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Bilirubin photoconversion induced by monochromatic laser radiation

Comparison between aerobic and anaerobic experiments *in vitro*

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Structural and geometric photoisomerization of bilirubin bound to human serum albumin was investigated. Solutions were irradiated with monochromatic light emitted by an Ar ion laser, the 457.9, 488.0 and 514.5 nm wavelengths being selected. Photoproducts were separated and analysed by h.p.l.c. Visible-absorption spectra of pure *ZZ*-bilirubin, *ZE*-bilirubin and lumirubin in the eluent were registered in the 350–550 nm region by collecting single fractions by h.p.l.c. Wavelength-dependence of bilirubin photoconversion was studied within photoequilibrium and up to a large decrement of the total concentration. Experiments were performed in aerobic and anaerobic conditions in order to assess the contribution of the photo-oxidation to the overall process. The presence of O_2 was found to increase the rate of bilirubin degradation and unexpectedly to favour lumirubin production. The ability of 514.5 nm irradiation to induce bilirubin cyclization was definitively confirmed.

INTRODUCTION

Bilirubin photochemistry has been widely studied by various authors because of its relevance to comprehension of the light-induced processes involved in the phototherapy of neonatal jaundice. Under exposure to the light, bilirubin undergoes three principal processes [1–7]: a fast and reversible configurational isomerization, a slower irreversible structural isomerization and a quantitatively less important photo-oxidation.

Initially, wavelengths falling near the absorption maximum of the bilirubin molecule were thought to be the most effective in its degradation [8–11], and accordingly daylight and blue lamps were usually employed in the clinical treatment of neonatal hyperbilirubinaemia. In a preliminary study performed *in vitro* on bilirubin solutions irradiated with monochromatic laser light [12] we found a good efficiency of longer wavelengths in bilirubin degradation. Similar experiments were extended successfully to the clinical procedure by employing green fluorescent lamps in the phototherapy of jaundiced babies [13–15].

Recently Ennever *et al.* [16] have shown that configurational photoisomers, mainly originated by shorter-wavelength (430–460 nm) irradiations, are not so important in the bilirubin degradation process as the structural isomer, lumirubin, which, being more polar and hydrophilic, is readily excreted during phototherapy. Green light seems to enhance the formation of lumirubin *in vivo*. Similar conclusions were also reached by Onishi and co-workers [17,18] in experiments based on the h.p.l.c. analysis of irradiated bilirubin solutions.

We have investigated the photolysis of bilirubin *in vitro* using, as exciting wavelengths, the monochromatic lines of an Ar ion laser and detecting the resulting configurational and structural isomers by h.p.l.c. analysis. Long-term irradiations were carried out until a drastic decrease in the bilirubin concentration was obtained. The contribution of photo-oxidation was also evaluated on the basis of experiments performed under aerobic and anaerobic conditions.

EXPERIMENTAL

Equimolar solutions (115 μM) of bilirubin and human serum albumin in phosphate buffer, pH 7.4, were prepared by adding a few drops of 10 mM-NaOH to 7 mg of crystalline bilirubin until it was completely dissolved; this solution was then rapidly added to 50 mM-phosphate buffer, pH 7.4, containing 8.30 g of human serum albumin/l to a total volume of 100 ml. The resulting solution was then stirred for 2 h to avoid the formation of micelles. Crystalline bilirubin (Serva, Heidelberg, Germany) and human serum albumin (fatty acid-free) (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used without any further purification. Solutions containing bilirubin were manipulated in the dark or in safety light at room temperature. Deoxygenated specimens were prepared and kept in a dry-box under N_2 atmosphere. Two series of small portions (1 ml) of this solution, saturated with pure O_2 and N_2 (99.99%) respectively, were irradiated in quartz cuvettes by using the 457.9, 488.0 and 514.5 nm exciting lines of an Ar ion laser (Coherent,

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Palo Alto, CA, U.S.A.) for different times of exposure. The laser beam was suitably expanded to provide a uniform irradiance across the cuvette surface. Irradiance measurements were performed with a power meter (model 362; Scientech, Boulder, CO, U.S.A.) that allowed a calibrated reading in the spectral range 400–1000 nm with $\simeq 5\%$ accuracy. The total energy absorbed by the solutions was evaluated by measuring the power of the incident beam and transmitted beam; the difference was then decreased by subtracting the amount scattered by the empty cell. The output power at different wavelengths was adjusted to give the same value of photon flux rate. Irradiated solutions were refrigerated at -10 °C and then added to 8 ml of methanol containing 0.1 M-di-n-octylamine acetate. The mixtures were stored at -24 °C for a few minutes and then centrifuged at 3000 *g* for 10 min. The supernatants were analysed by reversed-phase h.p.l.c. on a standard 0.46 cm × 25 cm C_{18} ODS column (Violet, Roma, Italy) equipped with a pre-column; the eluent was 0.1 M-di-n-octylamine acetate in methanol containing 6% water (pH 7.7) with a flow rate of 0.8 ml/min. A Waters model 510 apparatus equipped with a variable-wavelength-maximum UV-481LC (Waters, Millipore, Milford, MA, U.S.A.) detector set at 450 nm was employed. Multiple analyses were performed on each sample by successive injections of 25 μl to ensure quantitative reproducibility. Mean integrated areas ($\pm 2\%$) were used for calculations. Peak areas were calculated by a data-module 740 Waters integrator and normalized according to spectral data obtained on bilirubin and its isomers. For this, solutions of pure *ZZ*-bilirubin, *ZE*-bilirubin and lumirubin in the eluent (approx. 0.1 μM) were collected from h.p.l.c. and investigated in the 300–550 nm visible spectral region with the aid of a model λ 5 spectrophotometer (Perkin–Elmer, Norwalk, CT, U.S.A.) with quartz cuvettes of 1 cm path length.

RESULTS AND DISCUSSION

As mentioned above, h.p.l.c. analysis was performed with the u.v. detector set at 450 nm. Since each isomer of bilirubin has a different absorption maximum, the integrated peak areas cannot be directly correlated to the relative concentrations unless they are corrected by a factor that takes into account absorption coefficient and wavelength of the absorption maximum relative to each isomer.

Accordingly, we first registered the visible-absorption spectra of single fractions collected from h.p.l.c., namely *ZZ*-bilirubin, *ZE*-bilirubin and lumirubin. Then, for the *ZE*-photoisomer, we determined the isosbestic point (485 nm) in the spectra of mixtures of *ZZ*-bilirubin and *ZE*-bilirubin obtained by irradiating solutions of bilirubin in the h.p.l.c. eluent with small amounts of energy to avoid exceeding the photoequilibrium. Fig. 1 shows spectra normalized at an isosbestic point.

A molar absorption coefficient of 36200 $M^{-1} \cdot cm^{-1}$ was calculated for *ZE*-bilirubin in the eluent at the absorption maximum (464 nm). For non-irradiated bilirubin a value of 61300 $M^{-1} \cdot cm^{-1}$ (absorption maximum 452 nm) was found in the same solvent. From all these data a correction factor of 1.9 was obtained by which the integrated areas were multiplied in order to correspond directly with the concentration.

Strictly analogous results were also obtained from the chromatographic data, according to the method suggested in ref. [19]. From the analysis of the integrated areas performed on solutions irradiated within photoequilibrium, we calculated a mean correction factor of 1.85. The reliability of this value is ensured by the large number of measurements performed under different experimental conditions (different laser line, aerobic or anaerobic solutions).

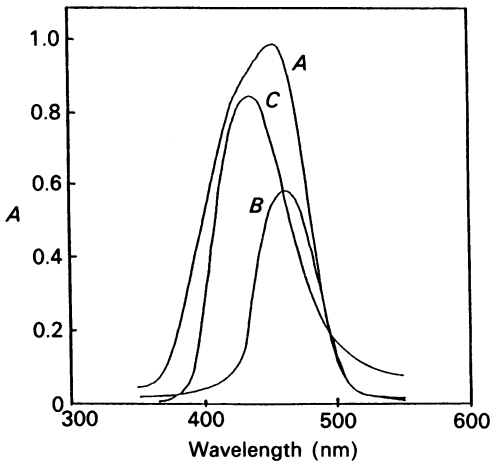

Fig. 1. Visible-absorption spectra of bilirubin and its photoisomers

Spectra of *ZZ*-bilirubin (*A*), *ZE*-bilirubin (*B*) and lumirubin (*C*) in methanolic 1 M-di-n-octylamine acetate were measured on pure fractions collected from h.p.l.c. Molar concentrations were approx. 0.1 μM. The absorption data were corrected to give equimolar spectra. For details see the text.

A more complex calculation, involving both spectroscopic and chromatographic data, was needed to obtain the proper correction factor (1.35) for lumirubin. The molar absorption coefficient of lumirubin in methanolic di-n-octylamine acetate was found to be 52700 $M^{-1} \cdot cm^{-1}$ (absorption maximum 436 nm).

As mentioned above, we have analysed by h.p.l.c. the photoconversion of bilirubin bound to human serum albumin as a function of irradiation wavelength and of energy supplied to the solutions. The most significant changes in the relative concentrations of photoisomers are shown in Fig. 2, where only three chromatograms for each wavelength are reported, obtained on aerobic solutions irradiated with low (within photoequilibrium), medium and high doses of energy. Although a direct correspondence of the concentration to the relative peak intensities is not achieved, it is clearly evident that the largest lumirubin production occurs when 514.5 nm radiation is employed.

A more detailed analysis, involving the corrected peak areas, was carried out on a larger number of measurements. The results are shown in Figs. 3 and 4, where integrated peak areas are plotted versus number of photons absorbed by the solution. The plots in Fig. 3 show the geometric photoisomerization of bilirubin under different experimental conditions. Photoequilibrium was reached with low-power laser radiations (approx. 1 mW/cm^2) for short times (5–100 s). Similar patterns were obtained from both aerobic and anaerobic

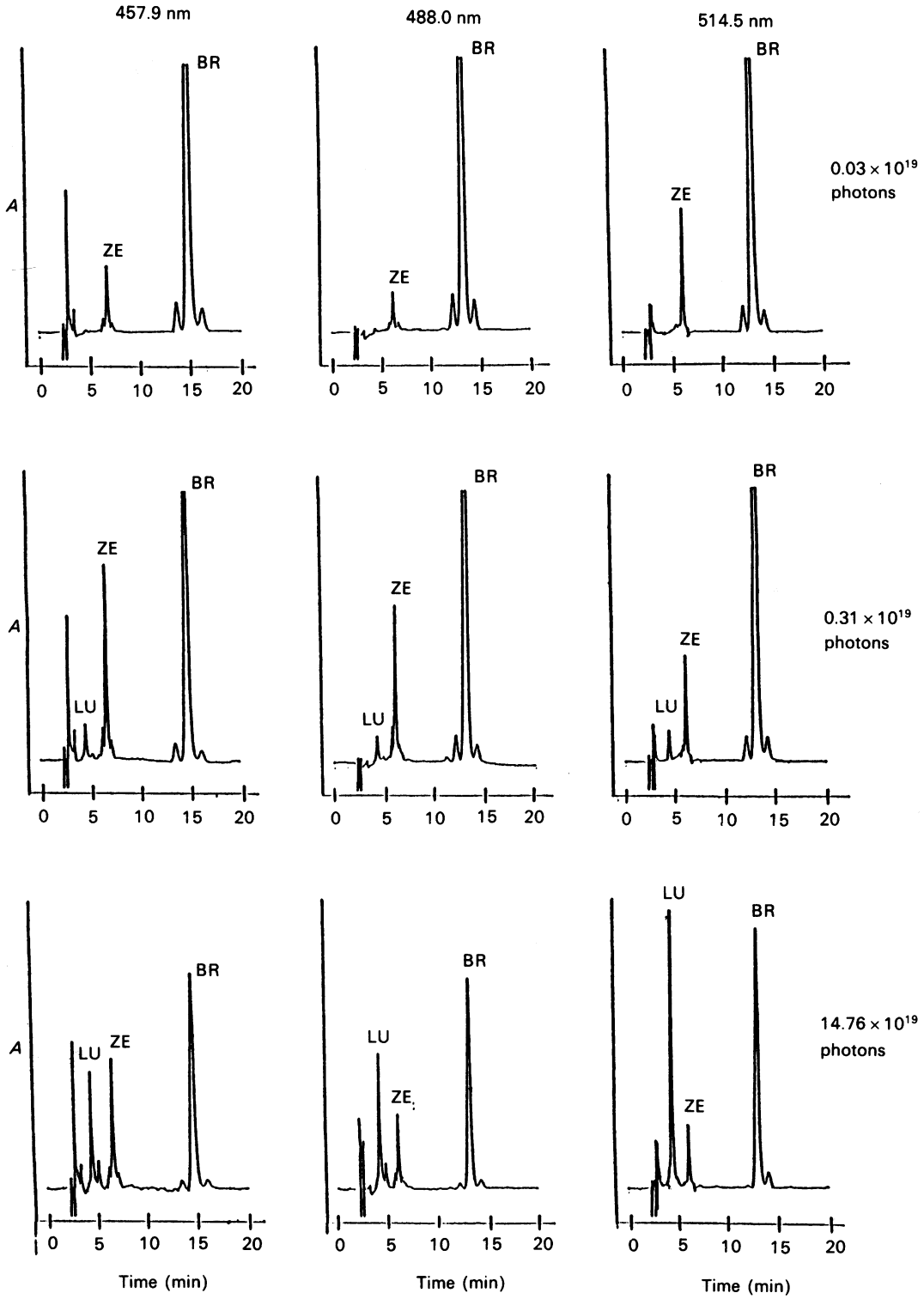

Fig. 2. Representative chromatograms of solutions irradiated with different laser lines under aerobic conditions

Irradiation wavelengths and number of photons absorbed by the solutions are indicated in the Figures. Labels BR, ZE and LU refer to *ZZ*-bilirubin, *ZE*-bilirubin and lumirubin respectively.

experiments, independently of the excitation wavelength. After an initial formation of the *ZE*-photoisomer, which corresponds to an equivalent decline of *ZZ*-bilirubin, changes in the relative concentrations occur only for doses of energy lower than 40×10^{16} photons (approx. 80 mJ). The *ZZ*-/*ZE*-bilirubin ratio remains practically constant for larger amounts of energy supplied to the solutions.

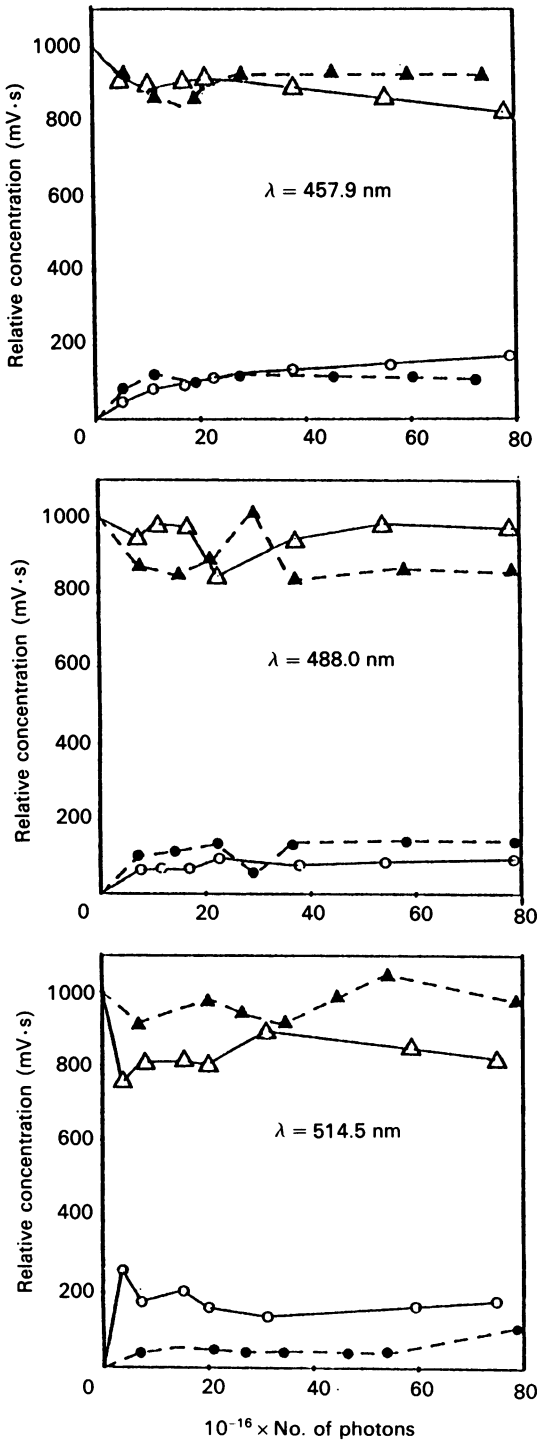

Fig. 3. Photochemical conversion of bilirubin within photoequilibrium

Relative concentrations are plotted versus number of photons absorbed by the solutions. Irradiation wavelengths are indicated in the Figures. Symbols: *ZZ*-bilirubin, △ (aerobic) and ▲ (anaerobic); *ZE*-bilirubin, ○ (aerobic) and ● (anaerobic).

The effect on bilirubin photodegradation of increasing doses of energy, exceeding the photoequilibrium to a maximum of approx. 110 J, is illustrated in Fig. 4. As shown in the plots on the left, which refer to aerobic solutions, larger decrements in *ZZ*-bilirubin concentration occur as the radiation wavelength increases; thus the 514.5 nm laser line results in the most efficient decrease in bilirubin concentrations, but at the same time it also produces the largest amount of lumirubin. No relevant difference exists among the curves relative to the *ZE*-photoisomer, whereas those corresponding to *EZ*-bilirubin indicate that this isomer is always present but at very low concentrations.

Plots relative to experiments performed under anaerobic conditions (right-hand-side panels) do not generally differ from those previously discussed. However, all the curves describing bilirubin decrement and lumirubin formation exhibit a lower slope, suggesting that both photoreactions are partially inhibited in the absence of O_2. Also in this case evidence exists for a largest efficiency of the 514.5 nm radiation. Concerning geometric isomers, no significant information can be drawn by the *ZE*-bilirubin curves, which remain practically unchanged in the three panels; the *EZ*-isomer is produced in small amounts only under 514.5 nm irradiation.

In general, our results agree with those obtained by Itoh & Onishi [20] and provide new experimental evidence of the efficiency of 'green light' that we claimed in prevous work performed *in vitro* and *in vivo* [12–15]. The use of the h.p.l.c. technique to separate and identify bilirubin photoproducts in solutions irradiated with strictly monochromatic laser lines allowed an exact measurement of the energy doses (number of photons) absorbed by the samples and a correct evaluation of the efficiency of each wavelength. Lumirubin formation was found to be strongly favoured by the 514.5 nm laser line, whereas the 457.9 nm laser line seems to be the most suitable radiation for the *ZZ*-/*ZE*-bilirubin conversion. It is difficult to decide on the role played by the *EZ*-isomer in the photodegradation, since it is not always present in the irradiated solutions and its formation is not clearly correlated to the other species. A possible explanation could be provided by the mechanism proposed in ref. [19]. In this hypothesis *EZ*-bilirubin is considered an intermediate in the process of cyclization of *ZZ*-bilirubin to lumirubin and accordingly it could be hardly detected because of its rapid conversion.

Most of the studies reported in the literature involve experiments performed on deoxygenated solutions to avoid the contribution of the photo-oxidation to the overall process. Instead, the parallel investigation that we have carried out on both oxygenated and deoxygenated samples, under the same photochemical conditions (Fig. 4), revealed some significant differences. First, the contribution of photo-oxidation can be directly evaluated by considering the different slopes of the corresponding bilirubin plots: the presence of O_2 induces a more rapid degradation. Another interesting feature is the unexpected larger production of lumirubin in the oxygenated solutions, especially under 514.5 nm irradiation. On the basis of our data, it is difficult to give a plausible explanation of the role played by the oxygen atom in the mechanism of endocyclization of bilirubin. We think that this question is worthy of a deeper investigation in view of its possible implication in the clinical procedure; in fact, if lumirubin represents the main pathway for bilirubin elimination, and its production depends on the O_2 concentration, particular attention should be paid to newborn humans with hypoxaemia submitted to phototherapy.

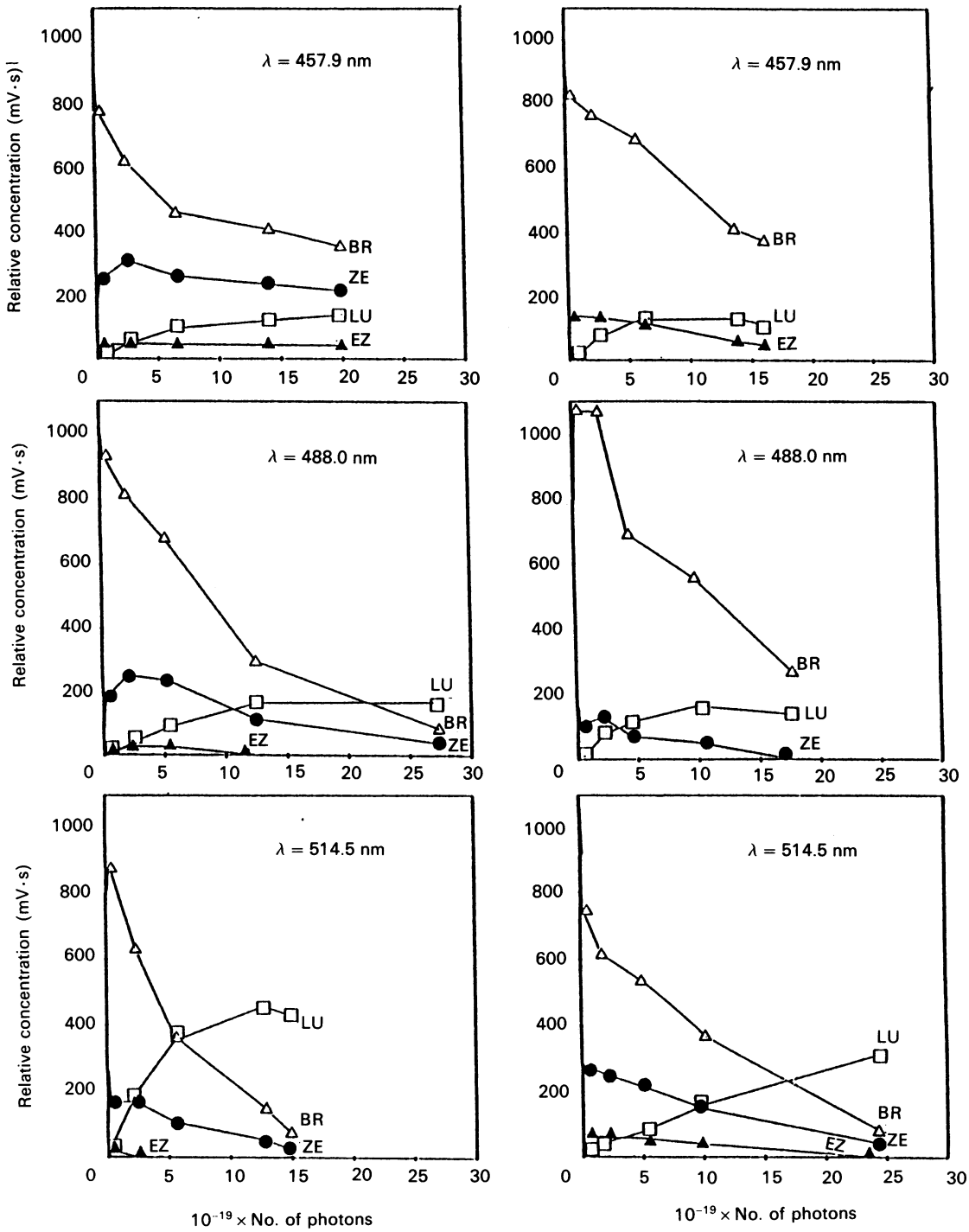

Fig. 4. Influence of increasing doses of energy on bilirubin photochemistry

Plots on the left-hand side and on the right-hand side refer to aerobic and anaerobic experiments respectively. Irradiation wavelengths are reported in the Figures. Symbols: △, BR, *ZZ*-bilirubin; ●, ZE, *ZE*-bilirubin; ▲, EZ, *EZ*-bilirubin; □, LU, lumirubin.

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