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Wavelength-dependence of the relative rate constants for the main geometric and structural photoisomerization of bilirubin IXα bound to human serum albumin

Demonstration of green light at 510 nm as the most effective wavelength in photochemical changes from (*ZZ*)-bilirubin IXα to (*EZ*)-cyclobilirubin IXα via (*EZ*)-bilirubin

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The kinetics for the quantitatively important reaction:

$$(EZ)\text{-Cyclobilirubin} \overset{k_5}{\leftarrow} (EZ)\text{-bilirubin} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} (ZZ)\text{-bilirubin} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ZE)\text{-bilirubin},$$

that is, the photochemical interconversion between bilirubin and its geometric and structural photoisomers bound to human serum albumin in aqueous solution when various wavelengths of monochromatic light were used, were assayed by h.p.l.c. In order to clarify the wavelength-dependence of the relative rate constants in the individual steps, a light-source with a half-bandwidth of 10 nm was used at increments of 20 nm, in the range from 410 nm to 550 nm. We describe for the first time studies on the wavelength-dependence of rate constants in geometric and structural photoisomerization reactions *in vitro* of (*ZZ*)-bilirubin or (*EZ*)-bilirubin bound to human serum albumin, especially the relative rate constants of cyclization of (*EZ*)-bilirubin into (*EZ*)-cyclobilirubin. Because studies *in vitro* have demonstrated that the wavelengths from 350 to 450 nm are mutagenic, the results obtained indicated that the safest and ideal light-source for phototherapy is green light of 510 nm, which keeps (*ZE*)-bilirubin concentrations as low as possible, as shown by a maximal value of k_2 at 510 nm and a relatively low value of k_1 at 510 nm. This light-source still ensures the substantial absorption of (*ZZ*)-bilirubin, which is the precursor of (*EZ*)-bilirubin, the intermediate in (*EZ*)-cyclobilirubin formation and, furthermore, as shown by the maximal value of k_5 and a considerable value of k_4 at 510 nm, promotes the cyclization of (*EZ*)-bilirubin derived from (*ZZ*)-bilirubin even though k_3 at 510 nm also shows a peak value.

INTRODUCTION

Bilirubin photochemistry has attracted attention in the light of its relevance to the widely employed phototherapy for neonatal unconjugated hyperbilirubinaemia (Cohen & Ostrow, 1980; Brown & McDonagh, 1980; McDonagh & Lightner, 1985). However, there is no general agreement as to the most desirable type of fluorescent lamp to be used in the treatment of neonatal jaundice by phototherapy. In a clinical comparison of green and white lights, a greater efficiency of the former over the latter was observed (Vecchi *et al.*, 1982). Moreover, studies *in vitro* demonstrating that wavelengths from 350 to 450 nm are mutagenic for prokaryotic and eukaryotic cells, and therefore potentially carcinogenic and teratogenic, have caused some concern with regard to the long-term safety of phototherapy (Speck & Rosenkranz, 1975; Parshad *et al.*, 1978, 1981; Bradley *et al.*, 1978; Burki & Lam, 1978; Sideris *et al.*, 1981). This concern has led to a recommendation that green light be used (Vecchi *et al.*, 1982; Pratesi *et al.*, 1985). At present, the precise relative contribution of geometric and structural photoisomers to the net effect of phototherapy is unknown, but in the Gunn rat the geometric photoisomers (Onishi *et al.*, 1984*b*) and in humans the structural photoisomers are the main contributors (Onishi *et al.*, 1980*b*, 1984*a*). These findings are seen as showing that the bilirubin photochemistry observed *in vivo* appears to resemble closely that observed *in vitro* (Itoh & Onishi, 1985; Onishi *et al.*, 1985). Since the photochemical and thermal interrelationship between bilirubin and its geometric and structural photoisomers (Scheme 1) as well as some of their biochemical characteristics (Table 1) have been established (Onishi *et al.*, 1981*b*, 1984*a*, 1985; Itoh & Onishi, 1985), we now describe for the first time studies on the wavelength-dependence of rate constants for the quantitatively important reaction:

$$(EZ)\text{-Cyclobilirubin} \overset{k_5}{\leftarrow} (EZ)\text{-bilirubin} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} (ZZ)\text{-bilirubin} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ZE)\text{-bilirubin}$$

that is, the geometric and structural photoisomerization reaction *in vitro* of (*ZZ*)- or (*EZ*)-bilirubin bound to human serum albumin, especially the relative rate

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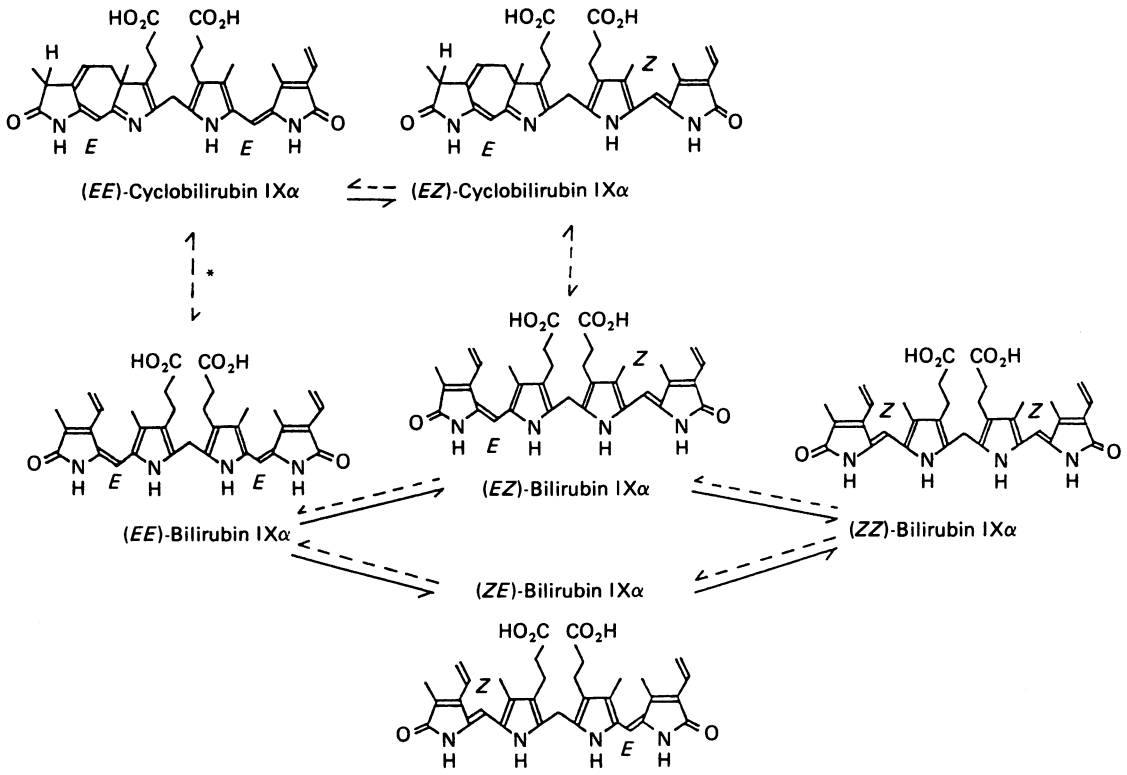

Scheme 1. Geometric and structural isomerization of (*ZZ*)-bilirubin IXα (from Onishi *et al.*, 1981*b*, 1985)

——, Photochemical and thermal conversion; ----, photochemical conversion only; *, theoretical pathway, but not confirmed. The steric configurations of the hydrogen atom and the methyl group at C-2 and the methyl group at C-7 of (*EZ*)- and (*EE*)-cyclobilirubins are not shown.

constant of cyclization of (*EZ*)-bilirubin into (*EZ*)-cyclobilirubin while bound to human serum albumin.

MATERIALS AND METHODS

Preparations for photochemical experiments

Aqueous solutions of (*ZZ*)-bilirubin and human serum albumin. Solutions of human serum albumin and bilirubin (43.5 μg/ml) were prepared as follows. Crystalline bilirubin (10 mg) was dissolved in 10.0 ml of 0.05 M-NaOH, and 50 μl of the solution was added to 1.0 ml of human serum albumin solution (20mg/ml) in 0.1 M-sodium phosphate buffer, pH 7.4. The mixture was further diluted with 100 μl of the same buffer. The solution was used to study the photochemical conversions of (*ZZ*)-bilirubin into (*ZE*)-bilirubin (k_1 and k_2) (eqn. 1) and/or (*ZZ*)-bilirubin into (*EZ*)-bilirubin and (*EZ*)-cyclobilirubin (*K*) (eqn. 2).

Bilirubin (Tokyo Kasei, Tokyo, Japan) was used without further purification. All other reagents used were of analytical grade.

Aqueous solutions of human serum albumin enriched with (*EZ*)- and (*ZE*)-bilirubin. Preparations highly enriched in (*EZ*)- and (*ZE*)-bilirubins (approx. 50 μg/ml), the latter not being eliminated by the techniques used, were obtained by solvent extraction of crude photolysis products. A trace of EDTA was added to a saturated bilirubin solution in distilled chloroform. The solution was deoxygenated with pure (99.99%) N_2 for 3 min. Under anaerobic conditions, four 2.0 ml portions of the solution, in stoppered 10 ml Pyrex glass tubes located 3 cm above the bank of four fluorescent tubes, were exposed to light for 10 min. The residues obtained after evaporation of the solvents during centrifugation *in vacuo* at room temperature were mixed with methanol. The supernatant obtained by the procedure described previously (Itoh & Onishi, 1985) was used for the photochemical conversion of (*EZ*)-bilirubin into (*ZZ*)-bilirubin (k_3) or (*EZ*)-cyclobilirubin (k_5) (eqn. 3).

Photochemical experiments

Except where noted otherwise, in all experiments the solutions were deoxygenated for 3 min with pure (99.99%) N_2. Several portions of the solution were irradiated in 10 mm-pathlength quartz cuvettes placed in the cell chamber of a Shimadzu spectrophotofluorimeter. While in the cell chamber, the sample was irradiated with a xenon arc lamp, light from which was made monochromatic by the spectrophotofluorimeter, at 20 nm bandwidth increments, in the range from 410 up to 550 nm. Each sample was analysed by h.p.l.c. Figs. 1(*a*) and 1(*b*) are shown as the representative chromatograms. Light-intensities were measured before and after irradiation at the surface of the cell with a Minolta T 1M illuminance meter without V filter, and the average was taken because the lamp did not have a constant emission (quanta/s) over the range used. Relative irradiance at the different wavelengths varied between 1431 and 2921. Except for photochemical experiments, all manipulations were carried out in the dark or under photographic safe-light and at room temperature. As described in the Results and

Table 1. Some biochemical characteristics of bilirubin IXα and its photoisomers in jaundice phototherapy

Abbreviations: a.d.s., absorption difference spectroscopy; e.c.i., electron chemical ionization; HSA, human serum albumin; +, demonstrated; −, not demonstrated; N.D., not determined.

	Classification and nomenclature				
	(*EE*)-Cyclobilirubin IXα A and B	(*EZ*)-Cyclobilirubin IXα A and B	(*EE*)-Bilirubin IXα	(*EZ*)- and (*ZE*)-Bilirubin IXα	(*ZZ*)-Bilirubin IXα
Separation of photoisomers					
H.p.l.c. *in vitro* (Onishi *et al.*, 1979, 1980, 1981)	Peaks 1A and 1B	Peaks 2A and 2B	Peak 0	Peaks 3A and 3B	Peak 5
H.p.l.c. *in vivo* (Onishi *et al.*, 1979, 1980, 1981)	Peak 1	Unknown pigment	Peak 0	Photobilirubin IXα	Bilirubin
T.l.c. *in vitro* (Stoll *et al.*, 1979, 1982; Bonnett *et al.*, 1984)	Photobilirubin III	Photobilirubins IIA and IIB		Photobilirubins IA and IB	Bilirubin
A.d.s. *in vitro* and *in vivo* (McDonagh *et al.*, 1979)			Photobilirubin	Photobilirubin	Bilirubin
H.p.l.c. *in vitro* and *in vivo* (McDonagh *et al.*, 1982*a*,*b*)	(*E*)-Lumirubin and (*E*)-isolumirubin	(*Z*)-Lumirubin and (*Z*)-isolumirubin	(4*E*,15*E*)-Bilirubin	(4*E*,15*Z*)- and (4*Z*,15*E*)-Bilirubin	Bilirubin
No. of components*	4	4	1	2	1
Configuration	Δ^4-*E*, Δ^{15}-*E*	Δ^4-*E*, Δ^{15}-*Z*	Δ^4-*E*, Δ^{15}-*E*	Δ^4-*E*, Δ^{15}-*Z*/Δ^4-*Z*, Δ^{15}-*E*	Δ^4-*Z*, Δ^{15}-*Z*
Molecular ion in mass spectrum by e.c.i.	N.D.	N.D.	585	585	585
$\lambda_{max.}$ (nm)					
In h.p.l.c. eluent	443	435	423	425	447
In chloroform	443	440	N.D.	446	451
Fluorescence in albumin-free aqueous solution	Positive	Positive ($\lambda_{exc.}$ 400 nm, $\lambda_{em.}$ 620 nm)	N.D.	Negative	Negative
Diazo reactivity in chloroform solution	Negative	Negative	N.D.	Positive (direct)	Positive (indirect)
Solubility	Water-soluble				Lipid-soluble
Stability					
In HSA solution	N.D.	Unstable	N.D.	Stable	Stable
In the bile	N.D.	Unstable	N.D.	Unstable	Stable
Affinity for HSA	Low				High
Excretory pathway of photoisomers during jaundice phototherapy	Liver and kidney	Liver and kidney	Kidney	Liver and kidney	—
Photoisomers in biological fluids during neonatal jaundice phototherapy					
In the serum	−	+	−	++	+++
In the bile	+	++	−	+	++
In the urine	+	+	+	+	+

* The enantiomeric conformers are not taken into account.

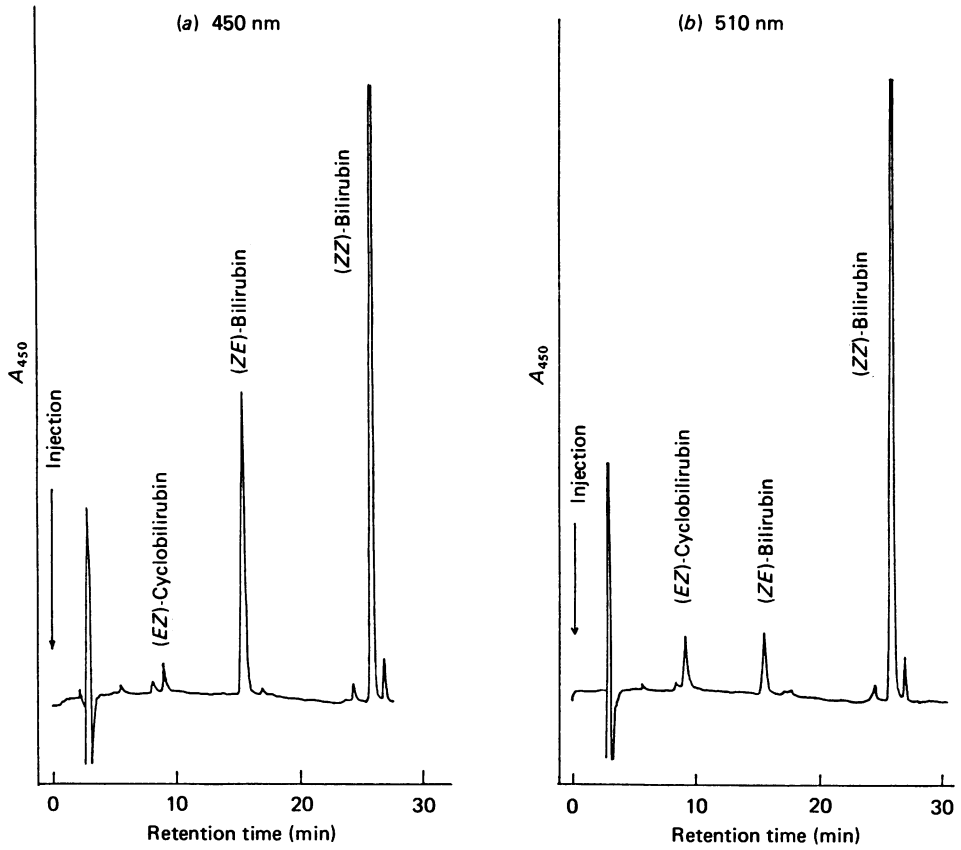

Fig. 1. Representative h.p.l.c. scan of aqueous solution of (*ZZ*)-bilirubin bound to human serum albumin obtained at various intervals during anaerobic irradiation by light of various wavelengths

Experimental details are given in the text. (*a*) and (*b*) show the chromatographic scans after a 10 min exposure to light of respectively 450 nm (blue) and 510 nm (green), i.e. the effects of narrow-band blue and green light on (*ZZ*)-bilirubin bound to human serum albumin.

discussion section, rate constants obtained were corrected with the same incident radiation energy per square centimetre as that of a wavelength function with 20 nm bandwidth in the region from 410 to 550 nm.

Equipment, preparation of the calibration curves, peak assignments except for (*EZ*)- and (*ZE*)-bilirubin IXα, and pigments and reagents were described previously (Onishi *et al.*, 1979, 1980*a*; Isobe & Onishi, 1981). Chromatographic operation was also as described previously (Itoh & Onishi, 1985). Peak assignments of (*EZ*)- and (*ZE*)-bilirubin IXα were made in accordance with the method reported by McDonagh *et al* (1982*a*,*b*).

Mathematical analysis (eqns. 1, 2 and 3)

In the following *A*, *B*, *C* and *D* are percentages of A, B, C and D respectively.

Kinetic study of (*ZZ*)-bilirubin–serum albumin complexes in solution.

Transformation of (*ZZ*)-bilirubin into (*ZE*)-bilirubin (eqn. 1). Kinetics for eqn. (1) are as follows:

$$\underset{(ZZ)\text{-Bilirubin}}{(B)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(ZE)\text{-bilirubin}}{(D)} \quad (1)$$

$$\frac{dB}{dt} = k_2 D - k_1 B$$

$$\frac{dD}{dt} = k_1 B - k_2 D$$

Transformation of (*ZZ*)-bilirubin into (*EZ*)-cyclobilirubin (eqn. 2). Kinetics for eqn. (2) are as follows:

$$\underset{(EZ)\text{-Cyclobilirubin}}{(C)} \overset{K}{\leftarrow} \underset{[(EZ)\text{-bilirubin}]}{(A)} \overset{K}{\leftarrow} \underset{(ZZ)\text{-bilirubin}}{(B)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(ZE)\text{-bilirubin}}{(D)} \quad (2)$$

$$\frac{dB}{dt} = k_2 D - (k_1 + K)B$$

$$\frac{dD}{dt} = k_1 B - k_2 D$$

$$K \ll K_5$$

Therefore:

$$K \fallingdotseq K_4$$

Kinetic study of photochemical experiments of (*EZ*)- and (*ZE*)-bilirubin-enriched human serum albumin in solution (eqn. 3). Kinetics for eqn. (3) are as follows:

$$\underset{(EZ)\text{-Cylobilirubin}}{(C)} \overset{k_5}{\leftarrow} \underset{(EZ)\text{-bilirubin}}{(A)} \overset{k_3}{\rightarrow} \underset{(ZZ)\text{-bilirubin}}{(B)} \quad (3)$$

$$\frac{dA}{dt} = -(k_3 + k_5)A$$

$$\frac{dC}{dt} = k_5 A$$

The relative rate constants (k_1, k_2, k_3, k_4, k_5 and K) for the above photochemical reactions were estimated as described previously (Itoh & Onishi, 1985; Onishi *et al.*, 1985).

RESULTS AND DISCUSSION

Reversible geometric photoisomerization of (*ZZ*)-bilirubin to (*ZE*)-bilirubin

Photoirradiation with light of each wavelength was applied to samples for short times, i.e. 0, 2.5, 5, 7.5 and 10 min, and the relative rate constants were estimated by the methods described previously (Itoh & Onishi, 1985; Onishi *et al.*, 1985)

Rate constant k_1 for geometric photoisomerization from (*ZZ*)-bilirubin to (*ZE*)-bilirubin. The rate constant k_1 for geometric photoisomerization from (*ZZ*)-bilirubin to (*ZE*)-bilirubin showed the highest value at 410 nm within the region investigated (410–550 nm) and gradually decreased with a slight shoulder peak at 490 nm as shown in Fig. 2. This is in agreement with the measurements reported by Ennever *et al.* (1984) on isomer composition obtained by irradiating bilirubin to photoequilibrium with 10 nm-bandwidth light.

Rate constant k_2 for photochemical reversion from (*ZE*)-bilirubin to (*ZZ*)-bilirubin. The most effective wavelength, as evaluated by the rate constants for photochemical reversion from (*ZE*)-bilirubin to (*ZZ*)-bilirubin, occurred at a wavelength of 510 nm. As shown in Fig. 2, the rate constants steeply decreased as the irradiating wavelengths were either decreased from 510 to 470 nm or increased from 510 to 530 nm. Light of wavelengths over 530 nm was ineffective in the photochemical reversion. The relative rate constants increased slightly as the irradiating wavelength was decreased from 470 to 410 nm.

Photobilirubin has been observed to revert to the original bilirubin at 510 nm irradiation (Lightner *et al.*, 1979). Donzelli *et al.* (1984) also reported that the greatest reversion efficiency is associated with the 514 nm line, followed by the 501 and 488 nm lines.

Transformation of (*EZ*)-bilirubin into (*EZ*)-cyclobilirubin or into (*ZZ*)-bilirubin while bound to human serum albumin

When photoirradiation at each light wavelength is applied for short times, i.e. 0, 2.5, 5, 10 and 20 min, the peaks of (*EZ*)- and (*ZE*)-bilirubin decreased single-exponentially, at least up to 20 min, with a half-life from 11.2 to 78 min, though the disappearance curves on the two wavelengths, i.e. 510 and 530 nm, were not linear. At the same time, peaks of (*ZZ*)-bilirubin and (*EZ*)-cyclobilirubin derived from (*EZ*)- and (*ZE*)-bilirubin appeared and gradually increased in parallel. Figs. 3(*a*) and 3(*b*) show the representative results obtained at 450 nm and 510 nm respectively. The relative rate constants were estimated by the methods described previously (Itoh & Onishi, 1985; Onishi *et al.*, 1985). The reactions are divided into two types, i.e. transformation

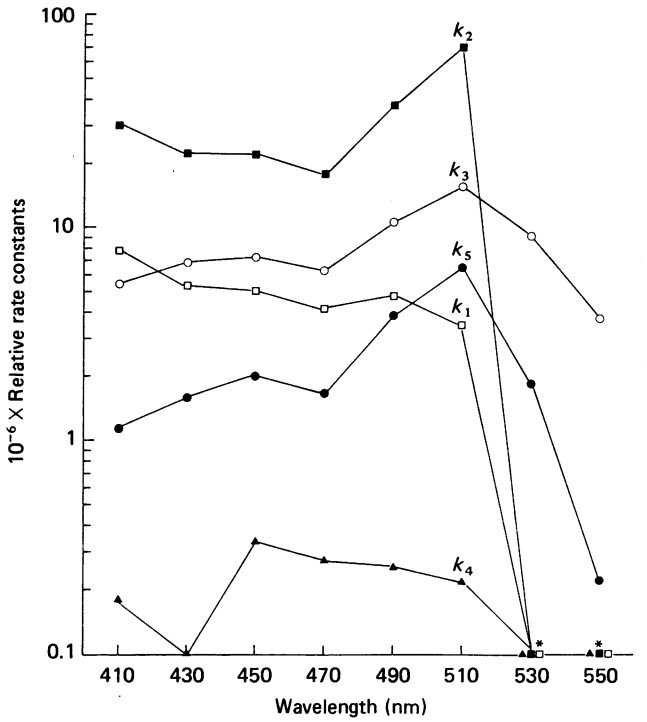

Fig. 2. Semi-logarithmic plotting of the individual relative rate constants corrected for differences in light-intensity as a function of irradiation wavelength

The light-source used for the photochemical reaction had a half-bandwidth of 10 nm at increments of 20 nm, in the range from 410 nm to 550 nm. Values of k_1, k_2 and k_4 at wavelengths of 530 and 550 nm were plotted as 0.1 instead of zero because of the logarithmic scale. Symbols: □, k_1; ■, k_2; ○, k_3; ▲, k_4; ●, k_5.

of (*EZ*)-bilirubin into either (*EZ*)-cyclobilirubin (eqn. 4) or (*ZZ*)-bilirubin (eqn. 5):

$$\underset{\text{(A)}}{(EZ)\text{-Bilirubin}} + h\nu \xrightarrow{k_5} \underset{\text{(C)}}{(EZ)\text{-cyclobilirubin}} \quad (4)$$

$$\underset{\text{(A)}}{(EZ)\text{-Bilirubin}} + h\nu \xrightarrow{k_3} \underset{\text{(B)}}{(ZZ)\text{-bilirubin}} \quad (5)$$

The most effective wavelength, as evaluated by the relative rate constants k_5 in the photochemical intramolecular cyclization reaction from (*EZ*)-bilirubin to (*EZ*)-cyclobilirubin (eqn. 4) or k_3 in the photochemical reversion reaction from (*EZ*)-bilirubin to (*ZZ*)-bilirubin (eqn. 5), was at 510 nm (Fig. 2). The relative rate constants gradually decreased as the irradiating wavelength was either decreased from 510 nm to 410 nm or increased from 510 nm to 550 nm. Light of wavelength above 550 nm was ineffective in the structural photoisomerization of bilirubin. However, the rate constant k_4 for geometric photoisomerization from (*ZZ*)-bilirubin to (*EZ*)-bilirubin at wavelength 450 nm was greater than that at 510 nm.

It was initially thought that the mechanism responsible for lowering of bilirubin concentration in the body is exclusively configurational *Z–E* photoisomerization (McDonagh, 1981; Lamola *et al.*, 1982). However, (*EZ*)-cyclobilirubin is now thought to be quantitatively the most important water-soluble photoproduct respon-

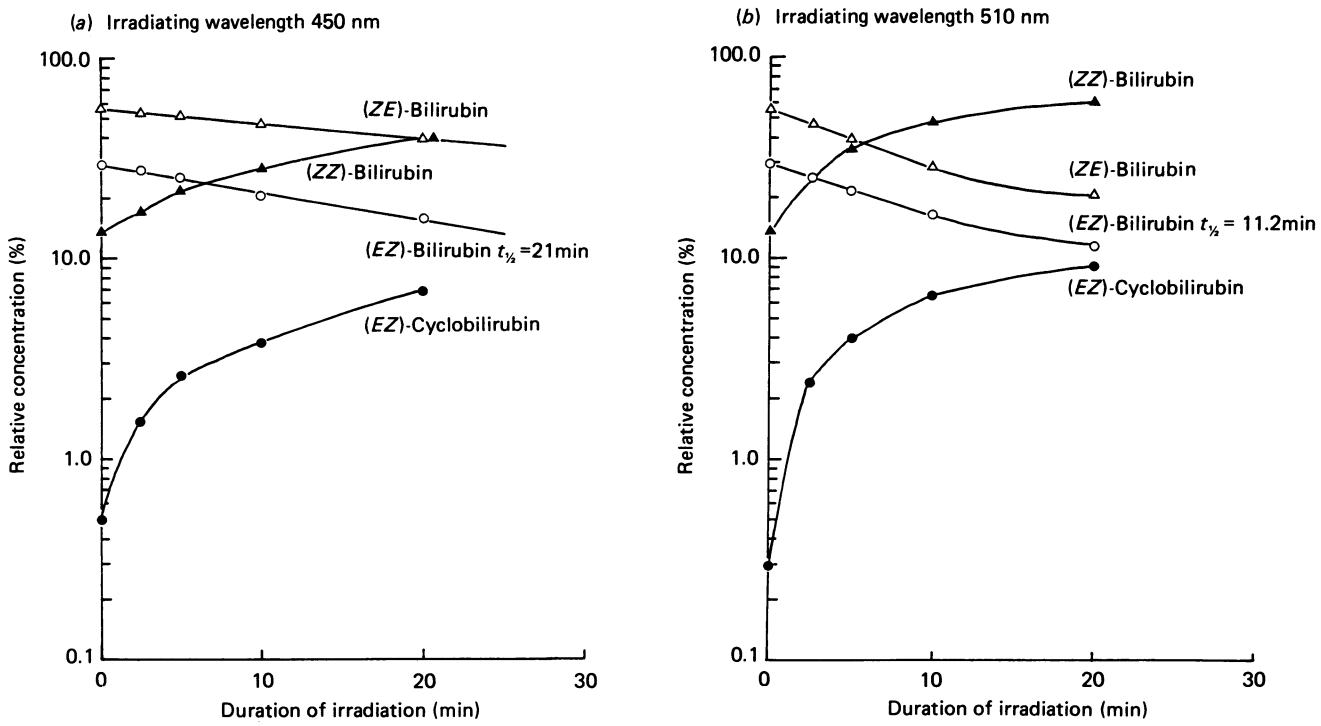

Fig. 3. Plotting of the photochemical transformation by light wavelengths of (*a*) 450 nm and (*b*) 510 nm as representative examples of (*EZ*)-bilirubin to (*EZ*)-cyclobilirubin and (*ZZ*)-bilirubin while bound to human serum albumin

Experimental details are given in the text. Time is plotted on a semi-logarithmic scale. Symbols: ○, (*EZ*)-bilirubin; ●, (*EZ*)-cyclobilirubin; ▲, (*ZZ*)-bilirubin; △, (*ZE*)-bilirubin.

sible for the rapid biliary and urinary clearance of bilirubin during phototherapy (Onishi *et al.*, 1980*b*, 1981*a*; Castarino *et al.*, 1985; McDonagh & Lightner, 1985). (*EZ*)-Cyclobilirubin, as photoreversible structural isomers of bilirubin (Isobe & Onishi, 1981), though many authors have described it as 'non-reversible' (Castarino *et al.*, 1985; Knox *et al.*, 1985; Pratesi *et al.*, 1985), attains much lower serum concentrations, but is more rapidly excreted. Thus structural isomerization, i.e. intramolecular enovinyl group cyclization with C-7 of the adjacent pyrrole ring established by Onishi *et al.* (1984*a*), is the most important process in jaundiced infants undergoing phototherapy. Since (*EZ*)-cyclobilirubin originates from (*ZZ*)-bilirubin via (*EZ*)-bilirubin as an intermediate (Itoh & Onishi, 1985), the rapid formation of (*ZE*)-bilirubin is counter-productive because it accumulates to as much as 20% of the total serum bilirubin concentration during phototherapy with a blue–white lamp (Onishi *et al.*, 1980*b*; Lamola *et al.*, 1981) and thus considerably decreases the number of (*ZZ*)-bilirubin molecules.

Visible-light phototherapy is the most common method for treating neonatal hyperbilirubinaemia. It is estimated that 2–6% of all infants born in the U.S.A. and U.K. receive phototherapy (Brown & McDonagh, 1980; Lewis *et al.*, 1982). However, recent studies *in vitro* showing that light of wavelengths between 350 and 450 nm is mutagenic for prokaryotic and eukaryotic cells, and therefore potentially carcinogenic, have caused concern because all commonly-used phototherapy lamps emit radiation in this region. Furthermore, a greater efficacy of green lamps compared with white fluorescent lamps has been reported (Vecchi *et al.*, 1982; Pratesi, 1983; Pratesi *et al.*, 1985). As a possible cause for this Donzelli *et al.* (1984) suggest that the green light penetrates deeper into the skin, and the best spectral range for efficient photodegradation of bilirubin may be the result of a balance between bilirubin absorption and skin penetration. The present study provides the first comparison of the efficacy of phototherapy-light wavelengths in the biologically highly important structural photoisomerization reactions of bilirubin *in vivo*, and accounts for the surprising clinical success obtained with green fluorescent lamps (Vecchi *et al.*, 1983). A short-wavelength source to excite (*ZZ*)-bilirubin to (*EZ*)- and (*ZE*)-bilirubin, although the formation of the latter is counterproductive, and long-wavelength light to promote both the transformation of (*EZ*)-bilirubin into (*EZ*)-cyclobilirubin and the reversion of (*ZE*)-bilirubin to (*ZZ*)-bilirubin are demonstrated. The data above prove that the safest and ideal light source for phototherapy is green light of wavelength 510 nm, which keeps (*ZE*)-bilirubin concentrations as low as possible at the site of formation, as shown in the maximal value of k_2 at 510 nm and relatively low value of k_1 at 510 nm, while still ensuring the substantial absorption of (*ZZ*)-bilirubin molecules, which are the precursors of (*EZ*)-bilirubin, the intermediary of (*EZ*)-cyclobilirubin. Furthermore, as shown in the maximal value of k_5 at 510 nm, green light of wavelength 510 nm promotes the cyclization of (*EZ*)-bilirubin derived from (*ZZ*)-bilirubin even though k_3 at 510 nm also shows a peak value.

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