Significance of the *endo*-vinyl group of bilirubin in photochemical reactions

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In photochemical experiments on bilirubin III α (no *endo*-vinyl group), IX α (one *endo*-vinyl group) and XIII α (two *endo*-vinyl groups) and in the photochemical, thermal and catalytical reversion of their photoproducts under anaerobic conditions, much more instability and complexity of photoproducts of bilirubin XIII α were observed than for those of bilirubin IX α or III α . On the basis of present and previous results of photochemical experiments *in vitro* and the fact that large amounts of (*EZ*)-cyclobilirubin IX α appear in the bile during phototherapy of neonatal hyperbilirubinaemia [Onishi, Kawade, Itoh, Isobe & Sugiyama (1980) *Biochem. J.* **190**, 527–532], it is concluded that the *endo*-vinyl group plays a crucial role in the photochemical reaction of bilirubin IX α . On reversed-phase high-pressure liquid chromatography of photoisomers, it was found that the retention times of geometric isomers and *E*-cyclized structural isomers were shortened compared with those of *Z*-isomer and *E*-isomer, respectively, as precursor substances.

Bilirubin photochemistry has attracted attention in the light of its relevance to the widely employed phototherapy for neonatal unconjugated hyperbilirubinaemia (Brown & McDonagh, 1980). The major mechanism of phototherapy for jaundice was initially postulated to be self-sensitized photooxygenation of bilirubin, which is a singlet-oxygen reaction (McDonagh, 1971). However, since considerable amounts of unconjugated bilirubin appear in the bile of Gunn rats (Ostrow, 1971) or newborn infants (Lund & Jacobsen, 1972) after irradiation with blue light, the geometric isomerization via rotation around the Δ^4 or Δ^{15} bridges of bilirubin IX α was proposed (Falk *et al.*, 1975; McDonagh, 1976; Lightner & Park, 1976; Bonnett *et al.*, 1978) and in part demonstrated (McDonagh *et al.*, 1979; Lightner *et al.*, 1979a,b; Onishi *et al.*, 1979; Falk *et al.*, 1982). Geometric isomerization now appears to be widely accepted as a possible mechanism. Therefore the configuration of Δ^4 and Δ^{15} bridges of bilirubin IX α has become the focus of attention. However, in photochemical experiments with bilirubin III α (no *endo*-vinyl group),

IX α (one *endo*-vinyl group) and XIII α (two *endo*-vinyl groups), we have observed much more lability and complexity for the photoproducts of bilirubin XIII α compared with those of bilirubin IX α or III α . Fairly recently it was demonstrated that the (*EZ*)-cyclobilirubins A and B are quantitatively the most important photoproducts excreted in the bile of newborn infants during phototherapy (Onishi *et al.*, 1980b). By n.m.r. spectrometry (Onishi *et al.*, 1981, 1984; Stoll *et al.*, 1982; McDonagh *et al.*, 1982a,b) and thermal-stability studies (Isobe *et al.*, 1983), these were proved to be not simple geometric isomers but cyclized bilirubin, formed via the participation of the *endo*-vinyl group of (*EZ*)-bilirubin IX α . Therefore the *endo*-vinyl group in the photochemical reaction of bilirubin IX α is considered to be of great significance. A comparison was made between the photochemical changes of bilirubin III α and XIII α in relation to those of bilirubin IX α . A preliminary report of these results has already appeared (Yokoyama *et al.*, 1983).

Materials and methods

Photochemical experiments

A trace of EDTA was added to bilirubin III α or XIII α (10 mg/dl) in dimethyl sulphoxide and

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

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then the solution was purged with pure (99.999%) argon. The process of photolysis was carried out in stoppered 10 ml Pyrex tubes located 3 cm above the canopy of four fluorescent lamps (20 W Matsushita blue-white fluorescent tubes). Light intensity, determined with Minolta Air-Shields Fluoro-lite-meter 451 (Minolta-Fujisawa), was $19.6 \mu\text{W}/\text{cm}^2$ per nm at the surface of the solution. Several 1.0 ml portions of the solution were exposed to light in Pyrex tubes which were successively removed and placed in the dark. Each portion was analysed by h.p.l.c. Purification of bilirubin III α and XIII α was carried out by the method of Wooldridge & Lightner (1978). All manipulations except photochemical experiments were carried out in subdued light. Chromatographic operation, preparation of a calibration curve of peak area, sample preparation, experimental conditions of photochemical, thermal and catalytical conversion of each photo-product of bilirubin III α or XIII α and numbering of each peak of bilirubin IX α separated by h.p.l.c. were carried out as described previously (Onishi *et al.*, 1980a; Isobe & Onishi, 1981).

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.

Results and discussion

Photochemical experiments on (ZZ)-bilirubin III α

The peaks separated by h.p.l.c. were numbered as shown in Figs. 1(a)–1(c). In the control experiment with a non-irradiated solution, only a large peak, peak 6, of (ZZ)-bilirubin III α was separated (Fig. 1a). After photoirradiation, the (ZZ)-bilirubin III α concentration decreased to constant level within 60 s, with the formation of two rather polar compounds, a very small peak, 0', and fairly large peak, 3'. More prolonged irradiation for up to 2 h did not result in further changes of peaks 0', 3' and 6, and no new peaks appeared. This result indicates that a photoequilibrium was reached and no further photochemical changes occurred. Chromatographic profiles at 1, 5, 10, 20, 30, 45 and 60 min were obtained, although only those at 5 and 60 min are shown in Figs. 1(b) and 1(c). Compounds corresponding to peaks 0' and 3' reverted to parent pigment (peak 6) slowly on warming and instantly on treatment with a trace of trifluoroacetic acid. By analogy with previous results for bilirubin IX α (Isobe *et al.*, 1983; Onishi *et al.*, 1984) peak 3' and 0' are identified as (EZ) = (ZE)-bilirubin III α and (EE)-bilirubin III α respectively (Scheme 1). Because the arrangement of substituents at the β -position in pyrrole rings of III α isomer is symmetric, the peak cannot be separated into two peaks by h.p.l.c. or t.l.c., unlike the photoisomers

Fig. 1. H.p.l.c. of solutions obtained at various intervals during anaerobic illumination of bilirubin III α in dimethyl sulphoxide solution

Experimental details are given in the text. (a) Shows the chromatographic scan for the control non-irradiated solution, and (b) and (c) show the scan after exposure to light for 5 and 60 min respectively.

Scheme 1.

→, Photochemical conversion; ⇌, photochemical, thermal and catalytical reversion.

Fig. 2. Changes in the concentration of bilirubin III α and its photoproducts plotted against time on a semilogarithmic scale

$10^4 \mu\text{V} \cdot \text{s}$ is equivalent to 14 ng of bilirubin III α .

Scheme 2.

→, Photochemical conversion; ⇌, photochemical, thermal and catalytical reversion; *, theoretical, but not confirmed, pathway.

pattern observed for bilirubin III α appears to be that the III α isomer has no *endo*-vinyl group, the group that participates in cyclization [as observed in the formation of (EZ)-cyclobilirubins A and B or photobilirubins IIA and IIB from bilirubin IX α].

Photochemical experiments on (ZZ)-bilirubin XIII α

The peaks were numbered as shown in Figs. 3(a)–3(b). In the control experiment with a non-irradiated solution, a single large peak, peak 4, of (ZZ)-bilirubin XIII α was separated (Fig. 3a). When photoirradiation was applied, peak 4 decreased. Chromatographic profiles at 1, 5, 10, 20, 30, 45 and 60 min were obtained, and a chromatographic profile at 60 min is shown in Fig. 3(b) as an example. A summary of these changes plotted against time on a semilogarithmic scale is shown in Fig. 4. Like the pattern of the photochemical changes of IX α and III α geometric isomers, the peaks of geometric isomers of bilirubin XIII α , peaks 0'' and 3'', i.e. (EE)- and (EZ)-isomers, initially increased rapidly, attaining the maximum within 1 min, and then decreased gradually approximately in parallel with changes of (ZZ)-isomer (Fig. 4). That the photoproducts corresponding to peaks 3'', 0'' and 1'', i.e. (EZ)-bilirubin XIII α , (EE)-bilirubin XIII α , and (EE)-monocyclobilirubin XIII α , are geometric isomers of the compounds corresponding to peaks 4, 3'' and 2'', i.e. (ZZ)-bilirubin XIII α , (EZ)-bilirubin XIII α , and (EZ)-monocyclobilirubin XIII α respectively, is confirmed by photochemical (Figs. 5, 6 and 7) and thermal and catalytical reversion of the photoproducts (results not shown). In comparison with the rate of geometric isomerization, cyclization of the above-mentioned geometric isomers proceeded at a lower rate (peaks 2'' and 1'' in Fig. 4). That the photoproducts corresponding to peaks 1'' and 2''

of bilirubin IX α (Stoll *et al.*, 1979; Isobe & Onishi, 1981). A summary of these changes plotted against time on a semilogarithmic scale is shown in Fig. 2. Thus it was confirmed by the present experiments that a compound equivalent to *E*-cyclobilirubin of bilirubin IX α (which is not converted to a cyclic isomer) (Isobe *et al.*, 1983; Onishi *et al.*, 1983) was not found in the photoproducts of III α isomer. Analogous to the case of bilirubin IX α (Onishi *et al.*, 1980a; Isobe & Onishi, 1981), a good quantitative correspondence was observed between the decrease of peak 6 and the increase of peaks 0' and 3'. The reason for the simple photochemical

Fig. 3. H.p.l.c. of solutions obtained at various intervals during anaerobic irradiation of bilirubin XIII α in dimethyl sulphoxide solution

(a) Shows the chromatographic scan for the control non-irradiated solution, and (b) shows the scans after exposure to light for 60 min.

Fig. 4. Changes in the concentration of bilirubin XIII α and its photoproducts plotted against time on a semilogarithmic scale

are 'cyclized' structural isomers is also confirmed by the photochemical reversion of peak 2'' to 3'', i.e. (EZ)-monocyclobilirubin XIII α to (EZ)-bilirubin XIII α , whereas no thermal nor catalytical reversion of (EZ)-monocyclobilirubin XIII α was observed (results not shown). However, no quantitative correspondence was noted between the decrease of peak 4 and the sum of the photoproducts. This is considered to be due to decomposition into brown pigment via the formation of an azacyclopentadiene ring (von Döbeneck, 1979; Onishi *et al.*, 1981, 1982). Thus, the total peak area estimated by h.p.l.c. as A_{455} also decreased. Because the XIII α isomer has two *endo*-vinyl groups at C-3 and C-17 which both may participate in the cyclization (Onishi *et al.*, 1981, 1982; Stoll *et al.*, 1982; McDonagh *et al.*, 1982a,b; Isobe *et al.*, 1983), six photoproducts of bilirubin XIII α are expected theoretically if one leaves aside epimeric isomers; in fact six peaks were observed. On reversed-phase h.p.l.c. of photoisomers, a remarkable regularity is found as follows; the retention times of geometric isomers with *E*-configuration, and *E*-cyclized structural isomers, are shortened by a rather regular period of time compared with those of the corresponding *Z*-isomer and *E*-isomer respectively. All values shown for the XIII α series in Table 1 are presented as the number obtained when the difference in retention time between a

Fig. 5. Photochemical reversion of photoproduct corresponding to peak 3'' (a) and (b) show the changes of peak 3'' during low (4.8 $\mu\text{W}/\text{cm}^2$ per nm) and high (24.8 $\mu\text{W}/\text{cm}^2$ per nm) illumination respectively. The relative percentages are given in an accumulative manner.

Fig. 6. Photochemical conversion of compound corresponding to peak 2'' into peak 1'' (a) and (b) show the changes of peak 2'' during low (4.8 $\mu\text{W}/\text{cm}^2$ per nm) and high (24.8 $\mu\text{W}/\text{cm}^2$ per nm) illumination respectively. The relative percentages are given in an accumulative manner.

pair of geometric isomers or a pair of structural isomers is divided by the difference in retention time between *E*-isomers or *E*-cyclized isomer respectively. All values for the III α or IX α series presented in Table 1 were derived in a similar way. Therefore, on the basis of this evidence, the peak with retention time of 33min is expected to be *EE*-dicyclobilirubin XIII α . However, a sufficient amount of this compound could not be obtained for thermal, photochemical or catalytic experiments.

Whereas previous interest has been focused on the *exo*-vinyl group, which shows photoaddition of alcohols or thiols (Garbagnati & Manitto, 1973) and furanoid-ring formation (Roos *et al.*, 1977), the *endo*-vinyl group has attracted little attention until recently, except for blue-green bile pigments of butterflies (Choussy & Barbier, 1977). Recently, the structure of cyclization products of bilirubin IX α was shown to involve participation of the *endo*-vinyl group (Stoll *et al.*, 1982; Onishi *et al.*,

Fig. 7. Photochemical conversion of peak 1'' photoproduct into peak-2'' photoproduct (a) and (b) show the changes of peak 1'' during low ($4.8 \mu\text{W}/\text{cm}^2$ per nm) and high ($24.8 \mu\text{W}/\text{cm}^2$ per nm) illumination respectively. The relative percentages are given in an accumulative manner.

Table 1. Relative differences in retention times between various pairs of geometric and structural photoisomers. The difference in retention time for each series (bilirubin XIII α , bilirubin III α and bilirubin IX α) is set to 1 in the Table. Abbreviations used: BR, bilirubin; MCBR, monocyclobilirubin; DCBR, dicyclobilirubin; CBR, cyclobilirubin.

	Geometric photoisomerization		Relative difference in retention time	Structural photoisomerization		Relative difference in retention time
	Z-Configuration	E-Configuration		E-Bilirubin	E-Cyclobilirubin	
Bilirubin XIII α ($n = 10$)	ZZ-BR	$\rightarrow$ EZ(ZE)	4.00 ± 0.54	ZE(=EZ)-BR $\rightarrow$ ZE-MCBR		1
	EZ(=ZE)-BR	$\rightarrow$ EE-BR	4.29 ± 0.61	EE-BR	$\rightarrow$ EE-MCBR	1.46 ± 0.21
	EZ-MCBR	$\rightarrow$ EE-MCBR	4.78 ± 0.65	EE-MCBR	$\rightarrow$ EE-DCBR	1.49 ± 0.09
Bilirubin III α ($n = 10$)	ZZ-BR	$\rightarrow$ EZ(=ZE)	1			
	EZ(=ZE)-BR	$\rightarrow$ EE	0.95 ± 0.24			
Bilirubin IX α ($n = 10$)	ZZ-BR	$\rightarrow$ EZ- and ZE-BR	4.09 ± 0.56	EZ-BR	$\rightarrow$ EZ-CBR	1
	EZ- and ZE-BR	$\rightarrow$ EE-BR	4.14 ± 0.48	EE-BR	$\rightarrow$ EE-CBR	1.54 ± 0.09
	EZ-CBR	$\rightarrow$ EE-CBR	4.68 ± 0.50			

1984). On the basis of photochemical experiments *in vitro* and the fact that large amounts of (EZ)-cyclobilirubin IX α appear in the bile during phototherapy (Onishi *et al.*, 1980b), it is concluded that the *endo*-vinyl group plays a crucial role in the photochemical reaction of bilirubin IX α . The proposed interrelationships between the bilirubin XIII α photoproducts, including those not yet confirmed, are given in Scheme 2.

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