Porphyrins* (Dolphin, D., ed.), vol. 6, pp. 403–409, Academic Press, New York

McDonagh, A. F. & Palma, L. A. (1977) in *Chemistry and Physiology of Bile Pigments* (Berk, P. D. & Berlin, N. I., eds.), pp. 81–92, U.S. Department of Health, Educational and Welfare, Washington, DC

McDonagh, A. F., Lightner, D. A. & Wooldridge, T. A. (1979) *J. Chem. Soc. Chem. Commun.* 110–112

McDonagh, A. F., Palma, L. A. & Lightner, D. A. (1980) *Science* **208**, 145–151

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

Onishi, S., Isobe, K., Itoh, S., Kawade, N. & Sugiyama, S. (1980*a*) *Biochem. J.* **190**, 533–536

Onishi, S., Kawade, N., Itoh, S., Isobe, K. & Sugiyama, S. (1980*b*) *Biochem. J.* **190**, 527–532

Ostrow, J. D. (1971) *J. Clin. Invest.* **50**, 707–718

Ostrow, J. D., Berry, C. S. & Zarembo, J. E. (1974) in *Phototherapy of the Newborn: An Overview* (Odell, G. B., Schaffer, R. & Simoupoulis, A. P., eds.), pp. 74–92, National Academy of Science, Washington, DC

Porto, S. O. (1970) in *Bilirubin Metabolism in the Newborn* (Bergsma, D., Hsia, D. Y. Y. & Jackson, C., eds.), pp. 83–89, Williams and Wilkins, Baltimore

Seligman, J. W. (1977) *Pediatr. Clin. North Am.* **24**, 509–527

Stoll, M. S. & Gray, C. H. (1977) *Biochem. J.* **163**, 59–101

Stoll, M. S., Zenone, E. A., Ostrow, J. D. & Zarembo, J. E. (1977) *Abstr. Annu. Meet. Am. Soc. Photobiol. 5th*, 97

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

Stoll, M. S., Zenone, E. A. & Ostrow, J. D. (1981) *J. Clin. Invest.* **68**, 134–141

Zenone, E. A., Stoll, M. S. & Ostrow, J. D. (1977) *Gastroenterology* **72**, 1180