High-pressure liquid chromatographic analysis of anaerobic photoproducts of bilirubin-IX α *in vitro* and its comparison with photoproducts *in vivo*

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To carry out photochemical experiments under conditions similar to those prevailing for neonatal bilirubin metabolism in jaundice phototherapy, we have studied photoproducts produced by the action of light on a bilirubin–albumin solution and further clarified the relationship between the photoproducts obtained from experiments *in vitro* and *in vivo*. (1) An accurate and sensitive separation method by high-pressure liquid chromatography for photoproducts of bilirubin under anaerobic irradiation of visible light is described. (2) There were two main photoproducts obtained from experiments both *in vivo* and *in vitro*. (3) Exact correspondence of retention time on high-pressure liquid chromatography, diazo-reactivity, thermal reversion and absorption-spectrum maxima was observed between unknown pigment and photobilirubin-IX α from biological fluids, and the comparable peaks 2 and 3 from experiments *in vitro*. (4) The behaviour of photoproducts in various solutions in the absence of light and O₂ is described. (5) A lower affinity of photoproducts, especially unknown pigment, for human serum albumin than with bilirubin-IX α for the albumin was demonstrated by the gel-filtration method.

Since the original report of Cremer and associates in 1958 (Cremer *et al.*, 1958), phototherapy has been successfully applied in the treatment of hyperbilirubinaemia of the newborn infant, and is now an established procedure in neonatal medicine. The major mechanism is thought to be photo-oxidation or photodegradation of bilirubin to other compounds that are easily excreted (McDonagh & Palma, 1977). The mechanism that has been studied more extensively is photo-oxidation of bilirubin *in vitro* in a variety of solvents to colourless products (Lightner, 1977; McDonagh, 1979). However, the fact that considerable amounts of unconjugated bilirubin appear in the bile of Gunn rats (Ostrow, 1971; Ostrow *et al.*, 1974) or newborn infants (Lund & Jacobsen, 1972) after irradiation with blue light is not consistent with this mechanism.

Therefore it seems reasonable to hypothesize that biliary excretion of bilirubin without conjugation represents the primary means of its elimination during phototherapy. McDonagh (1974) first suggested that bilirubin can be converted photochemically into a more polar, but less thermodynamically stable, isomer that does not need conjugation before excretion. By using X-ray crystallography, Bonnett *et al.* (1976) demonstrated that bilirubin-IX α is normally in the ZZ-confi-

guration at the C-5 and C-15 bridges, containing six internal hydrogen-bonds, all of which involve the carboxy groups.

The suppression of ionization of the two carboxy groups accounts for the insolubility of bilirubin in water in spite of its various polar functional groups, but if either, or both, of the C-5 and C-15 bridges had *E*- rather than the *Z*-configuration, the tightly knit system of six intramolecular hydrogen-bonds would no longer be formed (Bonnett *et al.*, 1978). Subsequently it has been reported that some configurational modification of the pigment by photo-energy probably occurs during phototherapy (McDonagh, 1976; Pedersen *et al.*, 1977; Lightner, 1977). More recently, McDonagh *et al.* (1979) demonstrated that bilirubin-IX α dimethyl ester undergoes rapid and reversible photoisomerization during irradiation with visible light, giving photobilirubin with *E*-configuration. This mechanism might involve light-induced conversion of bilirubin into unstable more polar photobilirubin that can be excreted via the liver into bile, and reversion to natural (ZZ)-bilirubin-IX α (Lightner *et al.*, 1979a,b). Stoll *et al.* (1979) arrived at similar conclusions by using t.l.c. These photochemical experiments with regard to photobilirubin were carried out exclusively in unphysiological conditions, such as in neutral or basic organic solvents. Reports on irradiation of human serum albumin and

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

bilirubin under aerobic conditions, possibly very relevant to conditions *in vivo*, have recently been described (Hutchinson & Mutopo, 1979; Rubaltelli & Jori, 1979), but concerned the photochemically covalent bonding of bilirubin with human serum albumin and photodegradation of certain amino acids residues in human serum albumin respectively. They did not investigate the geometric photoisomerization of bilirubin. Therefore, to carry out photochemical experiments in conditions similar to those prevailing for neonatal bilirubin metabolism in jaundice phototherapy, we have studied the photo-products produced by the action of light in a bilirubin-albumin solution and further clarified the relationship between the photoproducts obtained from experiments *in vitro* and *in vivo*.

Materials and methods

Photochemical experiments

Equimolar solutions (260 μM) of human serum albumin and bilirubin were prepared by adding 10 mg of crystalline bilirubin dissolved in 3.0 ml of 0.05 M-NaOH to 47.0 ml of human serum albumin solution (1.80 g/100 ml) that was dissolved in the 0.02 M-phosphate buffer, pH 7.4. 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). Except where noted otherwise, in all experiments the solutions were deoxygenated with pure (99.999%) argon. Light intensity, determined with a PR III phototherapy radiometer (Air-Shields), at the surface of the solution was 11.8 $\mu\text{W}\cdot\text{cm}^2\cdot\text{nm}$. Several 1.0 ml portions of solution were exposed to light in the tubes. One portion was placed in the dark as a control. At different periods of light exposure, Pyrex tubes were successively replaced in the dark. Each portion was analysed both by gel filtration and h.p.l.c. for photobilirubin as described below.

Gel filtration

Binding affinity of bilirubin and photoproducts to albumin was studied by gel filtration of each portion (0.2 ml), with a Sephadex G-25 (fine) column (12 cm $\times$ 12 mm) and 0.1 M-phosphate buffer, pH 7.4, as eluent. To elute all bilirubin and its photo-products left on the Sephadex, 0.2 ml of human serum albumin solution (60 mg/ml in 0.1 M-phosphate buffer, pH 7.4) was added (Onishi, 1970).

The stability and reproducibility of the h.p.l.c. method for photobilirubin

The solution of equimolar amounts of human serum albumin and bilirubin was irradiated for 15 min under anaerobic conditions. Of the solution of albumin-bilirubin complex 1 vol. was added to 4 vol.

of 60% (v/v) acetonitrile in 0.01 M-phosphate buffer, pH 7.4, containing 0.1% tetra-n-butylammonium hydroxide. The mixture was vortex-mixed for 30 s and centrifuged for 5 min at 1000 rev./min. The supernatant, which contained the same constituents as the eluent for h.p.l.c. for photobilirubin analysis, was kept in the dark at 17°C. Portions (0.4 ml) were removed at 0, 30, 60, 90, 120, 180, 240 and 300 min. Each portion was immediately separated by h.p.l.c. for photobilirubin analysis. The photobilirubin concentrations of each portion were plotted against time on a semilogarithmic scale and the exponential slope obtained was determined by the method of least squares, from which the half-life of photobilirubin was derived.

H.p.l.c.

Equipment. A Shimadzu LC-2FG liquid chromatograph with an SPD-1 detector and Chromatopac C-R1A was used for all h.p.l.c. procedures. Shimadzu PCH-05/S2504 columns (25 cm $\times$ 4.6 mm) were used. This column is packed with silica gel (5 μm particles) that is chemically bonded with hydrophobic hydrocarbon.

Chromatographic operation. The variable-wavelength detector was set at 455 nm. Separation was achieved at 17°C, with two or three columns in series. The flow rate was 0.8 ml/min. The mobile phase was a mixture of acetonitrile, water, sodium phosphate and tetra-n-butylammonium hydroxide. Separation for photobilirubin was best achieved by using a linear gradient of acetonitrile in 0.01 M-phosphate buffer, pH 8.0, containing 0.1% tetra-n-butylammonium hydroxide as counter ion [16–34% (v/v) in 120 min (Onishi *et al.*, 1979b)]. To avoid loss by oxidation the h.p.l.c. solvent systems were degassed before use. All manipulations were carried out in subdued light.

Preparation of calibration curve. The calibration curve of peak area, obtained from the electronically integrated peak area calculated by Chromatopac C-R1A, versus amount of bilirubin loaded was linear up to 1–5 μg of bilirubin. If the concentration is above this range, the value can be precisely estimated either by decreasing amounts of the sample injected into the chromatograph or by diluting the sample with the primary eluent. Calculation of each peak level was performed on the assumption that the molar absorption of all the bile pigments on h.p.l.c. scan was the same as that of (ZZ)-bilirubin-IX α .

Peak assignments. The following methods were used. One method was formation of azopigment derivatives by using diazotized ethyl anthranilate as described previously (Onishi *et al.*, 1979a, 1980a,b). The other method involved comparison with the retention time on h.p.l.c. either for conjugated bilirubin (Onishi *et al.*, 1980a) or for photobilirubin analysis (Onishi *et al.*, 1979b).

Sample preparation for h.p.l.c. Acetonitrile (3 vol.) was added to 5 vol. of the sample, which was vortex-mixed for 30s and then centrifuged for 5 min at 1000 rev./min. A portion (15 μ l) of the supernatant was injected into the chromatograph. The preparation was carried out within 6 min immediately before injection.

Pigments and reagents

Bilirubin (E. Merck, Darmstadt, Germany), tetra-n-butylammonium hydroxide (Wako, Osaka, Japan), crystalline human serum albumin (Kabi, Stockholm, Sweden), Sephadex G-25 (fine) (Pharmacia, Uppsala, Sweden) were used without further purification. Pentan-2-one (Tokyo Kasei, Tokyo, Japan) was distilled before use. Acetonitrile and all other reagents used were of analytical grade.

Results and discussion

Separation of photoproducts by h.p.l.c. for photobilirubin analysis

As shown in Figs. 1(a) and 1(b), a number was given to each peak according to the order of retention time. In the control experiment with a non-irradiated solution, a large peak 5 of (ZZ)-bilirubin-IX α , which had a retention time of 70 min, and two small peaks 4 and 6 of (ZZ)-bilirubin-XIII α and -III α were clearly separated (Fig. 1a). Assignments of these peaks 4, 5 and 6 were based on previous work (Wooldridge & Lightner, 1978; Onishi *et al.*, 1979a, 1980a,b). When photoirradiation was applied, the (ZZ)-bilirubin-IX α concentration decreased and peaks 1, 2 and 3 appeared with retention times at 14, 33 and 41 min respectively. At 180s, very small peaks 1 and 2 and a fairly large peak 3 appeared. After this, peak 3 did not change significantly with further irradiation. This would show that photoequilibrium was reached (McDonagh *et al.*, 1979). At 900s, peak 2 increased gradually (Fig. 1b) and at 1800s attained almost the same height as peak 3. However, peak 1 remained very small during the period of light exposure. Chromatographic profiles at 180s and at 1800s are not shown but a chromatographic profile at 900s is shown in Fig. 1(b) as a selected irradiated example. In comparison with the ZZ-configuration of bilirubins-IX α , -III α and -XIII α , the three peaks 1, 2 and 3 had a shorter retention time on reversed-phase h.p.l.c., so that they are more polar than the ZZ-configuration. A summary of these changes plotted against time on a semilogarithmic scale is shown in Fig. 2. Peak 3 was fractionated and subjected to h.p.l.c. while the fractionated solutions were kept in the dark at 17°C. The photoproduct peak gradually decreased and the (ZZ)-bilirubin-IX α peak increased correspondingly, i.e. after 30, 60 and 210 min of incubation, it had increased by 10.8, 23.8 and 64.9%

Fig. 1. H.p.l.c. of solutions obtained at various intervals during anaerobic illumination of bilirubin in alkaline human serum albumin solution

Experimental details are given in the text. (a) shows the chromatographic scan for the control non-irradiated solution and (b) shows the scan after exposure to light for 900s

respectively. A chromatographic profile at 210 min is shown in Fig. 3. At the higher temperatures of 37°C and 50°C, the rate of reversion was accelerated considerably. Thus, thermal reversion of the photoproduct to (ZZ)-bilirubin-IX α was demonstrated by h.p.l.c. In conclusion it can be stated that the photoproduct is one of the photobilirubins described by McDonagh *et al.* (1979). Since the peak can be divided into two peaks by h.p.l.c. (S. Onishi & S. Itoh, unpublished work), it most likely represents a mixture of the ZE and EZ isomers. It was also

Fig. 2. The changes in the concentration of bilirubin and its photoproducts plotted against time on a semi-logarithmic scale
Symbols: ○, total bilirubin; □, (ZZ)-bilirubin-IXα; △, (ZZ)-bilirubin-IIIα; ▽, (ZZ)-bilirubin-XIIIα; ■, photobilirubin-IXα; ●, unknown pigment.

confirmed that the photobilirubin was the IXα-isomer by the following observations. (1) Azo-dipyrroles derived from the photobilirubin consisted of equal amounts of *endo*- and *exo*-vinyl isomers. (2) The retention time of the photobilirubin was the same as that of (ZZ)-bilirubin-IXα from analysis of IXα-, IIIα-, and XIIIα-isomers of bilirubin by h.p.l.c. modified from the method of Wooldridge & Lightner (1978). As shown in Fig. 4, a photoproduct that had the same retention time as peak 3 was separated by h.p.l.c. from the serum of hyperbilirubinaemic infants during phototherapy. From another experiment *in vitro* on peak 3, involving thermal reversion, it was concluded that the serum photoproduct is photobilirubin-IXα.

With regard to peak 2, exact correspondence for retention time on h.p.l.c., diazo-reactivity and thermal reversion was observed with the 'unknown pigment' found in the biological fluids of hyperbilirubinaemic infants (Onishi *et al.*, 1980c) and with peak 2 obtained from the bilirubin-albumin complex

Fig. 3. H.p.l.c. of peak 3 with time while the fractionated solutions were kept in the dark at 17°C
Incubation in the dark at 17°C was for 210 min.

Fig. 4. H.p.l.c. scan of the serum of hyperbilirubinaemic infant during phototherapy

Table 1. Absorption maximum and diazo-reactivity of bilirubin and its photoproducts								
Peak	1	2		3		4	5	6
Substances	Unknown	Unknown pigment		Photobilirubin-IXα		(ZZ)-Bilirubin-XIIIα	(ZZ)-Bilirubin-IXα	(ZZ)-Bilirubin-IIIα
		<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>			
λ _{max.} (nm) in h.p.l.c. solvent	443–444	435–436	435–436	425	425	443	447	449
λ _{max.} (nm) in chloroform	?	440–442	442	446	440–453	448	451	455
Diazo-reactivity	?	—	—	+	+	+	+	+

photoirradiated *in vitro*. The available amounts were too small to investigate peak 1 in more detail. A summary of some chemical properties of photoproducts both *in vitro* and *in vivo* is shown in Table 1. As stated previously, photobilirubin-IX α obtained both *in vitro* and in the jaundiced subject during phototherapy corresponds to the (ZE)- and (EZ)-photobilirubin described by McDonagh *et al.* (1979) and to photobilirubin-I described by Stoll *et al.* (1979).

On the other hand, the unknown pigment has spectral and chemical properties that resemble the major photoproduct described by Berry *et al.* (1972) and Ostrow *et al.* (1974) and the photobilirubin-II described by Stoll *et al.* (1979). It also resembles, in part, the 415 nm-absorbing substance described by us, which was found in the urine of photo-treated hyperbilirubinaemic infants (Onishi *et al.*, 1969, 1971). The common properties include the atypical diazo-reactivity and the tendency to form brown insoluble residues in acid solutions.

Fig. 5. The half-life of photobilirubin in aqueous alkaline solution kept in the dark at 17°C

Explanation of the symbols is given in the legend to Fig. 1.

The stability and reproducibility of h.p.l.c. for photobilirubin analysis

As shown in Fig. 5, the half-life of photobilirubin in aqueous alkaline (pH 8.0) solutions kept in the dark at 17°C, the constituents of which were the same as the h.p.l.c. eluent used for photobilirubin analysis, was 6.5 h; i.e. the diminution of the photobilirubin peak during h.p.l.c. analysis was about 5%, since the retention time for photobilirubin-IX α is 40 min. Hence, the reversion of photobilirubin-IX α to bilirubin at 17°C in the h.p.l.c. eluent was considered to be very slow. On the other hand, in the dark at 17°C, no changes in peak height of the unknown pigment were detectable. Consequently, it was concluded that the reversal of photobilirubin-IX α during the separation by h.p.l.c. could be neglected and that the h.p.l.c. method for photoproducts is thus a reliable and sensitive way of determining photobilirubin-IX α and unknown pigment.

Behaviour of photoproducts in various solutions in the absence of light and O₂ (Table 2)

In all cases, the decay of photobilirubin-IX α was found to obey first-order kinetics. At 17°C, the half-life of photobilirubin-IX α in the above mentioned solution decreased from 390 to 135 min with decreasing pH values from 7.97 to 7.07. This agrees with the previous finding of McDonagh *et al.* (1979), but contradicts the result reported by Stoll *et al.* (1979). During the period of experiment, unknown pigment remained almost constant but the IX α -isomer decreased and III α - and XIII α -isomers increased gradually. At higher temperatures, the reversion of photobilirubin-IX α was accelerated and, moreover, unknown pigment also decreased with a half-life of 660 min. In contrast, in equimolar solutions of bilirubin-albumin complex kept in the dark at 37°C, unknown pigment decreased and photobilirubin did not change significantly. In contrast with the changes in the solution without albumin, IX α -isomer increased and III α - and XIII α -isomers decreased.

Change in the interaction of bilirubin with albumin by irradiation in anaerobic conditions

For each sample obtained from the experiment in Fig. 1, albumin-bound and free bilirubin were

Table 2. Reaction rate data for the anaerobic reversion in the dark in various polar media

Temperature (°C)	pH	Half-life (min)	
		Photobilirubin-IX α	Unknown pigment
17	7.97	390	Infinite
	7.67	180	Infinite
	7.07	135	Infinite
37	7.67	40	660

Fig. 6. Changes in the interaction of bilirubin and its anaerobic photoproducts with albumin

Symbols: O, total bilirubin; ●, free bilirubin and its photoproducts.

determined by gel filtration. In these anaerobic conditions, bilirubin was not photo-oxidized during irradiation, but photoisomerized unbound bilirubin, which was bound less firmly to albumin as determined by gel filtration, increased in proportion to the concentrations of photoproducts, especially unknown pigment (Fig. 6). This agrees with the previous report (Pedersen *et al.*, 1977) and could have practical significance because measurements of free bilirubin in serum are being used increasingly in the detection of neonatal jaundice, to help in deciding whether further phototherapy or exchange transfusion is necessary. We conclude that phototherapy results in the formation of hydrophilic isomers of bilirubin, e.g. photobilirubin-IXa and unknown pigment, which are loosely bound to albumin and, at least partially, excreted without previous conjugation (McDonagh *et al.*, 1980).

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