Short Communications

Singlet Oxygen in the Photo-Oxidation of Bilirubin in Hydroxylic Solvents

By R. BONNETT and J. C. M. STEWART

Department of Chemistry, Queen Mary College (London University),
Mile End Road, London E1 4NS, U.K.

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The treatment of jaundice in newly born and, especially, prematurely born infants (neonatal hyperbilirubinaemia) by phototherapy with visible light (Cremer *et al.*, 1958) has recently attracted increasing attention and comment (Bergsma *et al.*, 1970; Editorial, 1972). Little was known until very recently about the structures of the photodecomposition products of bilirubin, even for processes *in vitro*. Irradiation of bilirubin in chloroform–methanol under N_2 gives (20%) the product of Markovnikov addition of methanol to the *exo* (C8) vinyl group (Manitto, 1971). Most irradiations, however, have been done in O_2 (or air). Spectroscopic and chromatographic evidence was obtained in this laboratory (A. F. McDonagh & C. A. Vose, unpublished work) for the formation (10%) of methylvinylmaleimide on irradiation of bilirubin in aqueous buffer. This imide has been isolated (from a photo-oxidation in methanol) (Lightner & Quistad, 1972; Bonnett & Stewart, 1972), and it was also detected, along with five other monopyrrolic fragments, during an extended irradiation of bilirubin in chloroform (Gray *et al.*, 1972). Photo-oxidation of bilirubin in methanol gives methanol–propentdyopent adducts as major products (Bonnett & Stewart, 1972): three of these compounds have now been isolated.

Lightner & Quistad (1972) have suggested that biliverdin is the major product formed on short-term irradiation. However, in chloroform solutions biliverdin dimethyl ester resists photodegradation (McDonagh, 1972), and a singlet oxygen mechanism has been implicated for bilirubin photo-oxidation (McDonagh, 1971).

We now report observations about the reaction pathway of the photo-oxidation in methanol.

Methods

Photo-oxidations were done on solutions of bile pigment (IX α series, approx. $15\mu M$) in anhydrous methanolic 0.05M-NH $_3$ continuously flushed with O_2 at 18°C, and irradiated in a Pyrex vessel with a 500 W lamp (Philips Arga Photo B) about 10cm from the reactor in a standard configuration. The relative rates

of reaction in CH $_3$ OH and C 2 H $_3$ O 2 H (0.05M in NH $_3$, and saturated with air) were measured in cuvettes in a separate experiment (18°C, Pyrex filter). Kinetic data refer to initial rates of diminution of absorption at 450nm.

Photo-oxidation products were examined by t.l.c. (Merck silica gel HF irrigated with benzene–ethyl acetate–acetic acid, 50:50:1, by vol.). Bilirubin (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) appeared to contain traces of bile-pigment III α and XIII α isomers; C 2 H $_3$ O 2 H was purchased from Nuclear Magnetic Resonance Ltd., High Wycombe, Bucks., U.K.

Results and discussion

Under the standard conditions described above photo-oxidation of bilirubin, assessed by the diminution of absorption at 450nm, occurred with an apparent half-life of 21min, with only a minute increase in absorption, attributed to biliverdin, at 650nm. Under the same conditions biliverdin was essentially unchanged after 45min (Fig. 1). When a mixture of bilirubin and biliverdin was irradiated the former was photodegraded much more rapidly than the latter. We conclude that biliverdin, although formed, is not an intermediate on the main pathway.

The photodegradation proceeded extremely slowly in the absence of O_2 : in the presence of O_2 , but in the dark, a thermal reaction was detected, but again it proceeded so slowly in comparison with the photo-reaction that for present purposes it can be ignored. The reaction rate was decreased in the presence of 2,5-dimethylfuran (15mM; $t_{\frac{1}{2}} = 58$ min), a singlet oxygen trap (see below), and in the presence of β -carotene ($9.2\mu M$ in methanolic 0.05M-NH $_3$ containing 1% of tetrahydrofuran; $t_{\frac{1}{2}} = 35$ min), a singlet oxygen quencher (Foote *et al.*, 1970), but it was considerably increased in the presence of Rose Bengal ($1.5\mu M$; $t_{\frac{1}{2}} = 0.6$ min), a photosensitizer of singlet oxygen formation (Foote *et al.*, 1965). Inspection of thin-layer chromatograms from the last reaction indicated that the products were indistinguishable from those formed in the absence of added sensitizer.

Fig. 1. Effect of irradiation on (a) bilirubin and (b) biliverdin in the presence of O_2

Solutions ($15\mu M$) were irradiated in $0.05M-NH_3$ in methanol flushed with O_2 at $18^\circ C$ with a 500 W lamp. The numbers on the curves refer to time elapsed (min) from the beginning of each irradiation.

This result clearly accords with a mechanism involving singlet oxygen as a reactive intermediate (McDonagh, 1971; Bonnett & Stewart, 1972):

and is supported by two further observations.

(i) Photo-oxidation of 2,5-dimethylfuran (1.25 mmol) in methanolic $0.05M-NH_3$ (100 ml) is sensitized by the addition of bilirubin ($155\mu M$). The reaction (approx. 0.65 mol of O_2 uptake after 20h) was much slower than the corresponding reaction (Foote *et al.*, 1967) with Rose Bengal ($6.5\mu M$; approx. 0.82 mol of O_2 uptake in 1.5h) but a satisfactory yield (65%) of 2-hydroperoxy-5-methoxy-2,5-dimethyl-2,5-dihydrofuran was obtained. This suggests that bilirubin itself acts as a sensitizer of singlet oxygen formation (and thus has a triplet energy greater than 94 kJ/mol ; Rosen, 1970): the possibility that a degradation product of bilirubin

Fig. 2. Bilirubin photo-oxidation: rate enhancement in $C^2H_3O^2H$

o, In CH_3OH containing $0.05M-NH_3$; Δ , in $C^2H_3O^2H$ containing $0.05M-NH_3$. $a-x$, E_{450} at time t (min).

is the photosensitizer cannot, however, be entirely eliminated at this stage.

(ii) When bilirubin was irradiated in $0.05M-NH_3$ in $C^2H_3O^2H$ a fivefold increase in photodegradation, over that found for the CH_3OH control, was observed (Fig. 2). This result is consistent with the report (Merkel *et al.*, 1972) that the lifetime of singlet (${}^1\Delta_g$) oxygen in solution is dependent on the absorption of the solvent at 1270 and 1590 nm, and the prediction that a large increase in the lifetime of ${}^1\Delta_g$ oxygen should accompany the change to the deuterated solvent.

Whether or not a singlet oxygen mechanism operates for the photodegradation *in vivo* is a problem that awaits further experimentation. Were such a mechanism to operate *in vivo* an association of photosensitivity with hyperbilirubinaemia would be expected, but there appear to be few indications of this (Broughton, 1970). However, it is, we believe, important to distinguish between pigments, such as bilirubin, which are rapidly degraded by singlet oxygen, and those, such as uroporphyrins in certain porphyrias, which are not.

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