Structure and thermal interconversion of cyclobilirubin IX α

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One of the two main photoproducts in bilirubin metabolism during phototherapy in neonatal hyperbilirubinaemia is (*EZ*)-cyclobilirubin. However, it has not yet been possible to come to a final conclusion as to its chemical structure, despite the fact that much effort has been expended on the problem. The present paper demonstrates that (*EZ*)-cyclobilirubin is formed by the intramolecular cyclization of the C-3-vinyl group with the position at C-7 rather than at C-6, without δ -lactone-ring formation. The evidence comes from ^{13}C -n.m.r. spectra, which indicate that an oxygen-bound quaternary carbon atom is not present, and from ^1H -n.m.r. spectra, which indicate that the orientation of the methyl group at C-2 is equatorial; these findings are supported by mass spectra. The existence of both an epimeric relationship at C-7 between (*EE*)- and (*EZ*)-cyclobilirubins A and B and of steric isomers of the hydrogen atom and methyl group at C-2 is supported by the fact that the methyl-group protons at C-2 and C-7 are observed as a paired signal in ^1H -n.m.r. spectra, and that new signals at C-7, C-2 and C-3 β appear in ^{13}C -n.m.r. spectra, that mass spectra of (*EZ*)-cyclobilirubins A and B are extremely similar and that, furthermore, thermal interconversion between (*EE*)- and (*EZ*)-cyclobilirubins A and B is observed.

It has been reported that the main process responsible for hepatic bile-pigment excretion during phototherapy in the Gunn rat is single-photon *Z-E* isomerization, predominantly at the 15Z bridge, resulting in the formation of (*EZ*)- and (*ZE*)-bilirubin IX α , photobilirubin IX α (McDonagh *et al.*, 1982*a, b*). However, this reverts rapidly to (*ZZ*)-bilirubin IX α (Onishi *et al.*, 1981*a*; Stoll *et al.*, 1981), which is therefore readily absorbed by intestinal mucosa (Lester & Schmid, 1963; Corchs & Rodriguez Garay, 1966). This fact may diminish the effectiveness of phototherapy in the removal of bilirubin from the body. Meanwhile, during phototherapy of neonatal hyperbilirubinaemia, (*EZ*)-cyclobilirubin, which is photochemically cyclized bilirubin IX α and can be separated into two components, i.e. (*EZ*)-cyclobilirubins A and B, by t.l.c. (Stoll *et al.*, 1979) or h.p.l.c., predominated over the photobilirubin IX α , as previously described (Onishi *et al.*, 1980*b*). Moreover, the

serum concentration of the (*EZ*)-cyclobilirubin is considered to be the most sensitive indicator of biliary excretory function, inasmuch as the pigment is readily excreted into the bile. However, if there is impaired liver function, abnormal accumulation of the (*EZ*)-cyclobilirubin in the body may occur (Onishi *et al.*, 1982). The pigment has an azacyclopentadiene ring that can be polymerized, as described by von Döbeneck (1979), and may form a bilifuscin-like substance that is observed as a brown large- M_r substance and is considered to be the coloured component of the pigment of bronze-baby syndrome, one major complication of phototherapy. Thus (*EZ*)-cyclobilirubin has been recognized as having an important role both in the main process responsible for excretion and in the mechanism responsible for the bronze-baby syndrome (Onishi *et al.*, 1982; Meisel *et al.*, 1982). However, it has not yet been possible to come to a final conclusion as to its chemical structure. Although ^{13}C -n.m.r. spectra of bilirubin and its derivatives have been described (Severini-Ricca *et al.*, 1975; Hansen *et al.*, 1979; Kaplan & Navon, 1980, 1981), attempts to

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

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Table 1. *Classification and nomenclature of bilirubin IX α and its photoproducts in jaundice phototherapy*
Abbreviation: a.d.s., absorption difference spectroscopy.

Separation	(<i>EE</i>)-Cyclobilirubins IX α A and B	(<i>EZ</i>)-Cyclobilirubins IX α A and B	(<i>EE</i>)-Bilirubin IX α	(<i>EZ</i>)-* and (<i>ZE</i>)-Bilirubin IX α	(<i>ZZ</i>)-Bilirubin IX α
Onishi <i>et al.</i> (1979, 1980a,b, 1982) H.p.l.c., <i>in vitro</i> H.p.l.c., <i>in vivo</i>	Peaks 1A and 1B Peak 1	Peaks 2A and 2B Unknown pigment	Peak 0 Peak 0	Peaks 3A and 3B Photobilirubin IX α	Peak 5 Bilirubin
Stoll <i>et al.</i> (1981, 1982) T.l.c., <i>in vitro</i>	Photobilirubin III	Photobilirubins IIA and IIB	—	Photobilirubins IA and IB	Bilirubin
McDonagh <i>et al.</i> (1979, 1982a,b) A.d.s., <i>in vitro</i> and <i>in vivo</i> T.l.c., <i>in vitro</i> H.p.l.c., <i>in vitro</i> and <i>in vivo</i>	(<i>E</i>)-Lumirubin and (<i>E</i>)-isolumirubin	(<i>Z</i>)-Lumirubin and (<i>Z</i>)-isolumirubin	Photobilirubin (4 <i>E</i> ,15 <i>E</i>)-Bilirubin	Photobilirubin (4 <i>E</i> ,15 <i>Z</i>)-* and (4 <i>Z</i> ,15 <i>E</i>)-Bilirubin	Bilirubin

* Not detectable in human serum albumin-bilirubin complex.

Fig.1. Schematic structure of bilirubin

measure ^{13}C -n.m.r. spectra of (*EZ*)-cyclobilirubin, photobilirubin II, have been unsuccessful. Therefore we have undertaken an investigation of the interrelationships of four of its photoisomers, including one derived by cyclization via an endo-vinyl group of bilirubin IX α , and its chemical structure by using ^1H -n.m.r. and ^{13}C -n.m.r. spectroscopy. Although some reasonable doubt has been expressed about the identity of the various photoproducts (McDonagh *et al.*, 1980, 1982a,b), the relationships between the photoproducts and some of its biochemical characteristics are as shown in Table 1. In the present paper the predominant natural bilirubin isomer, (*ZZ*)-bilirubin IX α , is often simply referred to by the term bilirubin (Fig. 1). Also description of an isomeric form of bilirubin IX α has been omitted from Table 1.

Materials and methods

Separation of each peak

Isolation of (*EE*)- or (*EZ*)-cyclobilirubin was achieved by irradiation of bilirubin IX α in di-

methyl sulphoxide under anaerobic conditions, followed by reversed-phase h.p.l.c. separation and collection of each peak. The conditions for the reversed-phase h.p.l.c. were as described previously (Onishi *et al.*, 1979, 1980a). The separation of (*EZ*)-cyclobilirubins A and B was carried out by h.p.l.c. on a DuPont Zorbax-sil column (25 cm $\times$ 6.2 mm) with a linear gradient of ethanol in chloroform containing 0.1% (v/v) acetic acid [0–50% (v/v) in 25 min]. The solvent eluent of the separated (*EZ*)-cyclobilirubins A and B was removed with a rotary evaporator at room temperature.

Thermal conversion of each cyclobilirubin

Experiments on thermal conversion under anaerobic conditions in the dark were carried out in stoppered 10 ml Pyrex tubes, containing a solution consisting of (*EE*)-cyclobilirubins A and B and the reversed-phase h.p.l.c. eluent by which (*EE*)-cyclobilirubin was separated. In the experiment on the thermal conversion of (*EZ*)-cyclobilirubin A or B, each peak, separated as described above, was dissolved in 20% (v/v) acetonitrile in 0.01 M-phosphate buffer, pH 8.0, containing 0.1% tetra-n-butylammonium hydroxide, corresponding to the solvent by which (*EZ*)-cyclobilirubin was separated by reversed-phase h.p.l.c. In the experiment on the thermal conversion of (*EE*)-cyclobilirubin, the same solvent was used except that it contained 18% acetonitrile rather than 20% acetonitrile. The solution containing each cyclobilirubin was maintained in the dark at 50°C, and at intervals 100 μl portions were removed and analysed by reversed-phase h.p.l.c. The same experimental conditions, apart from the use of irradiation instead of heating,

were applied for photochemical interconversion. In all experiments the solutions were de-oxygenated with pure (99.999%) argon. Light-intensity, determined with a Minolta Air-Shields Fluoro-limeter 451, at the surface of the solution was $24 \mu\text{W}/\text{cm}^2$ per nm. All manipulations were carried out in subdued light, except for the photochemical treatment, and at room temperature, except for the thermal treatment. Photochemical and thermal experiments, use of chromatographic apparatus

and preparation of calibration curves of peak area were carried out as described previously (Onishi *et al.*, 1980a; Isobe & Onishi, 1981; Isobe *et al.*, 1983).

Pigments and reagents

Bilirubin (E. Merck, Darmstadt, Germany) and tetra-n-butylammonium hydroxide (Wako, Osaka, Japan) were used without further purification. Dimethyl sulphoxide, acetonitrile and all other reagents used were of analytical grade.

Fig. 2. H.p.l.c. of solution obtained at various intervals during incubation of (EE)-cyclobilirubin A in the dark at 50°C

Fig. 3. Plots of the thermal changes in (EE)-cyclobilirubin A (a) and in (EE)-cyclobilirubin B (b) with a time on a linear scale. The relative percentages are given in an accumulative manner as an area.

(a)

Fig. 4. 400 MHz ¹H-n.m.r. spectra of bilirubin IX α in C²HCl₃ (a) and (EZ)-cyclobilirubin A in C²HCl₃ (b). Distinguishing between the individual NH groups of ring B and ring C or of ring A and ring D is difficult.

N.m.r. spectra

^1H -n.m.r. spectra and ^{13}C -n.m.r. spectra were recorded on a Bruker WH400 instrument.

Mass spectrometry

Mass spectra were recorded with GCMS-6020 mass spectrometer. The conditions were: ion source, Cl ; reagent gas, NH_3 ; electron energy, 100 eV; electron current, 200 μA ; accelerating high voltage, 3.5 kV; ion-source temperature, 250°C.

Results and discussion

Thermal reversion of (*EE*)-cyclobilirubin A (or B) to (*EZ*)-cyclobilirubin A (or B)

When the solution of (*EZ*)-cyclobilirubin separated by h.p.l.c. was irradiated by blue light under anaerobic conditions, (*EE*)-cyclobilirubin, which is a more-polar substance than (*EZ*)-cyclobilirubin A, appeared abruptly (Isobe & Onishi, 1981). That photochemically converted (*EE*)-cyclobilirubin corresponds to '(*EE*)-cyclobilirubin A' is confirmed by the fact that (*EE*)-cyclobilirubin obtained in this manner was photochemically again converted into (*EZ*)-cyclobilirubin A. When the (*EE*)-cyclobilirubin A separated by h.p.l.c. was kept in the dark at 50°C, h.p.l.c. analysis of

samples showed that the (*EE*)-cyclobilirubin A decreased gradually and that (*EZ*)-cyclobilirubin A and a brown pigment appeared at the same time (Fig. 2). (*EE*)-Cyclobilirubin A disappeared and (*EZ*)-cyclobilirubin A appeared on treatment of the solution with trifluoroacetic acid.

Fig. 5. Working proposals (I) and (II) for the chemical structure of (*EZ*)-cyclobilirubin A

Table 2. ^1H -n.m.r. data of (ZZ)-bilirubin IX α and (*EZ*)-cyclobilirubin IX α

Proton	(ZZ)-Bilirubin IX α Chemical shift (p.p.m.)		(EZ)-Cyclobilirubin IX α Chemical shift (p.p.m.)	
	In C^2HCl_3	In $(\text{C}^2\text{H}_5)_2\text{SO}$	Major, in C^2HCl_3	Minor, in C^2HCl_3
2-Me	2.18	2.17	1.33 (d)	1.36 (d) J 7.7 Hz
H-2	—	—	3.24 (qd)	3.15 (qd) J 7.7, ~ 1 Hz
H-3 α	6.61	6.84	6.14 (ddd)	6.14
H-3 β	5.59	5.65	2.89 (dd)	2.90
H-3 β'	5.60 (dd)	5.65	2.73 (dd)	2.73
H-5	6.21 (s)	6.01	7.15 (s)	7.14
7-Me	1.95 (s)	—	1.41 (s)	1.37
8 α	3.01, 3.58	1.93 (t)	2.83 (m)	
8 β	2.89, 3.58	2.43 (t)	2.74 (m)	
H-10	4.08 (s)	3.99 (s)	3.85 (s)	J_{gem} 15.5 Hz [in $(\text{C}^2\text{H}_5)_2\text{CO}$]
H-12 α	2.81, 3.01	1.93 (t)	2.83 (m)	
H-12 β	2.58, 2.89	2.43 (t)	2.74 (m)	
13-Me	2.15	—	2.15 (s)	
H-15	6.13 (s)	—	6.14 (s)	
17-Me	2.16	—	2.15 (s)	
H-18 α	6.49	—	6.49 (dd)	J 17.9, 11.8 Hz
H-18 β	6.17	—	6.16 (dd)	J 17.9, 2.4 Hz
H-18 β'	5.37	—	5.38 (dd)	J 11.8, 2.4 Hz
Ring-A NH	10.69	10.48	8.36 (s)	
Ring-B NH	9.27	9.94	— (—)	
Ring-C NH	9.29	10.07	9.43 (s)	
Ring-D NH	10.80	10.51	10.73 (s)	
-CO $_2$ H	13.68	11.95	10.05 (s)	
•	13.68	11.95	10.05 (s)

Fig. 6. ^{13}C -n.m.r. spectra of (ZZ)-bilirubin in $[\text{2H}]$ dimethyl sulphoxide (a) and (EZ)-cyclobilirubin A in C_2HCl_3 (b) and its probable assignments

Scheme 1. Photochemical and thermal interrelationship of bilirubin IX α and its photoproducts

The steric configurations of the hydrogen atom and the methyl group at C-2 of (E,Z)-cyclobilirubins A and B and of (E,E)-cyclobilirubins A and B are not shown.

A similar experiment performed with (*EE*)-cyclobilirubin B gave comparable results. It may be noted that (*EE*)-cyclobilirubin has recently been separated into two peaks, i.e. (*EE*)-cyclobilirubins A and B, by an h.p.l.c. method.

A good quantitative correspondence between the disappearance of (*EE*)-cyclobilirubin A (or B) and the appearance of (*EZ*)-cyclobilirubin A (or B) and brown pigment was observed (Fig. 3).

Thermal interconversion of (EZ)-cyclobilirubins A and B

Analysis by h.p.l.c. of thermal interconversion of (*EZ*)-cyclobilirubins A and B in the dark at 50°C showed that the (*EZ*)-cyclobilirubin A (or B) decreased gradually and (*EZ*)-cyclobilirubin B (or A) and a small amount of brown pigment appeared simultaneously. Analogously to the case with (*EE*)-cyclobilirubin A (or B), a good quantitative correspondence of the disappearance of (*EZ*)-cyclobilirubin A (or B) and the appearance of (*EZ*)-cyclobilirubin B (or A) and a small amount of brown pigment was observed. Thermal reversion of (*EZ*)-cyclobilirubin A (or B) to (*EZ*)- and (*ZE*)-bilirubin in the dark at 50°C was not observed. This is an indication that there is an epimeric relationship between the A and B forms of (*EE*)- or (*EZ*)-cyclobilirubin, as described previously (Onishi *et al.*, 1981b).

Structure of (EZ)-cyclobilirubin (Table 2)

The ^1H -n.m.r.-spectra indicate that the C-3-vinyl group disappeared when (*EZ*)-cyclobilirubin is formed (Figs. 4a and 4b). The methine protons at δ 3.15 p.p.m. (C-2H, minor) and δ 3.24 p.p.m. (C-2H, major) and the methyl protons at δ 1.33 p.p.m. (C-2H, major) and δ 1.36 p.p.m. (C-2H, minor) derived from a $>\text{CH}-\text{CH}_3$ group were observed. The olefinic proton at δ 6.14 p.p.m. (C-3H) and the methylene protons at δ 2.83 and δ 2.72 p.p.m. were observed as an ABX system. A new signal at δ 1.37 p.p.m. (C-7Me, minor) and δ 1.41 p.p.m. (C-7H, major) represents the system $\text{Me}-\text{C}=\text{C}$. Therefore the (*EZ*)-cyclobilirubin is considered to be derived from cyclization between C-3 β and ring B by photoenergy. The evidence accords with either of the working proposals (I) and (II) for the chemical structure shown in Fig. 5 (Stoll *et al.*, 1982). Because in ^{13}C -n.m.r., as shown in Fig. 6, the quaternary-carbon signal was observed not at about δ 80 p.p.m. but at δ 55.25 p.p.m. (C-7, minor) and δ 55.40 p.p.m. (C-7, major), it is clear that an oxygen-bound quaternary carbon atom is not present. In addition, the appearance of methine signals at δ 40.47 p.p.m. (C-2, minor) and δ 40.09 p.p.m. (C-2, major) was observed. Detailed data of ^1H -n.m.r. relevant to the coupling system between ring A and ring B are given in Table 2.

Because the evidence provided in the present paper confirms both the formation of the intramolecular cyclization of the C-3-vinyl group with the position at C-7 rather than at C-6, without δ -lactone-ring formation, it is concluded that the structure of the (*EZ*)-cyclobilirubin is as shown in (I) Fig. 5 (Isobe *et al.*, 1983). As mentioned below, the data of the mass spectra of (*EZ*)-cyclobilirubin also support the conclusion. In addition to a peak at m/z 286, resulting from fragmentation at the central weak methylene bridge, the base peak, at m/z 243, may be derived by loss of both a methyl radical and a carbonyl group from the fragment. The next strongest peak at m/z 168, is due to subsequent loss of propionic side chain. The peak at m/z 269 is derived by a loss of $-\text{CH}_4$ from the fragment. It is concluded that the compound has no lactone ring. The spectrum of (*EZ*)-cyclobilirubin B was extremely similar to that of (*EZ*)-cyclobilirubin A. Therefore the result indicated that these compounds are isomeric. Their isomers are considered to account for the fact that the methyl-group protons at C-2 and C-7 were observed as a paired signal in ^1H -n.m.r. (Figs. 4b and 4c) and that new signals of C-7, C-2 and C-3 β appeared in ^{13}C -n.m.r., as shown in Fig. 6. Thus steric isomers of the hydrogen atom and the methyl group at C-2 of (*EZ*)- and (*EE*)-cyclobilirubins should surely exist, as with phycocyanobilin (Gossauer & Plieninger, 1979), but they could not be separated by t.l.c. or h.p.l.c. because, by virtue of the adjacent lactam carbonyl group, the hydrogen atom at C-2 readily exchanged and racemization of the molecule occurs (Gossauer & Plieninger, 1979). On the basis of these previous observations and the data provided from the present study, we conclude that the interrelationships between bilirubin IX α and its photoproducts, especially the A and B forms of (*EZ*)- and (*EE*)-cyclobilirubins, are as shown in Scheme 1.

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