

Kinetic study of photochemical and thermal conversion of bilirubin IX α and its photoproducts

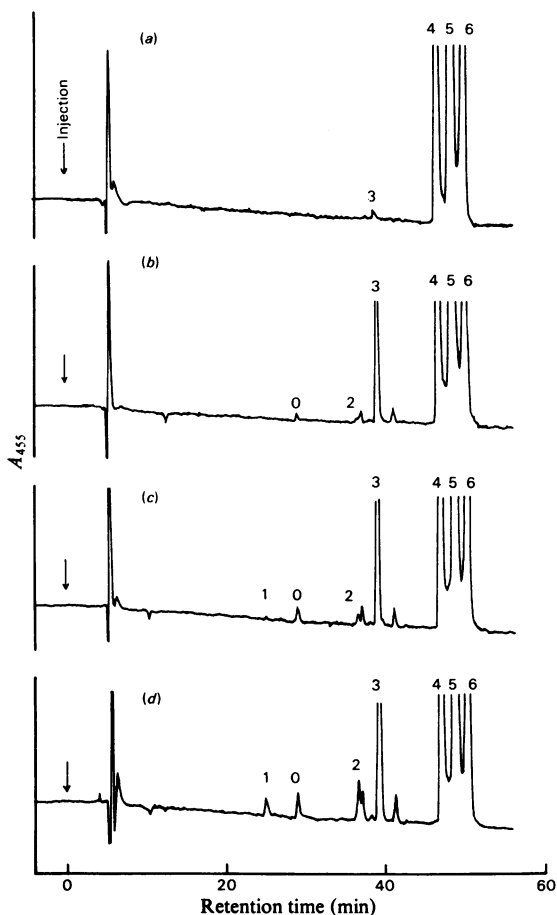
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A kinetic study of the photochemical and thermal conversion of photoisomers, especially peaks 0 and 3 [(*EE*)-bilirubin IX α and (*EZ*)- and (*ZE*)-bilirubin IX α], under anaerobic conditions, was performed by using reversed-phase high-pressure liquid chromatography. Peaks 0 and 3 are spontaneously, photochemically and thermally converted. Short-term photoirradiation of bilirubin gives a mixture containing not only the geometric isomers (*EE*)-, (*EZ*)- and (*ZE*)-bilirubin IX α , but also peak 2, (*EZ*)-cyclobilirubin IX α . On prolonged irradiation, cyclization and 15Z \rightarrow 15E isomerization lead to gradual accumulation of two pairs of photoproducts: (*EZ*)-cyclobilirubin IX α A and B and (*EE*)-cyclobilirubin IX α A and B.



The present view of the predominant pathway of bilirubin removal from the body in the course of phototherapy is both geometric and structural photoisomerization (Cohen & Ostrow, 1980; Onishi *et al.*, 1980*b*, 1981*a,b*; Stoll *et al.*, 1982). However, photochemical research on bilirubin was carried out mainly by t.l.c. or absorbance difference spectroscopy (McDonagh *et al.*, 1979; Lightner *et al.*, 1979*a,b*; Stoll *et al.*, 1981). Therefore quantitative data were rather difficult to obtain. Our work on this problem, reported previously, used an accurate and sensitive h.p.l.c. method (Onishi *et al.*, 1979, 1980*a,b*, 1981*a,b*; Isobe & Onishi, 1981). A summary is given below. Photobilirubin IX α in the serum of a hyperbilirubinaemic infant was demonstrated to increase significantly during phototherapy. Corresponding to these changes in the serum, the biliary unconjugated bilirubin concentration increased markedly, but photobilirubin IX α was not detected in the bile. Large amounts of

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

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

(a) shows the chromatographic scan for the control non-irradiated solution, and (b), (c) and (d) show the scans after exposure to light for 20, 40 and 60 s respectively.

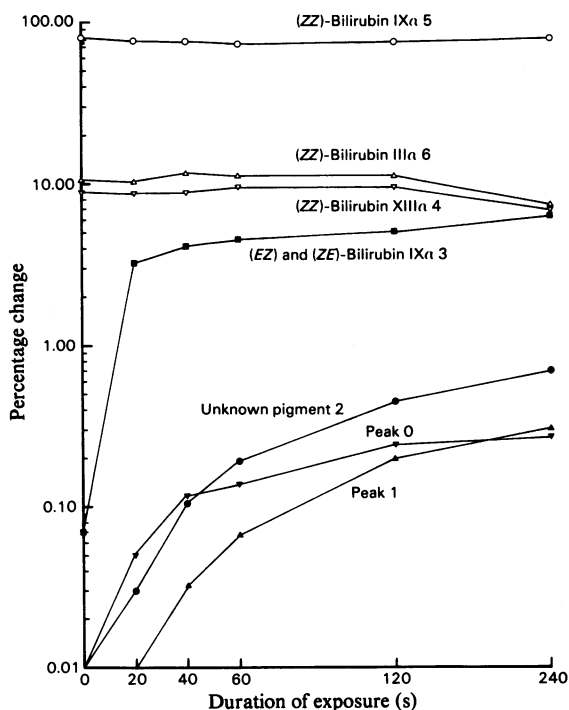


Fig. 2. Changes in the concentration of bilirubin and its photoproducts plotted against time on a semilogarithmic scale

Symbols: O, (ZZ)-bilirubin IX α ; Δ , (ZZ)-bilirubin III α ; ∇ , (ZZ)-bilirubin XIII α ; \blacksquare , (EZ)- and (ZE)-bilirubin IX; \bullet , unknown pigment; \blacktriangle , peak 1; \blacktriangledown , peak 0.

unknown pigment also appeared in the bile during phototherapy. Exact correspondence was observed between the two main photoproducts, unknown pigment and photobilirubin IX α , from biological fluids, and comparable main peaks 2 and 3 formed *in vitro*. By kinetic study of biliary excretion of the main two bilirubin photoproducts after injection into Gunn rats, the mean value of the half-time for the appearance of photobilirubin IX α in the bile estimated by the Sigma-minus method was 29.8 min and that for unknown pigment was 4.3 min. The thermal reversion of photobilirubin IX α to (ZZ)-bilirubin IX α in the bile at 37°C was very rapid, i.e. its half-life was 6.2 min. Rapid and definite interconversion among the three peaks 1, 2 and 3 and the parent pigment (bilirubin) occurred on irradiation with blue light. The chemical structure of the unknown pigment (Onishi *et al.*, 1981b, 1982) was shown to agree with endo-vinyl-cyclized (EZ)-bilirubin proposed by Stoll *et al.* (1982). Each of the above-mentioned bilirubin photoproducts, i.e. those contained in peaks 1, 2 and 3, were composed of two components, which may be called peaks 1A and 1B,

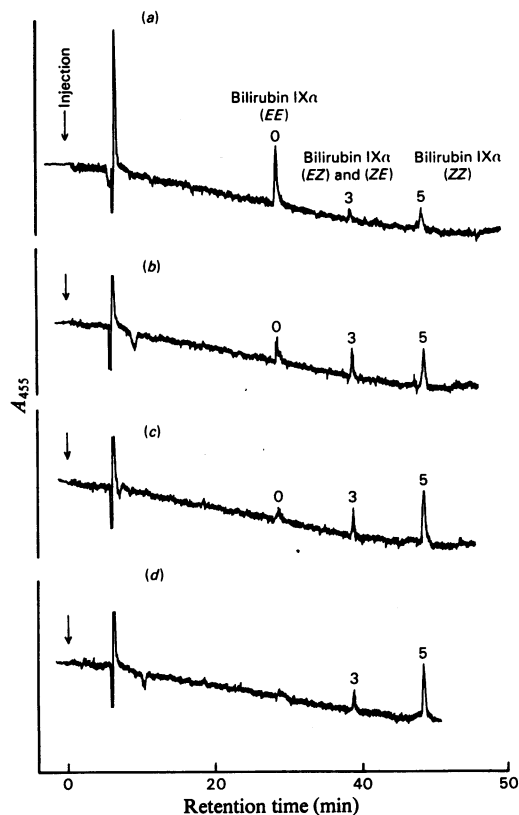


Fig. 3. H.p.l.c. of solutions obtained at various intervals during anaerobic irradiation of peak 0 in the h.p.l.c. eluent

(a) shows the chromatographic scan for the control non-irradiated solution; (b), (c) and (d) show the scans after exposure to light for 5, 10 and 15 s respectively.

peaks 2A and 2B and peaks 3A and 3B. Peaks 1A and 1B are the 15E geometric isomers of peaks 2A and 2B. Peaks 3A and 3B are the 4E,15Z and 4Z,15E geometric isomers of bilirubin. Moreover, peaks 1A and 1B are diastereoisomers and so are 2A and 2B, whereas 3A and 3B are geometric isomers. The pathophysiological significance of these photoproducts was also described previously. These facts contradict the view that the major mechanism of phototherapy in jaundice is exclusively geometric photoisomerization (McDonagh, 1981). In the present paper we have studied the relationship between all the bilirubin IX α photoproducts proposed by Stoll *et al.* (1982).

Materials and methods

Photochemical experiments

A trace of EDTA was added to bilirubin (340 μ M) in dimethyl sulphoxide. The process of photolysis

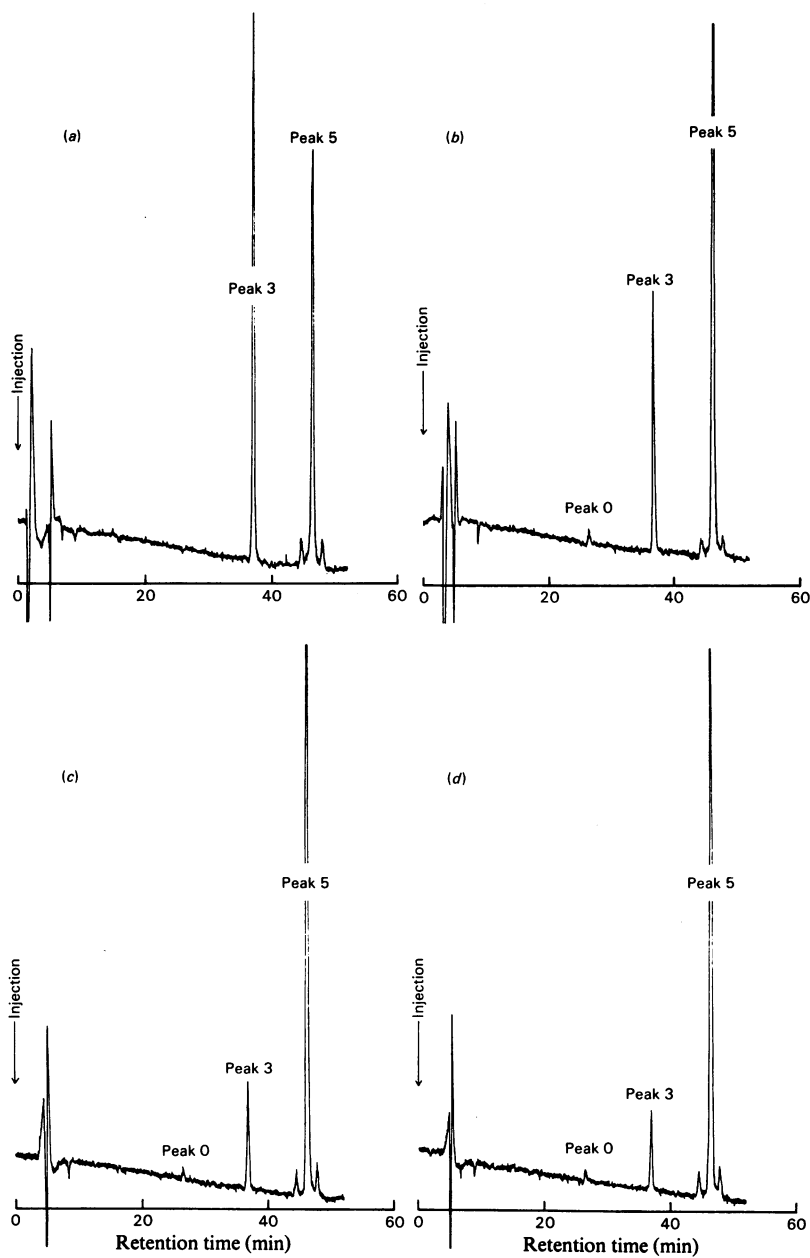


Fig. 4. H.p.l.c. of solutions obtained at various times during anaerobic irradiation of peak 3 in the h.p.l.c. eluent (a), (b), (c) and (d) correspond to those in Fig. 3.

under anaerobic conditions was carried out in stoppered 10ml Pyrex tubes located 3 cm above the canopy of four fluorescent tubes. Except where noted otherwise, in all experiments the solutions were deoxygenated with pure (99.999%) argon. Several 1.0ml portions of solution were exposed to light in the tubes. At different periods of light exposure,

Pyrex tubes were successively transferred to the dark. One sample was kept in the dark as a control. Each portion was analysed by h.p.l.c. for photo-products (Onishi *et al.*, 1980a; Isobe & Onishi, 1981). Light intensity, determined with a Minolta Air-Shields Fluoro-lite-meter 451, at the surface of the solution was $24 \mu\text{W} \cdot \text{cm}^{-2} \cdot \text{nm}^{-1}$.

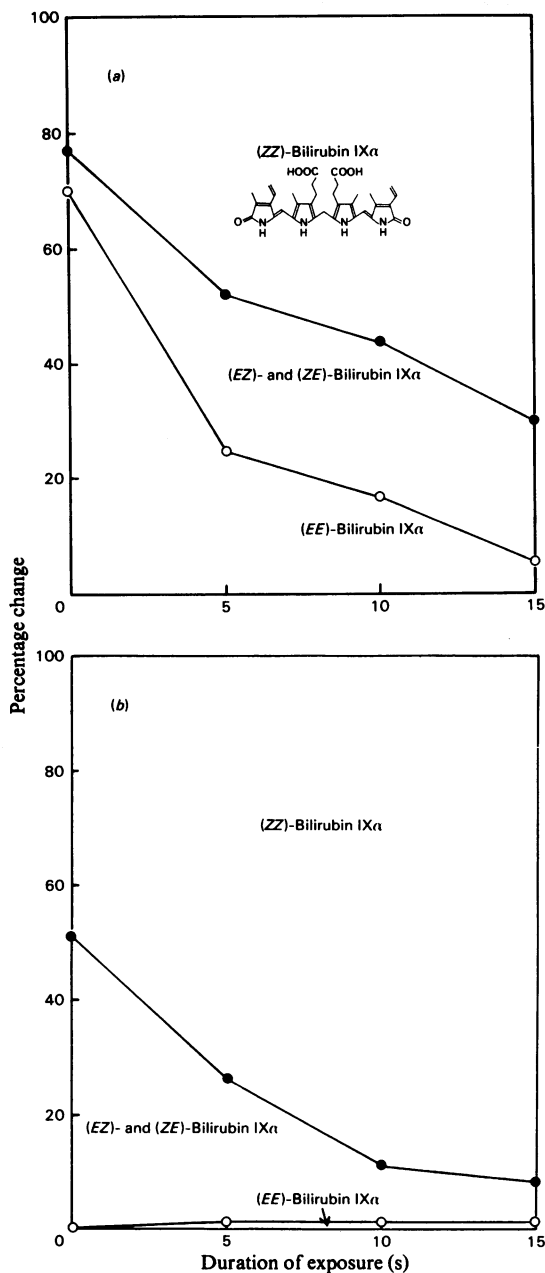


Fig. 5. Plot of the photochemical changes in peaks with time on a linear scale

(a) and (b) show the changes of peak 0 and peak 3 respectively. The relative percentages are given in an accumulative manner as an area.

Experiments of thermal reversion

Thermal reversion of each peak after elution was inhibited by cooling the collecting test tubes to -70°C . Experiments in thermal reversion were

performed in the dark under anaerobic conditions, followed by h.p.l.c. analysis. All manipulations were carried out in subdued light, except photochemical experiments, and at room temperature, except in the thermal experiments. Photochemical experiments, chromatographic operation and preparation of calibration curves were carried out as described previously (Onishi *et al.*, 1980a; Isobe & Onishi, 1981).

Results and discussion

Photochemical experiment of bilirubin IX α (Figs. 1 and 2)

The numbering of peaks 1, 2, 3, 4 and 6 separated by h.p.l.c. (Fig. 1) was carried out as described previously (Onishi *et al.*, 1980a,b). The number '0' was given to the peak with a retention time of 29 min. The peak was not detected in the experiment in equimolar solutions of human serum albumin and bilirubin (Onishi *et al.*, 1980a). This is derived from the use of albumin instead of dimethyl sulphoxide as a solvent. In the control experiment with non-irradiated solution (Fig. 1a), a large peak 5 of bilirubin IX α and two peaks 4 and 6 of bilirubin

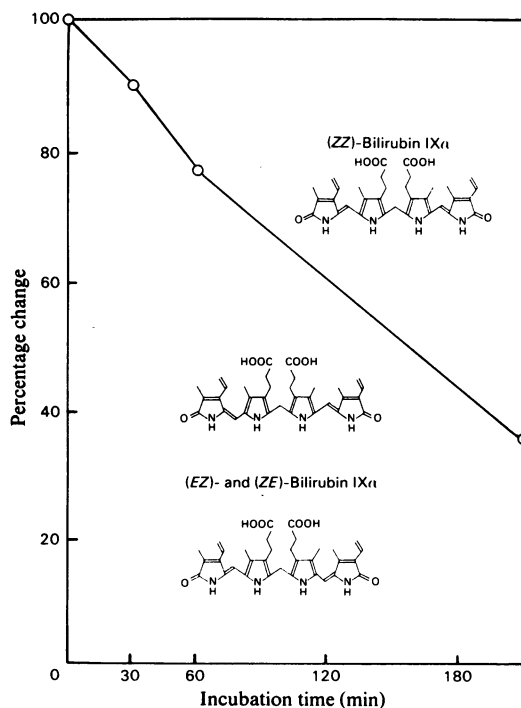
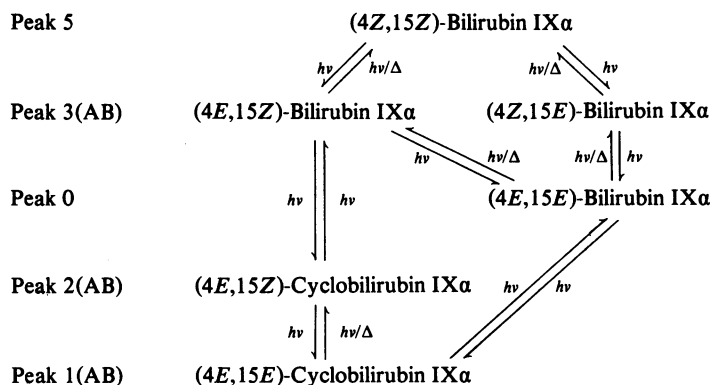


Fig. 6. Plot of the thermal changes in peak 3 with time on a linear scale

The relative percentages are given in an accumulative manner as an area.

Scheme 1. Interrelationship of bilirubin IX α and its photoproducts

XIII α and III α were clearly separated. When photoirradiation was applied for 20s (Fig. 1b), peaks 0, 2 and 3 appeared with retention times at 29, 37 and 39 min respectively. At 40s (Fig. 1c), a very small peak 1 with retention time at 25 min appeared, and then (Fig. 1d) peaks 1, 0 and 2 increased. The changes after this were almost the same as that described for the photochemical changes in the equimolar solution of human serum albumin and bilirubin (Onishi *et al.*, 1980a). It was demonstrated previously that peak 3 corresponds to photobilirubin IX α , a mixture of (*EZ*)- and (*ZE*)-bilirubin IX α (photobilirubin IA and IB), and peak 2 to unknown pigment, a mixture of (*EZ*)-cyclobilirubin IX α A and B (photobilirubin IIA and IIB) and peak 1 to (*EE*)-isomers of peak 2 (photobilirubin III) (Onishi *et al.*, 1980a,b; Stoll *et al.*, 1982). A summary of these changes, plotted as percentages against time on a semilogarithmic scale, is shown in Fig. 2. To investigate the relationship between peak 0 and other peaks, photochemical and thermal experiments were performed.

Photochemical reversion of peak 0 (Fig. 3) and peak 3 (Fig. 4)

In the control experiment with non-irradiated solution of peak 0, peaks 3 and 5 were already detected by h.p.l.c. (Fig. 3). This indicates that peak 0 spontaneously reverted into (*EZ*)- and (*ZE*)-bilirubin IX α and (*ZZ*)-bilirubin IX α . When photoirradiation was applied, the peak 0 decreased rapidly and peaks 3 and 5 increased, and then peak 0 almost disappeared within 15s. The same experiments were carried out for peak 3 as for peak 0 (Fig. 4). A summary of these changes plotted as percentages against time on a linear scale is shown in Fig. 5.

Thermal reversion of peak 0 and peak 3

Analysis by h.p.l.c. of thermal reversion of peak 0 in the dark at 50°C showed almost the same pattern

as that described for the photochemical reversion, except for slow changes. A good quantitative correspondence between the disappearance of peak 0 and the appearance of peaks 3 and 5 was observed in both the photochemical and thermal experiments. When the same experiments for peak 3 as for peak 0 were carried out, peak 3 decreased and peak 5 appeared and then increased, but peak 0 did not appear (Fig. 6). Furthermore, it was reported that h.p.l.c. analysis of the photoequilibrium mixture of bilirubin IX α dimethyl ester in organic solvents revealed three compounds, (*EZ*)-, (*ZE*)- and (*EE*)-isomers (McDonagh *et al.*, 1979; Lightner *et al.*, 1979a,b). Thus we conclude that peak 0 is a geometric isomer of peak 3, i.e. (*EE*)-bilirubin IX α .

Short-term photoirradiation of bilirubin IX α gives a mixture containing not only geometric isomers, separated in peaks 0 and 3A and 3B, but also peak 2, (*EZ*)-cyclobilirubin IX α (Scheme 1). On prolonged irradiation, both intramolecular endo vinyl cyclization and 15Z \rightarrow 15E isomerization lead to gradual accumulation of two pairs of photoproducts, peaks 2A and 2B and peaks 1A and 1B respectively, in addition to three geometric isomers of bilirubin IX α (Scheme 1). We substantiated the interrelationship of bilirubin IX α and its photoproducts proposed by Stoll *et al.* (1982) and Onishi *et al.* (1981b).

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