The effect of bilirubin photoisomers on unbound-bilirubin concentrations estimated by the peroxidase method

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Unbound bilirubin is oxidized to nearly colourless substances in the presence of H_2O_2 or ethyl hydroperoxide and horseradish peroxidase. To predict the risk of kernicterus (degenerated yellow pigmentation of nerve cells), this principle has been widely utilized for estimating the concentration of unbound bilirubin in hyperbilirubinaemic serum. However, the serum contains polar geometric photoisomers of bilirubin. Therefore, to clarify the effect of bilirubin photoisomer concentrations on unbound-bilirubin concentration, the concentration of bilirubin and its photoisomer and of unbound bilirubin in samples obtained from experiments *in vivo* and *in vitro* were simultaneously and individually estimated by h.p.l.c. and the peroxidase method. During photoradiation, both *in vivo* and *in vitro*, the serum polar (*ZE*)-bilirubin IX α concentration increased remarkably, but unbound-bilirubin values were not affected at all. However, during experiments *in vitro*, unbound bilirubin concentrations increased only when concentrations of the rather polar (*EZ*)- and (*EE*)-cyclobilirubin IX α increased considerably in a human serum albumin–bilirubin solution irradiated with blue light. Thus it is concluded that unbound-bilirubin concentrations, and consequently the initial rate of the peroxidase reaction, is not accelerated by the increase in either (*ZE*)-bilirubin or (*EZ*)-cyclobilirubin concentration within the clinically observed range.

INTRODUCTION

In blood plasma, bilirubin is almost exclusively bound to one primary binding site of the albumin molecule, with small amounts bound to one or two secondary binding sites (Brodersen, 1979). Studies *in vitro* indicate that bilirubin is markedly less toxic when bound to serum albumin than when free in solution. Thus one of the important functions of serum albumin is considered to be the protection of the newborn from the neurotoxic effects of bilirubin (Odell, 1959; Diamond & Schmid, 1966; Takahashi *et al.*, 1984). In order to protect infants, particularly premature infants, with hyperbilirubinaemia from kernicterus, the estimation of unbound-bilirubin concentration in the serum of hyperbilirubinaemic infants has been undertaken. For such estimates the phenomenon that bilirubin is oxidized to nearly colourless substances in the presence of either H_2O_2 or ethyl hydroperoxide and horseradish peroxidase, and the observation that binding of the bilirubin to serum albumin inhibits the process, has widely been utilized in experimental laboratories (Jacobsen, 1969; Nakamura & Lee, 1977; Brodersen, 1979; Brodersen *et al.*, 1979).

Phototherapy is the most common treatment for neonatal hyperbilirubinaemia. Bilirubin undergoes a number of photochemical reactions *in vitro* and *in vivo*, all of which yield photoisomers that are more polar than the native molecule (Onishi *et al.*, 1986). Many previous studies (Onishi, 1973; Cashore *et al.*, 1975; Ogawa *et al.*, 1976; Ebbesen & Jacobsen, 1980; Ahlfors *et al.*, 1982; Berde *et al.*, 1984; Gartner *et al.*, 1985) have already shown that phototherapy does not lead to any apparent detrimental effect on albumin binding of bilirubin and, furthermore, the expected increase in albumin binding capacity for bilirubin and the expected decreases in

unbound bilirubin in response to decreases in serum bilirubin concentration occurred. However, it was usually observed that, for a few hours after the initiation of phototherapy, the total bilirubin concentration does not decrease but rather increases and that the geometric-photoisomer concentrations increase significantly. Because the polar bilirubin photoisomers are considered to bind less firmly to serum albumin, the fraction of the unbound photoisomers is theoretically expected to increase, and this was demonstrated by the Sephadex-gel-filtration method (Onishi *et al.*, 1980a). However, different opinions have been expressed with regard to the peroxidase reaction rate. (a) with the geometric photoisomers Brodersen (1974) and Pedersen *et al.* (1977) observed that the geometric photoisomers are oxidized rapidly, but Lamola *et al.* (1983) reported that no significant difference is found in the unbound-bilirubin concentration for irradiated and dark specimens. (b) With the structural photoisomers Meisel *et al.* (1982, 1983) observed that the initial reaction rate is accelerated. Furthermore, it has been demonstrated that geometric photoisomers, mainly (*ZE*)-bilirubin, are always present to some extent in the serum of a hyperbilirubinaemic newborn infant under usual nursery illumination, and that during phototherapy the concentration increases significantly, to as much as 15% of the total serum bilirubin concentrations (Onishi *et al.*, 1980b; McDonagh *et al.*, 1982a,b; Lamola *et al.*, 1983; Ennever *et al.*, 1983). The structural photoisomers (*EZ*)-cyclobilirubin (Onishi *et al.*, 1980b, 1984), which is the same as photobilirubin II (Stoll *et al.*, 1982), and lumirubin (McDonagh *et al.*, 1982a,b), are almost undetectable in the serum before phototherapy but appear in very small quantities after phototherapy and increase significantly in patients with bronze-baby syndrome (Onishi *et al.*,

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1982; Meisel *et al.*, 1982). At present it is unclear to what extent the presence of these photoisomers in serum samples obfuscates measurements of 'unbound' bilirubin (McDonagh & Lightner, 1985). Bilirubin photoisomers present in serum obtained from hyperbilirubinaemic infants undergoing phototherapy may therefore give misleading results in evaluating the kernicterus risk. Thus, the question whether the peroxidase technique is a reliable method for measuring serum unbound bilirubin concentrations of hyperbilirubinaemic babies subjected to phototherapy is an important one. For this reason, in experiments *in vivo*, photoisomer levels and the total and unbound bilirubin levels in the serum of hyperbilirubinaemic infants undergoing phototherapy were simultaneously estimated by h.p.l.c. and the peroxidase method respectively. In experiments *in vitro*, changes in both individual photoisomer concentrations, and the total and unbound bilirubin concentrations in the irradiated human serum albumin-bilirubin solution were also estimated by h.p.l.c. and the peroxidase method respectively.

MATERIALS AND METHODS

Patients and experiments *in vivo*

Eight hyperbilirubinaemic infants undergoing phototherapy were studied. Blood samples from infants were obtained by heel puncture at 0, 60 and 120 min after the initiation of phototherapy. Phototherapy lamps were off during blood collection. Informed consent of parents was obtained in all cases. Plasma samples were separated by centrifugation and analysed immediately both by h.p.l.c. (Itoh & Onishi, 1985) and by unbound bilirubin analyser (Nakamura *et al.*, 1981). Bilirubin is oxidized to nearly colourless substances in the presence of H_2O_2 and horseradish peroxidase (EC 1.11.1.7). Binding of the bilirubin to serum albumin inhibits the process (Brodersen & Bartels, 1969). This phenomenon has been utilized for estimating the concentration of unbound bilirubin in the serum of jaundiced newborn infants (Jacobsen & Fedders, 1970). The principle of the unbound-bilirubin analyser we used is the same as that behind the above mentioned method, but is modified to the automated peroxidase micromethod. All the manipulations of the plasma samples in the chemical analysis were performed under dim red light. Total serum bilirubin levels were estimated by the method described by White *et al.* (1958).

Photochemical experiments *in vitro*

Solutions of human serum albumin-bilirubin complex were prepared by adding 1.2 ml of a solution

consisting of 15 mg of crystalline bilirubin (Tokyo Kasei, Tokyo, Japan), dissolved in 6.0 ml of 0.05 M-NaOH to 30 ml of human serum albumin (Sigma) solution (2.0 g/100 ml), dissolved in turn in 0.1 M-sodium phosphate buffer, pH 7.4. Photoirradiation was carried out in stoppered 10 ml Pyrex tubes located 3 cm above the canopy of four fluorescent lamps (20W Matsushita blue-white fluorescent tubes). The solutions were deoxygenated with pure (99.9%) N_2 . The light intensity at the surface of the solution, determined with a Minolta-Air-Shields Fluoro-Lite Meter 451, was $27.3 \mu W \cdot cm^{-2} \cdot nm^{-1}$. Several 0.5 ml portions of the solutions in tubes were exposed to light. After different periods of light exposure, the Pyrex tubes were successively placed in the dark. Each portion was analysed both by h.p.l.c. for photoisomers (Itoh & Onishi, 1985) and for unbound bilirubin (Nakamura *et al.*, 1981). Sample preparation, chromatography, preparation of calibration curves of peak areas and the assignment of each peak separated by h.p.l.c. were carried out as described previously (Onishi *et al.*, 1980a,b).

Solution of (ZZ)-bilirubin or (EZ)- and (ZE)-bilirubin

In order to investigate the affinity of peroxidase enzyme for (ZZ)-bilirubin or the geometric photoisomers, solutions were prepared as follows. For the preparation of 100% (ZZ)-bilirubin solution, 6.0 mg of bilirubin, dissolved in 0.1 ml of 0.1 M-NaOH and 50 μl of 0.1 M-EDTA, were diluted with 100 ml of 0.1 M-sodium phosphate buffer, pH 7.4. For preparation of solutions of 61% (EZ)- and (ZE)-bilirubin and 39% (ZZ)-bilirubin estimated by h.p.l.c., 9 μg of the bile-pigment mixture and 1 μl of 2.0 M-EDTA were diluted to 150 μl with 0.1 M-sodium phosphate buffer, pH 7.4.

RESULTS AND DISCUSSION

Table 1 shows the changes in the concentrations (mean $\pm$ S.D.) of total serum bilirubin, unbound bilirubin and photoisomers within 2 h after the initiation of phototherapy. As shown in Fig. 1, the most striking aspect of this study is that, unexpectedly, the acceleration of the peroxidase reaction rate of serum bile pigments, i.e. the increment of unbound-bilirubin concentration, is not observed in spite of the significant increase in (ZE)-bilirubin concentrations, namely 40% of the initial values. There have been many previous reports about the effect of phototherapy on the unbound-bilirubin levels or albumin-binding capacity, but in all cases the researchers, who reported either a decrease of the unbound bilirubin concentration or no change in binding capacity, made

Table 1. Changes in the concentrations of total serum bilirubin, unbound bilirubin and photoisomers in eight hyperbilirubinaemic infants before and during phototherapy

See the text for details of the analytical conditions. Abbreviations used: $[B_T]$, total bilirubin concentration; $[UB]$, unbound-bilirubin concentration; (EZ)-C, (EZ)-cyclobilirubin IX α ; (ZE)-BR, (ZE)-bilirubin IX α ; (EZ)-BR, (EZ)-bilirubin IX α ; (ZZ)-BR, (ZZ)-bilirubin IX α . Results are means $\pm$ S.D. ($n = 8$).

Duration (min)	$[B_T]$ (mg/dl)	$[UB]$ (μg /dl)	(EZ)-C (%)	(ZE)-BR (%)	(EZ)-BR (%)	(ZZ)-BR (%)
0	17.8 $\pm$ 2.7	0.41 $\pm$ 0.12	0.2 $\pm$ 0.5	9.0 $\pm$ 3.5	0.6 $\pm$ 0.4	89.5 $\pm$ 4.7
60	17.5 $\pm$ 2.9	0.39 $\pm$ 0.13	0.7 $\pm$ 0.6	12.5 $\pm$ 2.6	0.9 $\pm$ 0.3	85.0 $\pm$ 3.7
120	17.4 $\pm$ 2.4	0.37 $\pm$ 0.12	0.8 $\pm$ 0.6	13.9 $\pm$ 2.3	1.0 $\pm$ 0.3	83.6 $\pm$ 3.3

Fig. 1. Relationship between serum unbound bilirubin concentrations and the changes in percentage of (ZE)-bilirubin in the total serum bile-pigment concentrations at the initial stage when serum total bilirubin concentration shows no change

The mean $\pm$ S.D. of nine measurements is plotted. ●, Unbound bilirubin; ◆, (ZE)-bilirubin.

their measurements only after a significant decrease in the total bilirubin concentration (Onishi, 1973; Cashore *et al.*, 1975; Ogawa *et al.*, 1976; Ebbesen & Jacobsen, 1980; Ahlfors *et al.*, 1982; Berde *et al.*, 1984; Gartner *et al.*, 1985). In the present study the total serum bilirubin concentrations did not change significantly, but the geometric-photoisomer levels increased significantly. It is concluded that the increase in (ZE)-bilirubin IX α is not related to the initial rate of the peroxidase reaction. This may be because the oxidation rate, i.e. the affinity of peroxidase enzymes for the substrate (EZ)-/(ZE)-bilirubin, is much smaller than that for (ZZ)-bilirubin.

The concentrations of the bilirubin photoisomers and unbound bilirubin in each sample subjected to photoirradiation *in vitro* were measured by both the h.p.l.c. and peroxidase methods. Table 2 shows the changes in the concentrations of serum total bilirubin, unbound bilirubin and photoisomers during short photoirradiation. As shown in Fig. 2, when short photoirradiation for

Fig. 2. Unbound-bilirubin concentrations and the changes in percentage of (EZ)-bilirubin in the total serum bile-pigment concentrations in human serum albumin-bilirubin solution before and during short photoirradiation

The mean $\pm$ S.D. of five measurements is plotted. ●, Unbound bilirubin; ◆, (ZE)-bilirubin.

60 s was applied, (ZE)-bilirubin increased abruptly to as much as 19.8 ± 0.9 (mean $\pm$ S.D.) of the serum total bile pigments, and the acceleration of the peroxidase reaction rate of serum bile pigments was not observed, in spite of the significant increase in (ZE)-bilirubin concentrations. Table 3 shows the changes in the concentrations of the serum total bilirubin, unbound bilirubin and photoisomers during photoirradiation for 10 min. (EZ)- and (EE)-Cyclobilirubin appeared and increased significantly after 10 min of irradiation. However, (EZ)-bilirubin remained almost negligible (under 0.63% of the serum total bilirubin) during the period of light exposure (McDonagh *et al.*, 1982a). As shown in Fig. 3, the peroxidase reaction rate did not increase at all and was not related to either (ZE)-bilirubin or (EZ)-cyclobilirubin concentrations within the clinically observed range; it accelerated only when (EZ)- and (EE)-cyclobilirubin increased significantly, although it could not be ruled out that other factors (Brodersen *et al.*, 1979) might also have participated in the peroxidase reaction, as shown in Table 3.

Table 2. Changes in the concentrations of total serum bilirubin, unbound and photoisomers in human serum albumin-bilirubin solution before and during short photoirradiation *in vitro*

Abbreviation used: (EE)-C, (EE)-cyclobilirubin IX α . For definition of the other abbreviations, see the legend to Table 1. Results are means $\pm$ S.D. ($n = 5$).

Duration (s)	[B _T] (mg/dl)	[U _B] (µg/dl)	(EE)-C (%)	(EZ)-C (%)	(ZE)-BR (%)	(EZ)-BR (%)	(ZZ)-BR (%)
0	9.16 $\pm$ 0.15	0.61 $\pm$ 0.04	0	0	0	0	100 $\pm$ 0
15	9.16 $\pm$ 0.13	0.52 $\pm$ 0.08	0	0	17.4 $\pm$ 0.7	0.9 $\pm$ 0.2	81.7 $\pm$ 1.5
30	9.20 $\pm$ 0.25	0.55 $\pm$ 0.10	0.2 $\pm$ 0.2	0	19.8 $\pm$ 0.9	1.1 $\pm$ 0.1	79.2 $\pm$ 1.7
60	9.18 $\pm$ 0.08	0.57 $\pm$ 0.11	0.14 $\pm$ 0.2	0.56 $\pm$ 0.24	19.1 $\pm$ 1.4	1.2 $\pm$ 0.2	79.0 $\pm$ 2.6

Table 3. Changes in the concentrations of total serum bilirubin, unbound and photoisomers in human serum albumin–bilirubin solution before and during prolonged photoirradiation *in vitro*

For definition of abbreviations, see the legend to Table 1. Results are means $\pm$ S.D. ($n = 5$).

Duration (min)	[B _T] (mg/dl)	[UB] (μ g/dl)	(EE)-C (%)	(EZ)-C (%)	(ZE)-BR (%)	(EZ)-BR (%)	(ZZ)-BR (%)
0	9.16 $\pm$ 0.15	0.61 $\pm$ 0.04	0	0	0	0	100
5	9.08 $\pm$ 0.08	0.53 $\pm$ 0.13	1.2 $\pm$ 0.2	4.2 $\pm$ 0.3	18.8 $\pm$ 0.7	1.2 $\pm$ 0.1	73.8 $\pm$ 2.9
10	8.84 $\pm$ 0.17	0.70 $\pm$ 0.18	2.4 $\pm$ 0.4	9.2 $\pm$ 0.6	17.0 $\pm$ 0.8	1.0 $\pm$ 0.2	69.0 $\pm$ 3.1
15	8.24 $\pm$ 0.27	0.71 $\pm$ 0.14	3.6 $\pm$ 0.5	13.4 $\pm$ 0.5	16.1 $\pm$ 0.7	1.3 $\pm$ 0.2	64.2 $\pm$ 3.2
20	8.06 $\pm$ 0.09	1.31 $\pm$ 0.18	4.8 $\pm$ 0.1	18.3 $\pm$ 0.9	14.6 $\pm$ 1.0	0.5 $\pm$ 0.5	60.6 $\pm$ 3.6

Thus this evidence is not consistent with the fact that (ZE)-bilirubin binds to human serum albumin at the primary bilirubin-binding site with an affinity 2–3 times lower than that of (ZZ)-bilirubin (in the order of 10^7M^{-1}) estimated by c.d. spectroscopy (Lamola *et al.*, 1983) and therefore more (ZE)-bilirubin is dissociated compared with (ZZ)-bilirubin. In spite of this, the initial rate of the peroxidase reaction is not accelerated by the increase in either (ZE)-bilirubin or (EZ)-cyclobilirubin concentration within the clinically observed range.

In order to clarify the cause, a kinetic study of oxidations of (ZZ)-bilirubin or (EZ)- and (ZE)-bilirubin in the albumin-free solution (6.0 mg/100 ml) using the same concentrations of the enzymes and other substrates, i.e. under the same conditions, was carried out with the peroxidase method. The time course of the oxidation reaction of (ZZ)-bilirubin or the geometric photoisomers

is shown in Fig. 4. The A_{460} was recorded for a period of 2 min before addition of the reaction mixture and it remained constant, indicating the stability of the system. On addition of the reaction mixture, the absorbance initially decreased owing to dilution, and then proceeded to decline. When the (ZZ)-bilirubin was oxidized, the decline in A_{455} was steeper than for 61% (EZ)-/(ZE)-bilirubin and 39% (ZZ)-bilirubin as shown in Fig. 4. It was concluded that, because the reaction is performed under the same conditions except for the substrates, this difference may be because the oxidation rate, i.e. the affinity of peroxidase enzymes for the substrates (EZ)-/(ZE)-bilirubin, is much smaller than for (ZZ)-bilirubin.

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Fig. 3. Unbound bilirubin concentrations and the changes in the percentage of (EZ)- and (EE)-cyclobilirubin in the total serum bile-pigment concentrations in human serum albumin–bilirubin solution before and during prolonged photoirradiation *in vitro*

Because of overlapping of S.D., the lower bar of unbound bilirubin concentration at 5 min is omitted. ●, Unbound bilirubin; ▲, (EZ)-cyclobilirubin; ▼, (EE)-cyclobilirubin.

Fig. 4. Peroxidase method

The ordinate indicates the decrease in A_{455} . —, Time course of the maximal absorbance of albumin-free (ZZ)-bilirubin solution before and after addition of the reaction mixture at zero time. ---, That of albumin-free solution of 61% (EZ)-/(ZE)-bilirubin and 39% (ZZ)-bilirubin. For details, see the text.

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