ON THE FORMATION OF BILIVERDIN DURING PHOTOOXYGENATION OF BILIRUBIN *IN VITRO*

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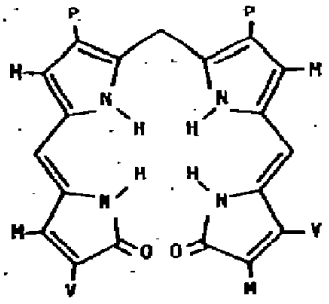
1. Introduction

In connection with a widely employed phototherapy for neonatal jaundice [1] we have been investigating [2, 3] the photooxygenation of bilirubin IXα (1), only a few of whose photo-destruction products have been characterized with certainty [2–6]. A green pigment, biliverdin (2) has been implicated as an intermediate in the photo-destruction of 1 by various investigators [5–8] on the basis of visible-ultraviolet spectroscopic evidence. Until this work, the green pigment in question had never been isolated, and its structure remained inconclusively proved [2]. Since McDonagh has shown that biliverdin (2) is a singlet oxygen quencher [9], the possibility and extent of its presence during the self-sensitized photooxygenation of bilirubin (1) [10] becomes an important criterion in evaluating the efficacy of the bilirubin phototherapy method [1]. We therefore wish to report on the *isolation* of 2 during the photooxidation of 1 and its *unequivocal identification* be means of its derivative, biliverdin dimethyl ester (3).

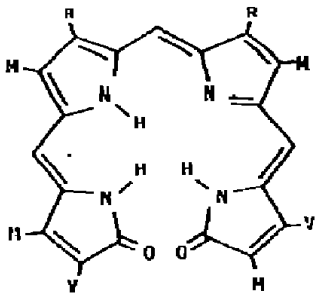
2. Materials and methods

A 0.40 mM methanolic solution of bilirubin IXα (1) [Matheson, Coleman and Bell] containing enough conc. NH_4OH (0.078 ml/100 ml methanol) to dissolve it was photolyzed in an immersion well apparatus using a 500 W Sylvania tungsten–halogen lamp (500 Q/CL) at 120 V while bubbling a slow stream of oxygen through the solution. Identically prepared solutions were irradiated separately for periods of 1, 2, 3, 4 and 7 hr. In each photolysis run, after the prescribed irradiation time, about 10 ml of 10% aq. HCl were added to neutralize the NH_4OH. (Unreacted bilirubin precipitated from solution following this



1

M = $-CH_3$
V = $-CH=CH_2$
P = $-CH_2CH_2COOH$
PM = $-CH_2CH_2COOCH_3$



2 R = P
3 R = PM

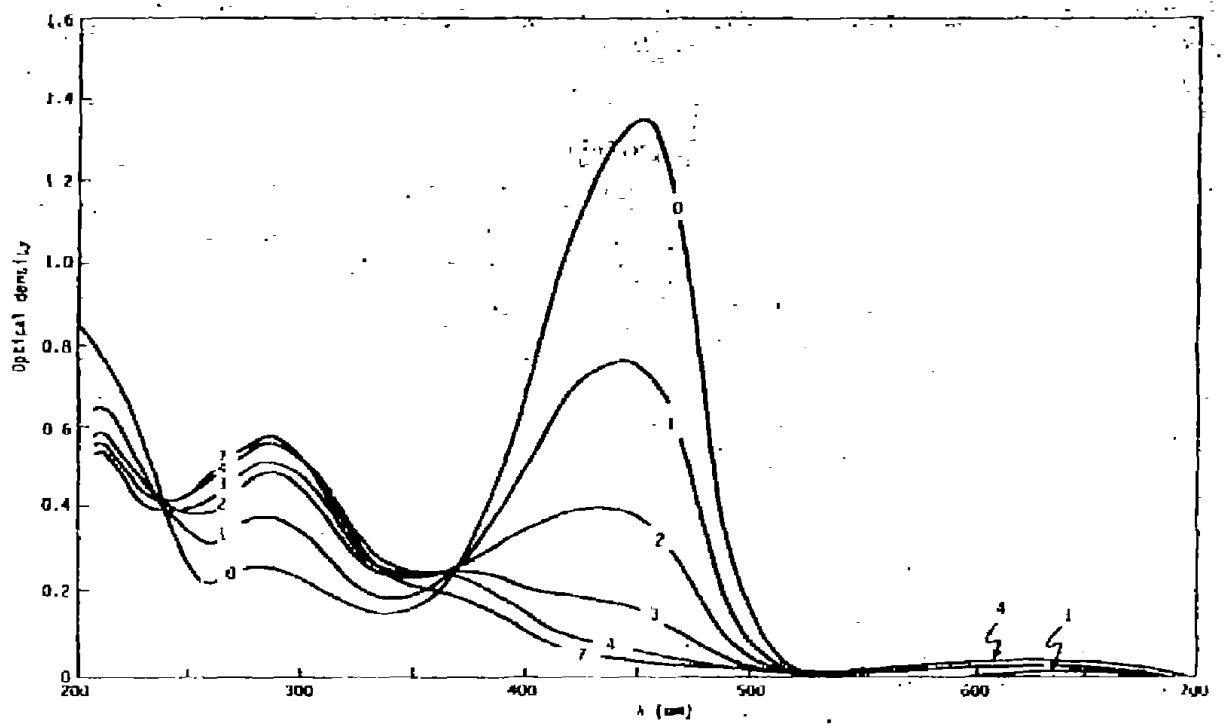


Fig. 1. Visible–UV spectral changes during bilirubin (1) photooxidation. Curves are labeled with irradation times (hours). The original solution (0 hr) is 0.4 mM in methanol + trace of NH_4OH. All curves are run after a concentration dilution of 1:22.5.

treatment of the 1- and 2-hr photooxidation runs). The solvent was evaporated on a flash evaporator, and the resulting solid was esterified using methanolic boron trifluoride etherate according to the procedure of Bonnett and McDonagh [11]. Biliverdin dimethyl ester (3) was separated as a dark blue-green band, R_f 0.43, from the crude esterification mixture by preparative thin-layer chromatography on silica gel F (M. Woelm, Eschwege, 1 mm, 24°) using 12% acetone in chloroform. The biliverdin dimethyl ester isolated following photooxidation of 1 [mol. wt. 610.2793, $C_{35}H_{38}N_4O_6$] had a mp 206–7° (lit. [11] mp 208–9°) which was undepressed in a mixture melting point with authentic material [12].

3. Results and discussion

As a control experiment to determine the efficiency of the esterification procedure applied to photoproduct 2, crude 2 was prepared in 90% yield by controlled oxidation of 1 [11] and purified by column chromatography on silica gel as reported earlier [13]. Samples of the purified 2 were esterified exactly as described for the photooxidation mixture. The yields of 3 were 49%.

The visible–ultraviolet spectral changes accompanying the photodecomposition of bilirubin IXα in methanolic NH_4OH are shown in fig. 1. After even a short irradiation time new absorbances in the vicinities of 280, 370 and 650 nm were readily apparent; whereas, the principal long wavelength (450 nm) peak of 1 was markedly diminished. The 280 nm absorbance has been associated with the two isomeric propentdyopent photo-products formed in moderately high yield (30–40%) from 1 [3,4]; the 370 and 650 nm absorbances have been associated with verdinoid pigment [6, 7, 14] which we have completely characterized as biliverdin IXα (2). In the course of 7 hr of irradiation, as 1 was more completely destroyed, both the 280 nm and 650 nm (+ 370 nm) absorbances grew and remained relatively constant (280 nm) or diminished somewhat (650 + 370 nm). Under our reaction conditions 2 was formed in a maximum amount of 15% (corrected) isolated yield after 3 hr during the course of photooxidation and was continously photo-destroyed [13] at a rate much slower than that of 1 (fig. 2). The maximum yield of 2 was achieved when 1 was nearly completely (85%) destroyed.

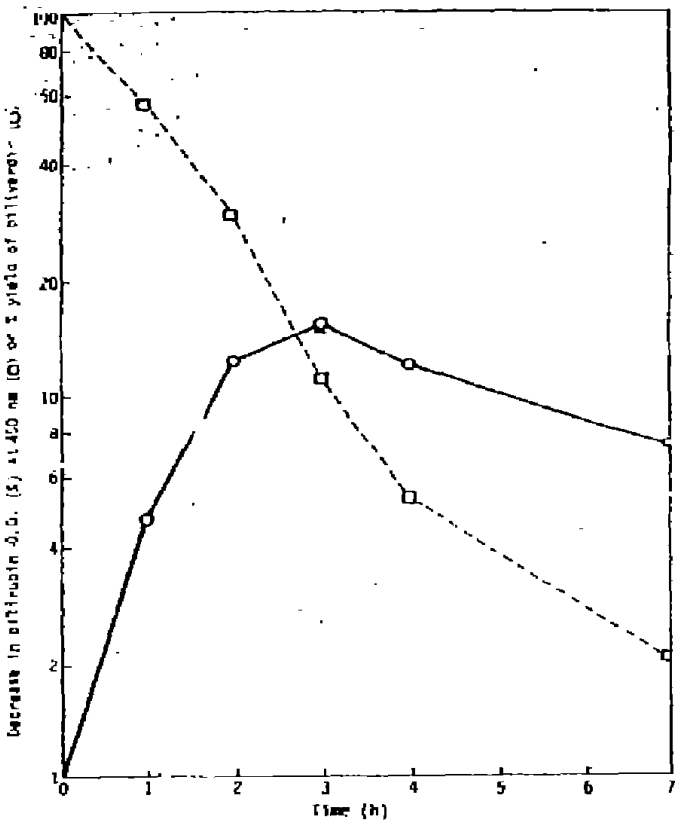


Fig. 2. Yield (%) of biliverdin (2) *vs* time of photooxidation of bilirubin (1) and decrease in optical density (100% at 0 hr) of 1 *vs* time during photooxidation. Bilirubin O.D. (□—□—□), biliverdin % yield (○—○—○).

We have also found that the yields of 2 from photooxygenation of 1 are solvent and concentration dependent. Thus, a change from methanolic NH_4OH to chloroform solvent afforded a surprisingly high (38% corrected) isolated yield of 2 after a photooxidation of a 0.7 mM solution of 1. The effect of bilirubin concentration variation on product yield may be seen in the following. For equivalent reaction times (as determined by a time study of the spectral decomposition curves), photooxygenation of a 0.4 mM methanolic ammonia solution of 1 gave an 11.5% (corrected) yield of 2; whereas, a 0.09 mM solution of 1 in the same solvent afforded a 4.8% yield. Similarly, in chloroform solvent a 0.7 mM solution of 1 gave a 38% (corrected) yield of 2; whereas, a 0.09 mM solution of 1 afforded a 16.7% yield. A lesser percentage yield of biliverdin was formed in the photooxidation of very dilute solutions of bilirubin as contrasted with more concentrated solutions, and a higher percent of biliverdin was formed in chloroform as compared to methanolic ammonia. The fact that more 2 was formed in chloroform than methanol supports the notion that it is formed *via* a free radical oxidation mechanism.

From our data we conclude that biliverdin (2) is *not* the principal precursor to the majority of the bilirubin photoproducts formed in protic solvents [3]. Rather, it arises in a competing side reaction and is considerably more photostable than bilirubin (1) under the conditions of photooxygenation [9]. It is noteworthy that biliverdin slowly photooxidizes to many of the same products as bilirubin, including methylvinylmaleimide [13], hematinic acid and propentdyopents [15].

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