EVIDENCE FOR SINGLET OXYGEN QUENCHING BY BILIVERDIN IX-α DIMETHYL ESTER AND ITS RELEVANCE TO BILIRUBIN PHOTO-OXIDATION

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Received June 8,1972

SUMMARY: Biliverdin IX-α dimethyl ester inhibits the sensitized and unsensitized photo-oxidation of bilirubin IX-α. Biliverdin IX-α is probably not an intermediate in the main pathway of bilirubin photo-oxidation in vitro.

Photo-oxidation of bilirubin IX-α yields a number of products of which only two, methylvinylmaleimide and biliverdin IX-α, have been identified (1,2). It has been suggested that the overall process follows the path

Bilirubin IX-α ⟶ Biliverdin IX-α ⟶ Products

with biliverdin IX-α being formed as an early intermediate (2). The reaction is markedly accelerated by the addition of known singlet oxygen sensitizers (e.g. methylene blue) (3,4) and there is strong evidence that even in the absence of added sensitizers the reaction involves singlet oxygen, arising from photosensitization by bilirubin itself (4). It is not known whether biliverdin IX-α also is a singlet oxygen sensitizer, but early studies on the photodynamic activity of this pigment implied that it might be (5,6).

In order to investigate further the role of biliverdin IX-α in the photodegradation of bilirubin, I have examined the relative stability of bilirubin IX-α and biliverdin IX-α dimethyl ester[+] towards sensitized and unsensitized photo-oxidation. In this report I wish to present evidence that biliverdin

[+] Biliverdin IX-α dimethyl ester has been more fully characterized and is more readily obtainable as a crystalline material of known purity than the free acid. Like bilirubin IX-α, it is soluble in chloroform. For these reasons the dimethyl ester, rather than biliverdin IX-α itself, was used in these preliminary studies.

IX-α dimethyl ester is an inhibitor of bilirubin photo-oxidation; that it is not a singlet oxygen sensitizer; and that biliverdin IX-α is not an intermediate in the main pathway of bilirubin photodegradation.

EXPERIMENTAL

Purified bilirubin IX-α [ε_{max} ($CHCl_3$) 62,600] , free from isomeric impurities, was prepared by preparative thin layer chromatography of commercial material (Pfanstiehl Laboratories) followed by crystallization (7). Biliverdin IX-α dimethyl ester was prepared by oxidation of bilirubin IX-α followed by methylation and crystallization (8); this material contained small amounts of biliverdin III-α dimethyl ester and biliverdin XIII-α dimethyl ester as impurities (8). Methylene blue trihydrate was obtained commercially (Sigma Laboratories). The solvent in all experiments was Analytical Reagent grade chloroform containing ethanol stabilizer.

For photo-oxidation experiments, solutions contained in 1-cm path-length quartz spectrophotometer cuvettes were purged for approximately 5 minutes with a slow stream of oxygen in the dark. The cuvettes were then stoppered, placed at a fixed distance from the light source, and exposed to filtered visible light from a high-pressure mercury lamp (Duro-Test R57, 400 Watt). Either a yellow filter (Corning Filter No. 3389) or an orange filter (Corning Filter No. 3480) was used as described below. After various time intervals the cuvettes were removed and absorption spectra were measured over the range 350-700nm.

Two sets of experiments were run. In the first set (Figs. 1 and 2) the incident light was restricted to wavelengths >390nm by use of the yellow filter and the photo-oxidation of bilirubin IX-α and biliverdin IX-α dimethyl ester, both independently and together in the same solution, was examined. The second set (Figs. 3 and 4) was similar except that an exogenous singlet oxygen sensitizer, methylene blue, was added and the orange filter was used to restrict the incident light to wavelengths >550nm.

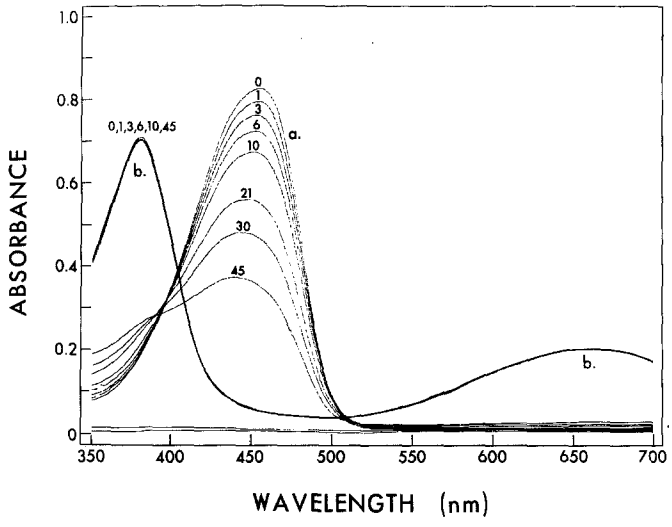

Figure 1. Photo-oxidation of bilirubin IX-α and biliverdin IX-α dimethyl ester with light >390nm.
a. Photo-oxidation of bilirubin IX-α (1.3×10^{-5}M) with biliverdin IX-α dimethyl ester solution (1.3×10^{-5}M) as an additional light filter.
b. Photo-oxidation of biliverdin IX-α dimethyl ester (1.3×10^{-5}M) with bilirubin IX-α solution (1.3×10^{-5}M) as an additional light filter.

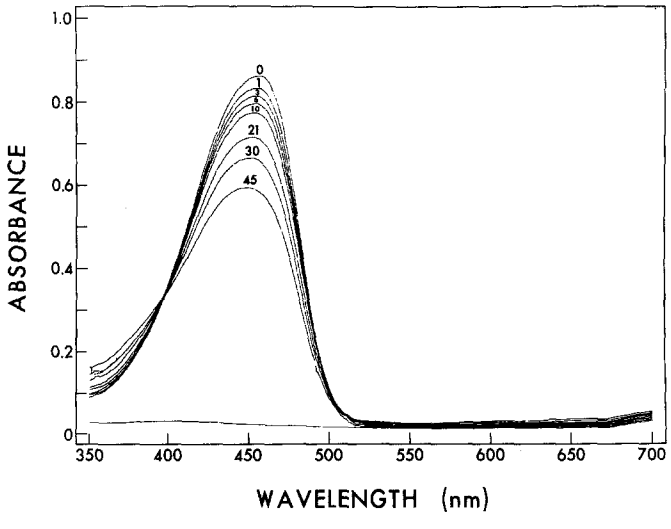

Figure 2. Photo-oxidation of bilirubin IX-α in the presence of biliverdin IX-α dimethyl ester with light >390nm.
Spectra were obtained by difference spectrophotometry. The sample solution contained bilirubin IX-α (1.3×10^{-5}M) and biliverdin IX-α dimethyl ester (1.3×10^{-5}M) and the reference solution contained biliverdin IX-α dimethyl ester (1.3×10^{-5}M) alone. Both solutions were irradiated simultaneously.

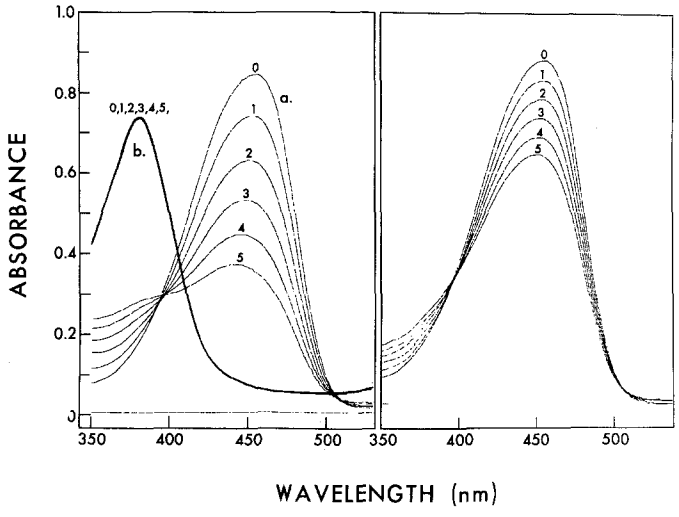

Figure 3 Figure 4

Figure 3. Photosensitized oxidation of bilirubin IX-α and biliverdin IX-α dimethyl ester with light >550nm.
a. Photo-oxidation of a solution containing bilirubin IX-α (1.3×10^{-5}M) and methylene blue (1.5×10^{-6}M) using biliverdin IX-α dimethyl ester solution (1.3×10^{-5}M) as an additional light filter.
b. Photo-oxidation of a solution containing biliverdin IX-α dimethyl ester (1.3×10^{-5}M) and methylene blue (1.5×10^{-6}M) using bilirubin IX-α solution (1.3×10^{-5}M) as an additional light filter.

Figure 4. Photosensitized oxidation of bilirubin IX-α in the presence of biliverdin IX-α dimethyl ester with light >550nm.
Spectra were obtained by difference spectrophotometry. The sample contained bilirubin IX-α (1.3×10^{-5}M), biliverdin IX-α dimethyl ester (1.3×10^{-5}M) and methylene blue (1.5×10^{-6}M) and the reference solution contained biliverdin IX-α dimethyl ester (1.3×10^{-5}M) and methylene blue (1.5×10^{-6}M). Both solutions were irradiated simultaneously.

Since the visible absorption spectra of bilirubin IX-α and biliverdin IX-α dimethyl ester partially overlap, when solutions containing both pigments are exposed to light each pigment will reduce the light intensity experienced by the other. To compensate for this "internal shielding effect," in some experiments solutions of the bile pigments themselves were used as additional light filters; for this purpose the "filter solution" contained in a 1-cm quartz cuvette was placed immediately in front of the cuvette containing the solution being studied.

RESULTS

When bilirubin IX-α was photo-oxidized using an equimolar solution of

biliverdin IX-α dimethyl ester as an additional light filter at least 60% of the pigment was destroyed in 45 min. (Fig. 1a).‡ At the end of this period the solution showed only slightly increased absorption near 380 and 660nm indicating that little biliverdin IX-α or other verdinoid products had been formed. Photo-oxidation of biliverdin IX-α dimethyl ester under similar conditions (Fig. 1b), but with bilirubin IX-α solution as the additional light filter, showed that over a similar period of time there was negligible destruction of the biliverdin ester.

When bilirubin IX-α was irradiated in the presence of an equimolar amount of biliverdin IX-α dimethyl ester and the reaction followed by difference spectrophotometry, the set of decay curves shown in Fig. 2 was obtained. This set of curves has two significant features. First, the spectra have similar contours to those obtained on irradiation of bilirubin IX-α alone and do not display anomalies near 380 and 660nm where the verdin has absorption maxima; since the verdin is not destroyed when irradiated alone (Fig. 1b), this indicates that it is also resistant to photo-oxidation in the presence of bilirubin IX-α. Second, only about 30% of the bilirubin IX-α was photo-oxidized after 45 min. as compared to 60% when the pigment was illuminated alone; this inhibition was not due to internal light shielding by the verdin since this had been more than compensated for in the solo run (Fig. 1a) by using a solution of the verdin as an external filter.

In an attempt to determine the relative reactivity of bilirubin and biliverdin IX-α dimethyl ester towards singlet oxygen the photosensitized oxidation of the two pigments was examined using methylene blue as sensitizer. In these experiments, to eliminate photosensitization by bilirubin IX-α, light falling outside the absorption band of bilirubin but within the methylene blue absorption region (561-688nm) was used.

On photosensitized oxidation of bilirubin IX-α (Fig. 3a) 60% of the pig-

‡ In all Figures numbers above the curves refer to duration of irradiation in minutes.

ment was destroyed in 5 min. Over a similar period of time there was no significant destruction of biliverdin IX-α dimethyl ester (Fig. 3b). In the presence of biliverdin IX-α dimethyl ester the rate of photosensitized oxidation of bilirubin IX-α again was diminished by about one-half (Fig. 4) so that after 5 min. only about 30% of the rubin had been destroyed.

DISCUSSION

The results clearly show that biliverdin IX-α dimethyl ester is relatively stable towards photo-oxidation and that it inhibits rather than accelerates the photo-oxidation of bilirubin IX-α. Therefore, under the conditions used, biliverdin IX-α dimethyl ester is not a photosensitizer.

The rate of bilirubin IX-α photo-oxidation is decreased by the verdin ester both in the presence and absence of methylene blue. This inhibition could be due either to quenching of bilirubin and methylene blue excited triplet or singlet states by the biliverdin ester, thus inhibiting singlet oxygen formation, or due to quenching of singlet oxygen itself. Although the present data do not discriminate between these two possibilities, the second mechanism seems more probable. From structural considerations and by analogy to bilirubin, biliverdin IX-α dimethyl ester would be expected to react rapidly with singlet oxygen in the absence of any quenching effects. Yet during the sensitized oxidation of bilirubin, the verdin was not attacked despite singlet oxygen levels sufficient to cause rapid destruction of the bilirubin. It seems likely, therefore, that the observed inhibition is due to quenching of singlet oxygen by the verdin rather than quenching of sensitizer, and that biliverdin IX-α dimethyl ester is more reactive as a quencher of singlet oxygen than as an acceptor. However, quenching of bilirubin and sensitizer excited states by biliverdin IX-α dimethyl ester cannot be ruled out at present.

There are numerous examples of the occurrence of biliverdin and related bilitriene pigments in living organisms (9). The finding that biliverdin IX-α dimethyl ester is an efficient photo-oxidation inhibitor leads to specula-

tion that perhaps some naturally occurring bilitrienes may have a protective function analogous to that of carotenoid pigments in plants (10). It is noteworthy that biliverdin IX-α dimethyl ester is approximately as effective as β-carotene in inhibiting the photo-oxidation of bilirubin IX-α (4).

It may be reasonably assumed that biliverdin IX-α and its dimethyl ester have similar photochemical properties. If this assumption is correct, then biliverdin IX-α cannot be an intermediate on the main pathway of bilirubin photo-oxidation, as suggested by Ostrow and Branham (2), since biliverdin IX-α dimethyl ester remains unscathed during a period of illumination which is sufficient to photo-oxidize 60% of an equimolar quantity of bilirubin IX-α to primarily non-verdinoid products. If biliverdin IX-α were an intermediate it would be expected to accumulate during the photo-oxidation of bilirubin IX-α to a much greater extent than is observed (Fig. 1a). However, since some biliverdin IX-α is formed on photo-oxidation of bilirubin (1,2), it follows that there must be at least two competing pathways for bilirubin photodegradation. One pathway leads to biliverdin and thence, by a much slower process (11), to further products; singlet oxygen may play a role in this pathway but this has not yet been established. The other pathway requires singlet oxygen (4) and does not involve biliverdin IX-α as an intermediate. Under the conditions of the present experiments the latter pathway is predominantly favored. It is possible, however, that under different conditions (different solvent, pH, and oxygen tension) the relative importance of each of these pathways might be altered.

Bilirubin photo-oxidation is of interest primarily because of its probable relevance to the mechanism by which phototherapy reduces hyperbilirubinemia in jaundiced infants (3,12). In this regard it should be noted that in vivo any pathway involving photo-oxidation of bilirubin to biliverdin may be of little therapeutic benefit because the biliverdin thus formed would be expected to be reduced back to bilirubin by biliverdin reductase (13,14), thereby setting up an abortive closed loop system.

The present findings thus suggest that in the main pathway of bilirubin photo-oxidation, *in vitro* and *in vivo*, biliverdin is a red herring.

ACKNOWLEDGMENTS

This work was aided by a grant from the United Cerebral Palsy Research and Educational Foundation and by USPHS Grant AM-11275. Lighting equipment and additional support was provided by the Duro-Test Corporation, New Jersey. I gratefully acknowledge the cooperation of Professor Rudi Schmid and stimulating discussions with Professor David Lightner.

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