Photo-oxidation of Bilirubin in Hydroxylic Solvents

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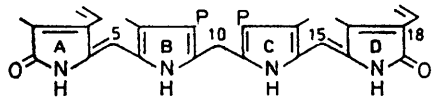
Photo-oxidation of bilirubin in methanol containing anhydrous ammonia (0·2%) rapidly destroys the substrate. Methylvinylmaleimide, three methanol–propentdyopent adducts, and a water–propentdyopent adduct have been isolated. Arguments are presented which allow structures to be assigned to the propentdyopent adducts, and the absence of a fourth methanol–propentdyopent isomer is rationalised. Equilibration between a pair of unsymmetrically substituted methanol–propentdyopent adducts occurs on irradiation (of the free acids), on treatment with zinc acetate, and on treatment with 10% acetic acid in chloroform. A dipyrrylmethane dialdehyde obtained from an aqueous photolysate of bilirubin has been characterised. Mechanistic aspects of the photo-oxidation are discussed.

JAUNDICE in the newborn (neonatal hyperbilirubinemia) can be markedly diminished by irradiating the infant with visible light.[1] This jaundice, due to unconjugated bilirubin in the serum and tissues, is regarded as dangerous because—at least above a certain threshold (*ca.* 15 mg of bilirubin per 100 ml of blood)—bilirubin appears to be highly toxic to developing neural tissue. One classical treatment involves blood transfusion: but, when it is realised that neonatal hyperbilirubinemia is most prevalent in the prematurely born, the advantages of a mild simple phototherapeutic method are evident, and phototherapy is now widely employed.[2]

The nature of the photoproducts is of considerable interest. Preliminary observations by Ostrow[3] had shown that the photoreaction gave a complex mixture of products *in vitro*, but at the outset of the present work the structures of the photoproducts were unknown. Manitto and his colleagues[4] have examined the reaction in an inert atmosphere, and report slow photoreactions in which alcohols and thiols undergo Markovnikov addition to the *exo*- (C-18) vinyl group of bilirubin (I).



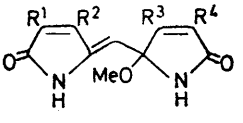
(I) Bilirubin (P = $CH_2{\cdot}CH_2{\cdot}CO_2H$ throughout the paper; the 1—24 numbering system is used)

We took the view that the photobleaching *in vivo* was likely to be a photo-autoxidation, and therefore studied the reaction in a hydroxylic solvent (methanol) in the presence of oxygen.[5]

Isolation and Identification of Photoproducts.—Oxygen was bubbled through a solution of bilirubin (*ca.* 10^{-3}M) in anhydrous methanol [containing anhydrous ammonia (0·2%) to solubilise the bilirubin] in a water-cooled Pyrex cell irradiated with tungsten lamps (2 × 500 W). Electronic spectroscopy showed that the bilirubin ($\lambda_{max.}$ *ca.* 450 nm) was rapidly destroyed and that a new band appeared at *ca.* 280 nm,† but that very little biliverdin ($\lambda_{max.}$ *ca.* 650 nm) was formed. In the dark only a small change in the 450 nm band was observed.

T.l.c. of the photolysate revealed a complex mixture. The most mobile component was isolated by column chromatography on silica, followed by sublimation.[6] It proved to be methylvinylmaleimide, obtained in 7% yield (based on the formation of two mol of this imide from each bilirubin molecule). This imide has also been isolated by Lightner and Quistad[7] from a methanolic photolysate, and has been detected, along with several other monopyrrolic fragments (including hematinic acid imide and methylethylmaleimide) after a long-term photolysis of bilirubin in chloroform.[8]

The major products (*ca.* 60% on a weight recovery basis) were a group of three components (A′—C′ in order of decreasing mobility) which gave a positive indication in a pentdyopent test. They could be separated with difficulty, but components A′ and B′ appeared to undergo interconversion. Component C′ was isolated and formulated[5a] as one of the four isomers of the methanol–propentdyopent adduct (II). Compound A′ was also isolated and shown to be an isomeric adduct, but its instability encouraged us to transfer our attention[5b,c] to the corresponding methyl esters. Brief methylation of the crude photolysate with diazomethane, followed by preparative t.l.c., gave three



(II) R^1 = P, R^2 = Me; R^3 and R^4 = Me, vinyl
R^4 = P, R^3 = Me; R^2 and R^1 = Me, vinyl
(the suffix a refers to P = $CH_2{\cdot}CH_2{\cdot}CO_2Me$ throughout the paper)

crystalline esters A—C corresponding to the three acids A′—C′ (II). Their properties are summarised in Table 1.

Mass spectrometry and (for esters A and C) elemental

† For displayed spectra see ref. 5*b*.

[1] R. J. Cremer, P. W. Perryman, and D. H. Richards, *Lancet*, 1958, (i), 1094.

[2] For reviews see J. Lucey, M. Ferreiro, and J. Hewitt, *Pediatrics*, 1968, **41**, 1047; J. D. Ostrow, *Progr. Liver Diseases*, 1972, **4**, 447; 'Bilirubin Metabolism in the Newborn,' eds. D. Bergsma, D. Y. Y. Hsia, and C. Jackson, Williams and Wilkins, Baltimore, 1970.

[3] J. D. Ostrow in 'Bilirubin Metabolism,' ed. I. A. D. Bouchier and B. H. Billing, Blackwell, Oxford, 1967, p. 117.

[4] P. Manitto, *Experientia*, 1971, **27**, 1147; P. Manitto and D. Monti, *J. Pediatrics*, 1973, **83**, 109.

[5] Preliminary reports, (*a*) R. Bonnett and J. C. M. Stewart, *J.C.S. Chem. Comm.*, 1972, 596; (*b*) R. Bonnett, *Ann. New York Acad. Sci.*, 1973, **206**, 722; (*c*) Invited Lecture, Autumn Meeting, Chemical Society, Norwich, 1973.

[6] R. Bonnett and A. F. McDonagh, *Chem. and Ind.*, 1969, 107.

[7] D. A. Lightner and G. B. Quistad, *Nature New Biology*, 1972, **236**, 203.

[8] C. H. Gray, A. Kulczycka, and D. C. Nicholson, *J.C.S. Perkin I*, 1972, 288.

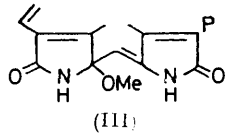
analysis established the molecular formula $C_{18}H_{22}N_2O_5$. Loss of methanol (or methoxyl) from the molecular ion was prominent in each case. The identification of these compounds as isomeric methyl esters (IIa) was confirmed by (i) a positive pentdyopent test in each case, (ii) the similarity of their i.r. spectra, (iii) the general assignments of the n.m.r. spectra (see Table), (iv) the electronic spectra which, although not the same, had maxima in the region of 280 nm found for other methanol–propentdyopent adducts,[9,10] and (v) the oxidation of esters A and C with chromic acid (B was not examined because it equilibrates with A; see later) to give methylvinylmaleimide and methyl 4-methyl-2,5-dioxo-Δ^3-pyrroline-3-propionate (hematinic acid imide methyl ester).

TABLE 1

Properties of the isomeric methanol–propentdyopent esters

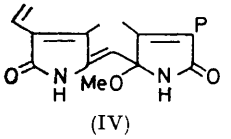
Compound		A	B	C
M.p. (°C)		200—205 (decomp.)	150—156 (decomp.)	180—181 (decomp.)
Pendyopent test ($\lambda_{max.}$/nm)		524	524	526
Fluorescence on t.l.c. plate			Blue-white	
$\lambda_{max.}$(MeOH)/nm (ε)		292(26,200)	296(25,300)	271(22,400)
τ ($CDCl_3$)	NH	1·74, 3·98	1·80, 4·16	1·76, 4·02
	vinyl H (m)	3·64 (2H)	3·58 (2H)	3·52 (1H)
		4·50 (1H)	4·53 (1H)	4·44 (2H)
	meso-H	5·29	5·28	5·22
	CO_2Me	6·36	6·38	6·36
	OMe	6·86	6·88	6·88
	CMe	8·00, 8·11	7·98, 8·18	8·02 (6H)
Structural assignment		(IIIa)	(IVa)	(Va)

An unsymmetrically substituted propentdyopent adduct would be expected to equilibrate, for example under acidic conditions, through the intermediate mesomerically stabilised cation (Scheme 1). Although an example of this equilibration does not appear to have been reported before, if it occurred during the photolysis or during work-up, four isomers [(III)—(VI)] of



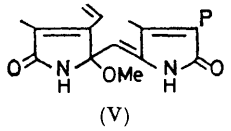
(III)

and



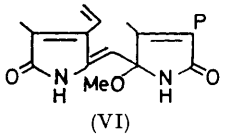
(IV)

from rings C and D



(V)

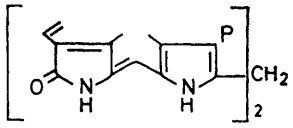
and



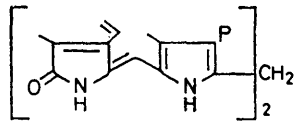
(VI)

from rings A and B

the composition $C_{18}H_{22}N_2O_5$ would be expected [5b,c] from bilirubin. However, the more symmetrical bilirubin isomers (VII) (bilirubin IIIα) and (VIII) (bilirubin XIIIα) would be expected to give only two products each [(III) and (IV) from (VII); (V) and (VI) from (VIII)].



(VII) Bilirubin IIIα

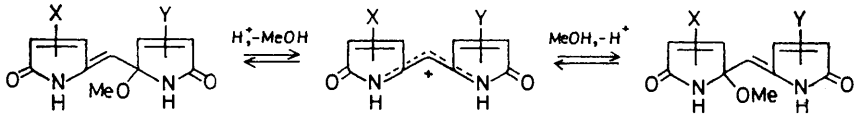


(VIII) Bilirubin XIIIα

After a careful search we have been able to isolate only three isomeric methanol–propentdyopent methyl esters from the photolysis of bilirubin (I); we attribute our inability to detect the fourth to an unfavourable equilibrium (see later). Our assignment of structures to the individual isomers is based on the following considerations.

(i) Isomers A and B were readily equilibrated, for example by refluxing with zinc acetate in methanol or by irradiating the free acids in methanol under the conditions of the preparative experiment. The equilibration also proceeded slowly in the dark in 10% acetic acid in chloroform and could be followed by n.m.r. spectroscopy as indicated in Figure 1. (Isomer C was unaffected under these conditions.) Hence A and B are either the pair (IIIa),(IVa) or the pair (Va),(VIa), but not necessarily respectively.

(ii) Only one of the isomers (B) emitted bluish-white fluorescence on silica when irradiated with u.v. light. We associate this behaviour here with the isolated chromophore (IX): thus it is shown by methylvinylmaleimide but not by methylethylmaleimide.[6] This



SCHEME 1

cannot be regarded as decisive evidence, but suggests that B is either (IVa) or, possibly, (VIa).

(iii) The more symmetrically substituted bilirubin isomers (VII) and (VIII) were prepared by the method of McDonagh and Assisi.[11,12] Photo-oxidation of (VII) gave isomers A′ and B′, whereas photo-oxidation of (VIII) gave only C′. This key result establishes that A and B are (IIIa) and (IVa), not necessarily respectively, and C is (Va) or (VIa).

(iv) In this series the vinyl group adjacent to the imide carbonyl group [as in (IX)] is characterised by two deshielded protons [see structure (IX), τ values for methylvinylmaleimide]. A vinyl group at a β-position not adjacent to an imide carbonyl group shows the ' normal ' pattern of proton signals (one-proton multiplet

[9] F. Pruckner and H. von Dobeneck, *Z. phys. Chem. (Leipzig)*, 1941, **190**, 43.

[10] R. Bonnett, M. J. Dimsdale, and G. F. Stephenson, *Chem. Comm.*, 1968, 1121.

[11] A. F. McDonagh and F. Assisi, *F.E.B.S. Letters*, 1971, **18**, 315.

[12] A. F. McDonagh and F. Assisi, *J.C.S. Chem. Comm.*, 1972, 117.

at low field, two one-proton multiplets at high field). This is illustrated in the n.m.r. spectra of the two

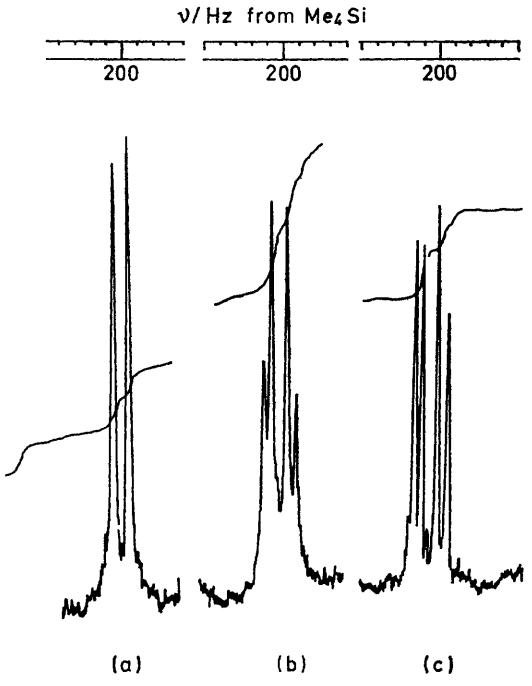
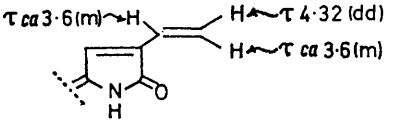


FIGURE 1 Equilibration of methanol–propentdyopent methyl esters in 10% $C(^2H)_3CO_2(^2H)$ in $C(^2H)Cl_3$ at room temperature. *C*-Methyl region of n.m.r. spectrum at (a) $t = 0$, pure isomer A (IIIa); (*b*) $t = 4$ days; (c) $t = 8$ days, equilibrium mixture of A (IIIa) and B (IVa)

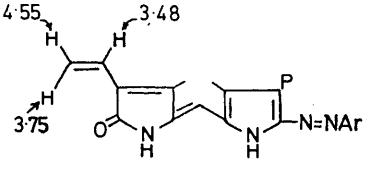
isomeric azobilirubin derivatives (Xa) and (XIa) observed by Jansen and Stoll.[13]

and interpretation here is difficult because these are non-planar, non-rigid systems. Nevertheless the separation of the signals would be expected to be greater for

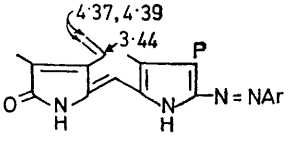


(IX)

B (IVa) than for A (IIIa), as is found (Figure 1). In C (Va) both *C*-methyl groups are internal substituents on a conjugated system and resonate at lower field (fortuitously both at τ 8·02 in chloroform).

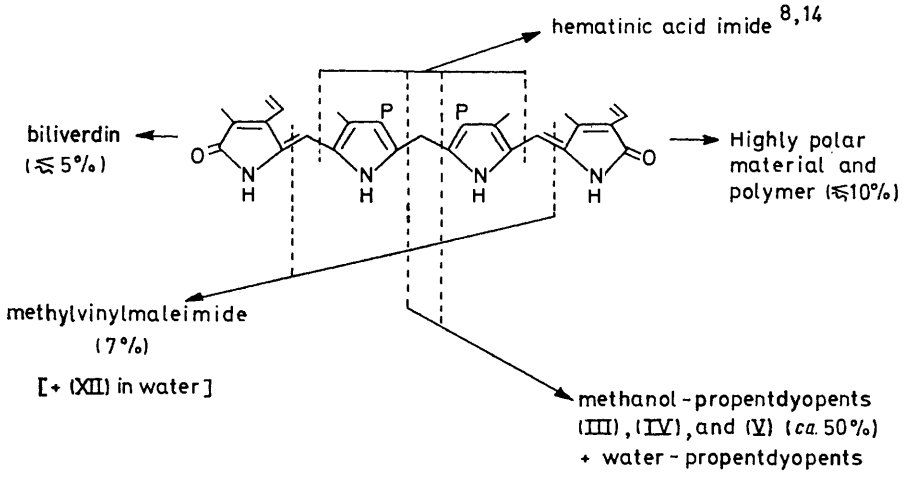


(X) *exo* vinyl group
[τ values refer to methyl ester (Xa)]



(XI) *endo* vinyl group
[τ values refer to methyl ester (XIa)]

Overall the evidence strongly indicates that A is (IIIa), B (IVa), and C (Va). This contradicts the conclusion of Lightner and Quistad[14] who, from a similar reaction, reported two esters which appear to be A and C (although m.p.s and molar absorption coefficients are much lower than those observed here) to which they assign structures (VIa) and (IVa), respectively. Our failure to detect the isomer (VIa) we attribute to unfavourable steric factors. Models show



SCHEME 2 Photo-oxidation of bilirubin in methanol

Table 1 shows that, on this criterion, A and B have *exo*-vinyl groups whereas C has an *endo*-vinyl group. This accords with the evidence from (iii). The chemical shifts of the *C*-methyl groups are similar to one another, that the *endo*-vinyl group of structures (VI) can interact strongly with the *meso*-hydrogen atom; in the isomer (V) this interaction is removed. This destabilisation of one component does not apply to the (III) ⇌ (IV) pair since *both endo*-substituents are methyl groups.

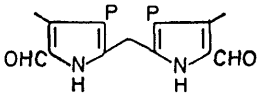
[13] F. H. Jansen and M. S. Stoll, *Biochem. J.*, 1971, **125**, 585; see Figure 2 for displayed spectra (vinyl regions) of (Xa) and (XIa). We use *exo* here to refer to the ' outside ' β-positions of a linear polypyrrole, and *endo* to refer to ' inside ' β-positions.

[14] D. A. Lightner and G. B. Quistad, *F.E.B.S. Letters*, 1972, **25**, 94.

The more polar components of the photolysate consisted of (i) a small quantity of water–propentdyopent methyl ester [the compound isolated crystalline was the hydroxy-analogue of (Va)], and (ii) a minor amount (*ca.* 10% on a weight recovery basis) of a mixture of at least three components. These were not further investigated but may well include products of addition at vinyl groups.

Thus on photo-oxidation in methanol, the bilirubin system breaks up about the three *meso*-bridges as shown in Scheme 2.

Cleavage about C-5 and -15 might be expected [15] to give, besides methylvinylmaleimide, the dialdehyde (XII) derived from rings B and C. This compound was not detected in methanolic photolysates but was observed



(XII)

as a minor product of photo-oxidation in aqueous solution and was isolated as the dimethyl ester (XIIa), identified by comparison with an authentic specimen.[16] This dialdehyde appears also to have been observed as a product of the alkaline degradation of bilirubin.[17]

Mechanistic Aspects.—Biliverdin, the dehydrogenation product of bilirubin, has been proposed [7,18] as an intermediate in the photo-oxidation. In methanol, however, biliverdin is rather resistant to photo-oxidation [19] and we conclude that, although it is formed in a minor process (*ca.* 5%), biliverdin is not an intermediate in the main pathway to cleavage products in this solvent. The relative importance of dehydrogenation does depend on solvent, however. In anhydrous freshly distilled chloroform the formation of biliverdin becomes important and phosgene is formed at the same time. It is thought [5a] that this dehydrogenation involves a radical abstraction at the C-10 methylene bridge, *i.e.* it follows a Type I photo-oxidation pathway [20] involving organic radicals and triplet oxygen. In chloroform stabilised with alcohol the radical dehydrogenation is expected to be inhibited (as is the formation of phosgene).

McDonagh's suggestion [15] that the photo-oxidation of bilirubin is a self-sensitised singlet oxygen reaction was supported by experiments with solutions in reagent grade chloroform (*i.e.* presumably containing alcohol stabiliser). Singlet oxygen is thought to be an intermediate in the photo-bleaching of bilirubin in methanolic solution on the following grounds.

(i) The reaction rate is increased by the addition of Rose Bengal: [19] t.l.c. reveals the same product distribution as is found in the reaction in the absence of added sensitiser.

(ii) The reaction rate is decreased by adding β-carotene [19] (a singlet oxygen quencher [21]) or 2,5-dimethylfuran [19] (a singlet oxygen trap). The effect of these additives is shown in Figure 2, which also indicates that the photo-oxidation is first-order in bilirubin under the conditions employed. Cyclopentadiene does not appear to be effective as a competitive inhibitor,[5b,c] but is known [22] to be a less reactive dienophile towards singlet oxygen than is 2,5-dimethylfuran.

(iii) Because deactivation of the electronically excited singlet oxygen (by energy transfer to appropriate overtone and combination bands of C–H and O–H stretching modes of the methanol solvent) is reduced when the methanol is completely deuteriated (since the overtone bands no longer fall at appropriate energies), the lifetime of $^1\Delta g$ oxygen is longer in $C(^2H)_3O(^2H)$ (τ expected to be >35 μs) than it is in CH_3OH (τ 7 μs).[23] Kearns has predicted that, as a consequence, the rate of a reaction involving singlet oxygen in a rate-determining

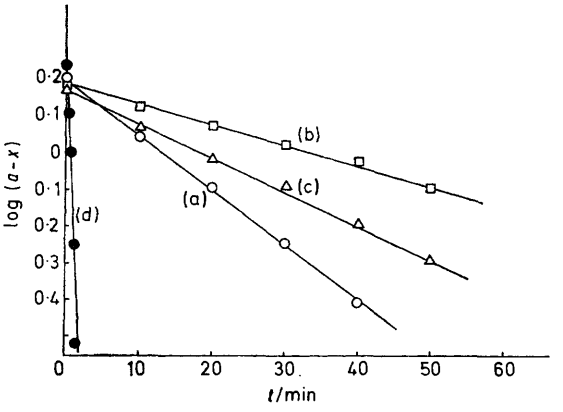


FIGURE 2 Photo-oxidation of bilirubin (*ca.* $1{\cdot}5 \times 10^{-5}$M) in methanol (0·05N in NH_3), flushed with oxygen and irradiated in a Pyrex vessel at 18° with a 500 W lamp (Phillips Arga Photo B) in a standard configuration. Rates of degradation of bilirubin were judged by diminution of the 450 nm band; (a) no additive; (b) with added 2,5-dimethylfuran ($1{\cdot}5 \times 10^{-2}$M); (c) with β-carotene ($9{\cdot}2 \times 10^{-6}$M) and 1% tetrahydrofuran; (d) with Rose Bengal ($1{\cdot}5 \times 10^{-6}$M)

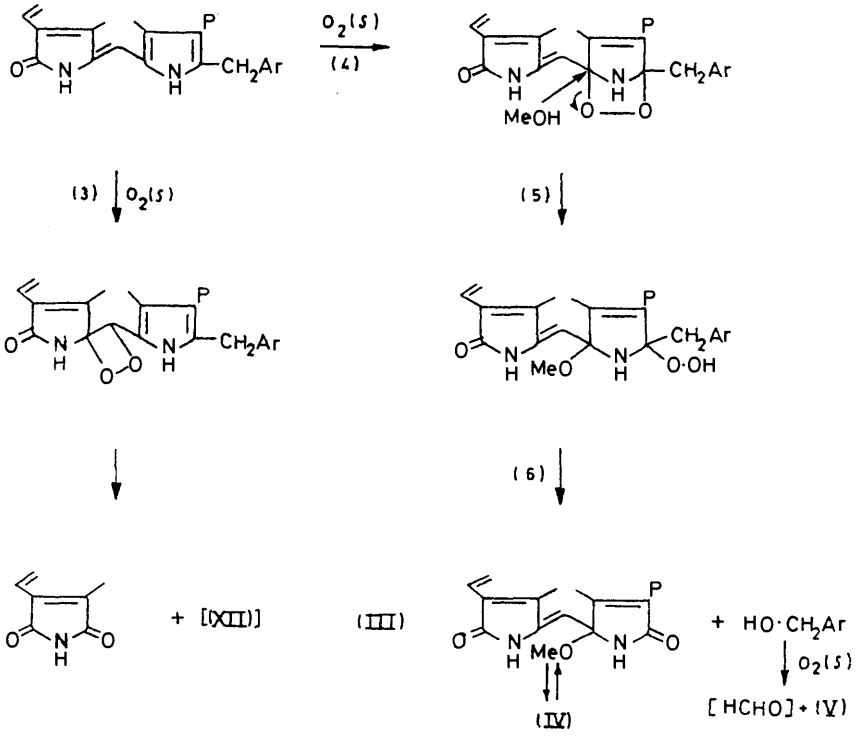
step is about ten times greater in $C(^2H)_3O(^2H)$ than it is in CH_3OH, and has suggested that this is a good criterion for a singlet oxygen reaction. We have found that photo-bleaching of bilirubin shows a five-fold rate increase in changing the solvent from methanol to [$^2H_4$]methanol,[19] which accords with a singlet oxygen mechanism.

That bilirubin can act as a sensitiser has been demonstrated by employing it to photosensitise the oxygenation of 2,5-dimethylfuran.[19] Bilirubin is, however, a much less efficient photosensitiser than is Rose Bengal partly because, unlike Rose Bengal, it is itself readily

[15] A. F. McDonagh, *Biochem. Biophys. Res. Comm.*, 1971, 1306.
[16] P. S. Clezy, C. J. R. Fookes, and A. J. Liepa, *Austral. J. Chem.*, 1972, **25**, 1979.
[17] J. D. Ostrow, D. C. Nicholson, and M. S. Stoll, *Gastroenterology*, 1971, **60**, 186.
[18] J. D. Ostrow and R. V. Brantham, *Birth Defects: Original Article Series*, 1970, **6**, 93.
[19] R. Bonnett and J. C. M. Stewart, *Biochem. J.*, 1972, **130**, 895.
[20] G. O. Schenck, *Ind. and Eng. Chem.*, 1963, **55**(6), 40.
[21] C. S. Foote, Y. C. Chang, and R. W. Denny, *J. Amer. Chem. Soc.*, 1970, **92**, 5216.
[22] C. S. Foote, S. Wexler, W. Ando, and R. Higgins, *J. Amer. Chem. Soc.*, 1968, **90**, 975.
[23] P. B. Merkel, R. Nilsson, and D. R. Kearns, *J. Amer. Chem. Soc.*, 1972, **94**, 1030; P. B. Merkel and D. R. Kearns, *J. Amer. Chem. Soc.*, 1972, **94**, 7244.

attacked by singlet oxygen. A similar explanation has been offered [19] for the observation that bilirubin (in superficial tissue in jaundice), unlike for example, uroporphyrin (in superficial tissue in certain porphyrias), does not appear to be associated with photo-sensitivity in man.

The present view of the reaction in methanol is shown in Scheme 3. Step (2) of this mechanism implies that



SCHEME 3 Suggested mechanism for the photo-oxidation of bilirubin in methanol

the triplet energy of bilirubin is greater than 94 kJ mol^{-1}. The magnitude of E_T for bilirubin is not known, and phosphorescence spectra of bilirubin do not appear to have been observed. Bilirubin in methanol (containing, as usual, a trace of ammonia to effect solubilisation) does not luminesce visibly at room temperature; [24] in a glass at 77 K a greenish emission is observed which fades as the glass melts. The emission is strong in polar solvents (*e.g.* acetone–methanol, dimethylformamide) but weak or absent in less polar ones ($CHCl_3$, CS_2, PhCl). Fluorimetry reveals a structureless broad band with a maximum at 525 nm (228 kJ mol^{-1}). This may represent fluorescence (with a large Stokes shift, not unexpected for such a non-rigid structure) but could also be a phosphorescent emission: lifetime measurements should settle this point.

Step (3) of the mechanism [15] parallels known reactions of enamines with singlet oxygen.[25] Step (4) involves a 1,4-cycloaddition to the pyrrole-type ring B (or C), followed by methanolysis at the bis-allylic centre [step (5)].* A formal analogy exists between step (6) and the Hock cleavage of hydroperoxides,[26] and, overall, the generation of the 5-methoxy-lactam structure of (III) and its isomers finds analogy in the sensitised photo-oxidation of simple alkylpyrroles.[27]

$$\text{Bilirubin } S_0 \xrightarrow{h\nu} \text{Bilirubin } S_1 \xrightarrow{\text{ISC}\dagger} \text{Bilirubin } T_1 \qquad (1)$$

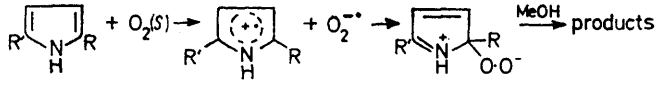
E_{S_1} *ca.* 265 kJ mol^{-1}. † Intersystem crossing.

$$\text{Bilirubin } T_1 + O_2(T) \longrightarrow \text{Bilirubin } S_0 + O_2(S) \qquad (2)$$

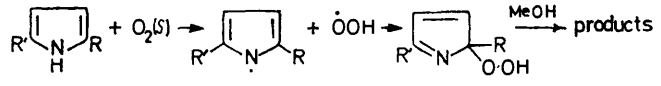
$E_T = ?$ $\qquad$ $E_{S_1} = 94$ kJ mol^{-1}

It is concluded that the singlet oxygen hypothesis of bilirubin photo-oxidation is well supported for methanolic solutions. The reaction is remarkably sensitive to

* Alternative steps avoiding 1,4-cycloaddition may be written, for example involving charge transfer:



or imino hydrogen abstraction:



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[25] C. S. Foote and J. W. P. Lin, *Tetrahedron Letters*, 1968, 3267.
[26] K. Gollnick, *Adv. Photochem.*, 1968, **6**, 1.
[27] L. K. Low and D. A. Lightner, *J.C.S. Chem. Comm.*, 1972, 116.

solvent, however. Thus in carbon disulphide, in which solvent singlet oxygen is reported [23] to be long-lived (τ 200 μs), there is no marked increase in the rate of photobleaching. Since a rate enhancement is observed if other photosensitisers (*e.g. meso*-tetraphenylporphyrin) are present this indicates that bilirubin cannot function efficiently as a photosensitiser in carbon disulphide. Possibly carbon disulphide quenches electronically excited bilirubin (*cf.* absence of luminescence in this solvent). A specific interaction between solvent and ground-state molecules may be of more importance, and is indicated by the absorption spectrum, where the maximum (470 nm) appears at much longer wavelengths than it does in chloroform or methanol ($\lambda_{max.}$ *ca.* 450 nm for each). The sensitivity of this photoreaction towards change of solvent, including aqueous systems of biological interest, is being examined further.

EXPERIMENTAL

General experimental conditions and conventions used in presenting data [*e.g.* $\lambda_{max.}$ in nm (ε in parentheses); $\nu_{max.}$ in cm^{-1}] were given previously.[28] Irradiations were carried out on *ca.* 10^{-3}M-solutions flushed with oxygen in a Pyrex vessel with a water-cooled (15—18 °C) outer jacket. The light source was a 500 W lamp (Philips Arga Photo B) set about 10 cm from the reactor in a standard configuration. Except where otherwise stated the solvent (methanol) contained anhydrous ammonia (0·2%; from liquid NH_3) to solubilise the bilirubin. The following t.l.c. systems were used with plates prepared from silica gel HF_{254} (Merck): (i) methanol–propentydyopent free acids: ethyl acetate–benzene–acetic acid (80 : 30 : 2); (ii) methanol–propentdyopent methyl esters: ethyl acetate–benzene (1 : 1) (preparative work) and (7 : 3) (analytical work). Quoted R_F values refer to analytical conditions.

The pentdyopent test was carried out as follows. A speck of propentdyopent adduct in methanol (1 drop) was treated with aqueous 20% potassium hydroxide (5 drops) followed by solid sodium dithionite (*ca.* 1—2 mg). A reddish pink colour [$\lambda_{max.}$ (H_2O) 519 nm for tetraethyl-(methanol–propentdyopent)] developed rapidly in the cold or on gentle warming.

Photo-oxidation of Bilirubin in Methanol without Esterification.—(i) *Methylvinylmaleimide.* Bilirubin (208 mg) in anhydrous methanol (500 ml; containing 0·2% NH_3) was irradiated (2 × 500 W) with oxygen flush for 24 h. The solvent was removed (35 °C) to give a pale brown glass. Chromatography on silica gel (Hopkin and Williams MFC; 10 cm × 4 cm diam) with ethanol-free chloroform gave methylvinylmaleimide (6·4 mg, 7% based on formation of 2 mol. equiv. of the imide) (M^+, 137·048. Calc. for $C_7H_7NO_2$: *M*, 137·048). The n.m.r. and mass spectra agreed with the literature.[6]

(ii) *Methanol–Propentdyopents* (as the propionic acids). Bilirubin (204 mg) was irradiated as described above (but with one 500 W lamp). The products, separated by preparative t.l.c. (200 × 200 × 1 mm), were as follows in order of decreasing R_F value: (a) *methylvinylmaleimide* (2·5%) (estimated spectroscopically); (b) *methanol–propentdyopent isomer A'* (R_F 0·34, non-fluorescent), crystallised (yield 22 mg, 19%) from methanol, m.p. (gradual decomp.) >150° (Found: M^+, 332·137. $C_{14}H_{20}N_2O_5$ requires *M*, 332·137); positive pentdyopent test ($\lambda_{max.}$ 526 nm); $\lambda_{max.}$ (MeOH) 286 (21,000); $\nu_{max.}$ (KBr) 3365, 3210, 3060—3080, 1692, 1660, 1258, 1010, 995, 935, 925, and 700; τ (100 MHz; C_5D_5N) low field signals partially obscured by solvent, 3·30 (m, $=CH_2$), 4·38 (impurity), 5·03 (s, *meso*-H), 6·96 (s, OMe), 7·12br (m, $CH_2{\cdot}CH_2$), 8·08br (s, CMe), and 8·15 (s, CMe); *m/e* (150°) 333 (28%), 332 (100), 317 (10), 314 (8), 302 (10), 301 (44), 300 (5), 299 (30), 281 (15), 271 (15), 258 (18), 257 (13), 256 (18), and 255 (30); (c) *methanol–propentdyopent isomer B'* (R_F 0·29, blue-white fluorescence) (29 mg, 25%); positive pentdyopent test ($\lambda_{max.}$ 525 nm); (d) *methanol–propentdyopent isomer C'* (R_F 0·21, non-fluorescent) (36 mg, 31%), m.p. (gradual decomp.) >160° (Found: M^+, 332·137); positive pentdyopent test ($\lambda_{max.}$ 527 nm); $\lambda_{max.}$ (MeOH) 270 (26,000) (redetermined values [5a]); $\nu_{max.}$ (KBr) 3360, 3270, 3080, 1715, 1702, 1685, 1655, 1266, 1253, 1010, 986, and 933; τ (100 MHz; C_5D_5N) 0·26br (s, NH), 0·62br (s, NH), 3·45br (s + dd, *J* 11 and 18 Hz, H_2O? + vinyl CH), 4·30 (d, *J* 18 Hz, vinyl CH), 4·67 (d, *J* 11 Hz, vinyl CH), 4·92 (s, *meso*-H), 6·98 (s, OMe), 7·16br (s, $CH_2{\cdot}CH_2$), 8·02 (s, CMe), and 8·13 (s, CMe); τ [220 MHz; $(CD_3)_2SO$] 0·90br (s, NH, exchangeable), 1·39br (s, NH, exchangeable), 3·44 (dd, *J* 11 and 17 Hz, vinyl CH), 4·44 (d, *J* 17 Hz, vinyl CH), 4·54 (d, *J* 11 Hz, vinyl CH), 5·06 (s, *meso*-H), 7·02 (s, OMe), 7·63 (m, $CH_2{\cdot}CH_2$), 8·10 (s, CMe), and 8·18 (s, CMe); *m/e* (155°) 333 (19%), 332 (90), 317 (10), 314 (5), 303 (5), 302 (25), 301 (60), 300 (100), 299 (35), 287 (5), 286 (8), 285 (29), 284 (12), 283 (5), 282 (10), 281 (10), 275 (5), 274 (10), 273 (30), 272 (20), 271 (35), 259 (18), 258 (6), 257 (18), 256 (33), 255 (62), 254 (30), and 253 (10).

The more polar bands were rechromatographed with ethyl acetate as irrigant to give a further quantity (10 mg) of isomer C' together with water–propentdyopent isomer C' (R_F 0·10) (13 mg).

Photo-oxidation of Bilirubin in Methanol: Isolation of Methanol–Propentdyopent Adducts as Methyl Esters.—Bilirubin (101 mg) in methanolic ammonia (500 ml) was irradiated as before (1 × 500 W; 24 h). The khaki-brown solution was concentrated. The residue was dissolved in methanol and treated with an excess of diazomethane in ether, the excess being destroyed (HOAc) after 1·5 min. The solvent was removed and the components were separated by preparative t.l.c. [200 × 200 × 1 mm, ethyl acetate–benzene (1 : 1), extraction from the adsorbent with methanol]. The following were obtained, in order of decreasing mobility: (i) methylvinylmaleimide (2·3% estimated spectroscopically).

(ii) *Methanol–propentdyopent methyl ester isomers A and B.* Although they separate under analytical conditions [R_F (A) 0·27, non-fluorescent; R_F (B) 0·24, blue-white fluorescence] these components were not clearly separated in the preparative run. The mixed isomers (amorphous; 19 mg, 32%) were dissolved in methanol (2 ml). Isomer A (8·7 mg) crystallised as fine white *needles*, m.p. 200—205° (decomp.) (Found: C, 62·15; H, 6·25; N, 7·95%; M^+, 346·152. $C_{18}H_{22}N_2O_5$ requires C, 62·4; H, 6·4; N, 8·1%; *M*, 346·153); $\nu_{max.}$ (KBr) 3380, 3200, 3160, 1731, 1694, 1656, 1252, 1175, 1144, 1070, 1003, and 700; τ ($CDCl_3$) 1·74br (s, NH), 3·64 (m, 2 vinyl H), 3·98br (s, NH), 4·50 (dd, 1 vinyl H), 5·29 (s, *meso*-H), 6·36 (s, CO_2Me), 6·86 (s, OMe), 7·38 (s, $CH_2{\cdot}CH_2$), 8·00 (s, CMe), and 8·11 (s, C-Me); *m/e* (140°) 347 (24%), 346 (100), 331·129 (17; $C_{17}H_{19}N_2O_5$ requires 331·129), 316 (8), 315 (30), 314·126 (14; $C_{17}H_{18}N_2O_4$ requires 314·127), 300 (10), 299 (42), 288 (10), 284 (10),

[28] R. Bonnett and A. F. McDonagh, *J.C.S. Perkin I*, 1973, 881.

283 (41), 272 (6), 271 (15), 259 (6), 257 (9), 256 (9), and 255 (35).

Careful chromatography of material from the mother liquors enriched in isomer B gave the latter (4·8 mg) as *microcrystals*, m.p. 150—156° (decomp.) (from ethyl acetate) as a homogeneous (t.l.c.) substance (Found: M^+, 346·152); $\nu_{max.}$ (KBr) 3340, 3280i, 3200i, 1733, 1690, 1655, 1256, 1180, 1100, 1052, 1005, and 697; τ ($CDCl_3$) 1·8br (s, NH), 3·58 (m, 2 vinyl H), 4·16br (s, NH?), 4·53 (dd, 1 vinyl H), 5·28 (s, *meso*-H), 6·38 (s, CO_2Me), 6·88 (s, OMe), 7·40 (s, CH_2·CH_2), 7·98 (s, CMe), and 8·18 (s, CMe); *m/e* (130°) 347 (7%), 346 (30), 331 (4), 316 (7), 315 (25), 314 (15), 299 (13), 288 (5), 284 (10), 279 (14), 260 (20), 259 (100), 256 (11), and 255 (25).

(iii) *Methanol–propentdyopent methyl ester isomer C.* This (R_F 0·17, non-fluorescent) was obtained as an apparently homogeneous solid (23 mg, 38%) which crystallised from methanol–ethyl acetate to give colourless *rosettes* (9·4 mg), m.p. 180—181° (decomp.) (Found: C, 62·05, 61·85; H, 6·50, 6·50; N, 7·2, 7·95%; M^+, 346·152); $\nu_{max.}$ (KBr) 3360, 3280, 3200i, 3080, 1732, 1704, 1688, 1652, 1260,1182, 1142, 1100, 1075, 1045, 1110, and 685; τ ($CDCl_3$) 1·76br (s, NH, exchangeable), 3·52 (m, 1 vinyl H), 4·02br (s, NH, exchangeable), 4·44 (m, 2 vinyl H), 5·22 (s, *meso*-H), 6·36 (s, CO_2Me), 6·88 (s, OMe), 7·38br (s, CH_2·CH_2), and 8·02 (s, 2 × CMe); *m/e* (165°) 347 (20%), 346 (95), 331 (5), 316 (9), 315 (35), 314 (35), 313 (5), 299 (17), 284 (5), 283 (24), 282 (8), 273 (8), 272 (9), 271 (13), 260 (20), 259 (100), 258 (9), 257 (9), 256 (15), 255 (44), 254 (17), and 253 (7).

(iv) *Water–propentdyopent methyl ester isomer C.* This (R_F 0·08) was obtained crystalline (2·2 mg), m.p. 209—212° (decomp.) (from ethyl acetate) (Found: M^+, 332·138. $C_{17}H_{20}N_2O_5$ requires M, 332·137); $\lambda_{max.}$ (MeOH) 272; positive pentdyopent test, slowly until warmed ($\lambda_{max.}$ 524 nm); *m/e* (180°) 333 (7%), 332 (28), 317 (8), 316(38), 315 (31), 314 (100), 313 (13), 302 (6), 301 (34), 300 (30), 299 (16), 285 (26), 284 (15), 283 (45), 282 (6), 259 (7), 258 (35), 257 (9), 256 (19), 255 (24), 254 (30), 253 (14), and 252 (9). Methanolysis (10% HOAc–MeOH; reflux; 1 h) gave a product identical (t.l.c.) with the methanol–propentdyopent methyl ester isomer C.

(v) *More polar components.* These (9 mg) were combined. This fraction consisted of three major constituents which were not studied further.

Comparative Photo-oxidation of Bilirubin IIIα, Bilirubin IXα, and Bilirubin XIIIα.—The isomeric bilirubins were prepared after the procedure of McDonagh and Assisi [12] as follows. Concentrated hydrochloric acid (5 ml) was added to a solution of bilirubin (50 mg) in dimethyl sulphoxide (45 ml) and the mixture was kept at room temperature for 1 min with gentle shaking. The solution was poured into water (1 l). The orange precipitate was collected on Whatman GF/A glass fibre paper, and washed in turn with water and methanol. The isomeric bilirubins were extracted with chloroform and separated by t.l.c. (Merck silica gel G; 200 × 200 × 1 mm; 2% AcOH in $CHCl_3$) to give small quantities of bilirubin IIIα (R_F 0·76), bilirubin IXα (R_F 0·69), and bilirubin XIIIα (R_F 0·60).

Solutions of the individual bilirubins in methanol (50 ml) were irradiated at 18° for 4 h with oxygen flushing. The initial and final absorption maxima are given in Table 2. T.l.c. of the products showed that the IXα isomer gave the same distribution of products as observed before. The XIIIα isomer gave methylvinylmaleimide and methanol–propentdyopent isomer C′ (R_F 0·21) and water–propentdyopent isomer C′ (R_F 0·12) only. The IIIα isomer gave methanol–propentdyopent isomer A′ (R_F 0·34) and isomer B′ (R_F 0·29) together with a trace of material having the same R_F value as isomer C′. This is probably the water–propentdyopent derived from isomer A′ since after methanolysis (10% AcOH–MeOH; 30 min; reflux) this weak spot was no longer apparent.

TABLE 2

Bilirubin	Initial $\lambda_{max.}$/nm (absorbance)	Final $\lambda_{max.}$/nm
XIIIα	448 (0·64)	273 (i)
IXα	452 (1·32)	289
IIIα	454 (0·63)	293

Oxidation of Methanol–Propentdyopent Methyl Esters.—Methanol–propentdyopent methyl ester isomer A (2·5 mg), stirred in acetone (1 ml) at 0°, was treated with a solution of chromium trioxide (14 mg in 2 ml of 2N-H_2SO_4) dropwise over 5 min. The solution was stirred for 1 h at room temperature and then extracted with ethyl acetate (2 × 10 ml). The extract was treated with a little solid anhydrous sodium sulphate and sodium hydrogen carbonate, filtered, and taken to dryness. T.l.c. [silica gel HF254; EtOAc–PhH (1 : 10)] showed, besides a trace of starting material, only methylvinylmaleimide and hematinic acid imide methyl ester. To confirm its identity the latter oxidation product was isolated by preparative t.l.c. in the same solvent system; *m/e* 197 (15%, $C_9H_{11}O_4$), 165 (100), and 137 (90). Methanol–propentdyopent methyl ester isomer C (2·2 mg) was oxidised in the same way, and gave the same oxidation products.

Interconversion of Methanol–Propentdyopent Isomers.—(i) *Irradiation of the free acids.* Methanol–propentdyopent (isomer A′, absorbance 0·90 at $\lambda_{max.}$ 288 nm) in methanol (50 ml) containing Rose Bengal (absorbance 0·20 at 550 nm) was subjected to photo-oxidation. After 100 min $\lambda_{max.}$ had shifted to 296 nm, with no loss of intensity. T.l.c. showed that methylvinylmaleimide was not detectable, but that both isomers A′ and B′ were present.

In a similar experiment with methanol–propentdyopent (isomer C′) only a slight shift in $\lambda_{max.}$ was observed (273 ⟶ 268 nm). T.l.c. revealed only the starting material.

(ii) *Treatment of the methyl ester with zinc acetate.* Methanol–propentdyopent methyl ester (isomer A; 0·4 mg) in methanol (0·5 ml) was refluxed with saturated methanolic zinc acetate (5 ml) for 5 min. The solution became yellow. T.l.c. indicated the presence of isomers A (the starting material) and B (the fluorescent isomer), together with some material of lower R_F value.

The experiment was repeated with isomer C. In this case no second (fluorescent?) product was detected.

(iii) *Treatment of the methyl esters with* [$^2H_4$]*acetic acid–*[^2H]*chloroform.* The methyl ester (isomer A; 7·3 mg) in $C(^2H)Cl_3$ (0·5 ml) containing $C(^2H)_3CO_2(^2H)$ (0·05 ml) was kept in the dark at 22°, and the n.m.r. spectrum was recorded at intervals. The signals at τ 8·01 and 8·12 decreased, and new signals, attributed to isomer B, appeared at 7·98 and 8·19. After 8 days approximately equal amounts of A and B were present (Figure 1).

No change in the n.m.r. spectrum of isomer C was observed under these conditions.

Isolation of 5,5′-Diformyl-3,3′-bis(2-methoxycarbonylethyl)-4,4′-dimethyldipyrrylmethane from an Aqueous Photolysate of Bilirubin.—Bilirubin (100 mg) in aqueous 0·08N-ammonia (500 ml) was irradiated for 26 h as before. The

solution was concentrated (to *ca.* 50 ml), extracted with chloroform (2 × 25 ml), and taken to dryness. The residue in methanol (20 ml) was methylated (CH_2N_2–Et_2O; 2 min), and again taken to dryness. The residue was extracted with chloroform–methanol (1 : 1; 25 ml) and the extract was subjected to preparative t.l.c. on silica gel GF_{254} irrigated with ethyl acetate. From the complex mixture (R_F 0·59, 0·43, 0·27, 0·15, 0·06, and 0·0) the component with R_F 0·06 was shown to be water–propentdyopent methyl ester isomer C (11·5 mg), m.p. 209—212°. The component with R_F 0·43 was separated from a yellowish contaminant by crystallisation from methanol to give the dialdehyde (XIIa) (4·5 mg, 6·5%), m.p. 179·5—180°, mixed m.p.[16] 178·5—179·5°, M^+ 402·178 ($C_{21}H_{26}N_2O_6$ requires *M*, 402·179); τ ($CHCl_3$) 0·54 (s, CHO), 5·98 (s, CH bridge), 6·34 (s, OMe), 7·23 and 7·48 (2 t, CH_2·CH_2), and 7·74 (s, Ar*Me*).

Luminescence of Bilirubin.—When irradiated with a medium-pressure mercury lamp (365 nm) bilirubin did not visibly luminesce in fluid solution at room temperature in any of the freshly distilled solvents tested. A yellowish-green emission was observed in frozen solutions (77 K) in the following solvents: acetone–methanol (1 : 1), dimethylformamide, and methanol containing 0·2% anhydrous ammonia; but not in the following: carbon disulphide, chloroform, chlorobenzene, and benzene. The emission from the methanol glass was recorded ($\lambda_{max.}$ 525 nm) on a Beckman DK-2A spectrofluorimeter.

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