## Singlet oxygen reactivity of bilirubin and related tetrapyrroles

D. A. Lightner[1] and Y.-T. Park

*Department of Chemistry, University of Nevada, Reno (Nevada 89557, USA), 23 September 1977*

*Summary.* The rates of chemical reactivity ($k_R$) and physical quenching ($k_Q$) of singlet oxygen by bilirubin IX*α*, mesobilirubin IX*α*, bilirubin IX*α* dimethyl ester, aetiobilirubin IV*γ*, biliverdin IX*α*, biliverdin IX*α* dimethyl ester, aetiobiliverdin IV*γ* and an oxodipyrromethene have been determined. The $k_R$ and $k_Q$ values approach the diffusion threshold for the bilirubin-like substrates, but $k_R < k_Q$ by about a factor of $10^3$ for the verdins. A reaction mechanism involving superoxide ion is suggested. Bilirubin appears to quench singlet oxygen by an electron-transfer mechanism.

The phototherapy method[2] for treating physiologic jaundice in newborn infants owes its success, in part, to photodestruction of bilirubin IX*α* (BR)[3,4]. BR can undergo photodestruction in vitro under either anaerobic[3,5] or aerobic[3-7] conditions. Photodestruction with visible light is more rapid under aerobic conditions[5], and the reaction involves BR-sensitized production of singlet oxygen ($^1O_2$) followed by rapid reaction with it[8,9]. Since the light selection in phototherapy is invariably visible or blue light[10,11], it becomes important to know the quantitative aspects of BR photoreactivity and its reactivity toward $^1O_2$, especially since $^1O_2$ is implicated in photodynamic action[11,12]. In this work we report on the rate constants for quenching ($k_Q$) and reaction ($k_R$) of $^1O_2$ with BR and related substances in chloroform and methanol.

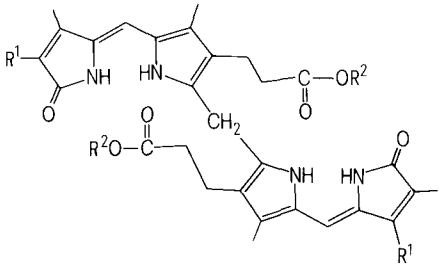
*Materials and methods.* Bilirubin IX*α* (**1**) was obtained from MCB and crystallized from chloroform-methanol. It contained less than 3% of the III*α* and XIII*α* isomers as determined by T. A. Wooldridge using high pressure liquid chromatography on silica (Zorbax-Sil) with $CHCl_3$-1% acetic acid. Mesobilirubin IX*α* (**2**) was prepared by G. Bisacchi using a catalytic hydrogenation of **1** in methanolic ammonia with 5% Pd(C). Its dimethyl (**3**) ester was prepared by reaction with diazomethane[13]. Aetiobilirubin IV*γ* (**4**) and aetiobiliverdin IV*γ* (**8**) were supplied by Prof. K. M. Smith (University of California, Davis), and pure biliverdin IX*α* (**6**) was supplied by Prof. A. F. McDonagh (University of California, San Francisco). Biliverdin IX*α* dimethyl ester (**7**) was prepared by D. C. Crandall[14] and oxodipyrromethene (**5**) was synthesized by G. B. Quistad[15]. The methanol and chloroform solvents used were distilled reagent grade from MCB.

Singlet oxygen ($^1O_2$) was produced by excitation of Rose Bengal (RB) (Matheson) or Rose Bengal-18-crown-6 ether (RBCE)[16] (eqs. 1 and 2) with monochromatic light, 10-nm bandpass, from a Bausch and Lomb Model 33-86-07 monochromator and a 15-W tungsten-halogen source. RB was used to produce $^1O_2$ in methanol solutions by excitation at 557 nm ($\lambda_{max}$) and RBCE was used to produce $^1O_2$ in chloroform solution by excitation at 560 nm ($\lambda_{max}$). These excitation wavelengths are sufficiently far removed from the BR long wavelength $\lambda_{max}$ (450 nm) that excitation of the dye-sensitizer with 10-nm bandpass light involves no direct excitation of BR.

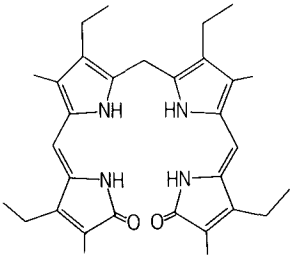
$$^1RB_0 \text{ or } ^1RBCE_0 \xrightarrow[\lambda = 557 \text{ or } 560 \text{ nm}]{h\nu} {}^3RB \text{ or } {}^3RBCE \qquad (1)$$

$$^3RB \text{ or } {}^3RBCE + {}^3O_2 \longrightarrow {}^1O_2 + {}^1RB_0 \text{ or } {}^1RBCE_0 \qquad (2)$$

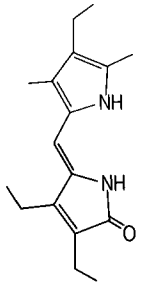
The $^1O_2$ produced in this way may decay ($k_d$), be physically quenched ($k_Q$) by substrate (S), or chemically react ($k_R$)



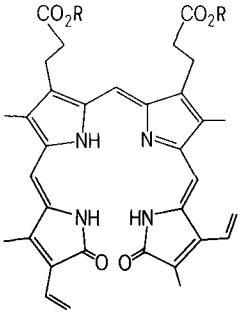
**1** $R^1 = CH{=}CH_2, R^2 = H$
**2** $R^1 = CH_2CH_3, R^2 = H$
**3** $R^1 = CH{=}CH_2, R^2 = CH_3$



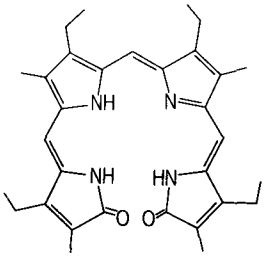
**4**



**5**



**6** R = H
**7** R = $CH_3$



**8**

Physical ($k_Q$) and reactive ($k_R$) rate constants for substrate singlet oxygen reactivity

| Substrate ($10^{-5}$ M) | Value $\times 10^9 M^{-1} sec^{-1}$ in $CHCl_3$[a] $(k_Q+k_R)$ | $k_R$ | Value $\times 10^9 M^{-1} sec^{-1}$ in $CH_3OH$[b] $(k_Q+k_R)$ | $k_R$ |
|---|---|---|---|---|
| **1** Bilirubin IX*a* | 2.8 | 0.38 | 2.1[c] | 0.28[c] |
| | 2.5[d] | 0.43[d] | – | – |
| | 2.5[e,f] | 0.17[e,f] | – | – |
| | – | 0.01[g,h] | – | – |
| | – | 0.10[h,i] | – | – |
| **2** Mesobilirubin IX*a* | 2.8 | 0.59 | 2.5[c] | 0.79[c] |
| | – | 0.06[h,i] | | |
| **3** Bilirubin IX*a*-dimethyl ester | 1.8 | 0.67 | 2.3 | 0.55 |
| | – | 0.01[h,i] | 3.3[j] | 0.55[j] |
| **4** Aetiobilirubin IV$\gamma$[k] | 3.0 | 2.3 | 2.2[j] | 0.85[j] |
| **5** Oxodipyrromethene | 4.3 | 3.0 | 2.7 | 1.4 |
| | | | 2.1 | 1.0 |
| | – | 0.7[h,i] | – | – |
| **6** Biliverdin IX*a* | 1.7 | 0.0019 | 8.4 | 0.0024 |
| | 3.3[e] | $\leq 0.003$[e] | – | – |
| **7** Biliverdin IX*a*-dimethyl ester | 1.4 | 0.0012 | 3.3 | 0.0016 |
| | 0.8[h,l] | 0.0006[h,l] | – | – |
| **8** Aetiobiliverdin IV$\gamma$[k] | 1.8 | 0.0043 | 5.5 | 0.0035 |

[a] 18-Crown-6 Rose Bengal sensitizer. [b] Rose Bengal sensitizer. [c] With 0.2% (vol.) conc. $NH_4OH$. [d] Data of Foote and Ching[17]. [e] Data of Stevens and Small[22]. [f] $CCl_4$ solvent. [g] Data of Matheson et al.[31]. [h] Freon -113 solvent using Nd-YAG laser direct production of $^1O_2$ at high $^3O_2$ pressures. [i] Recent data of I.B.C. Matheson. [j] $CH_3OH$:$CHCl_3$ - 9:1 (v/v) solvent. [k] Sample provided by Prof. Kevin M. Smith, University of California at Davis. [l] Data of Matheson and Toledo[32].

with S (eqs. 3-5). Singlet oxygen reactivity ($k'_Q+k'_R$) with the dye sensitizer is much slower than $k_d$, $k_Q$ or $k_R$[12,17].

$$^1O_2 \xrightarrow{k_d} {}^3O_2 \quad (3)$$

$$S + {}^1O_2 \xrightarrow{k_Q} {}^3O_2 + S \quad (4)$$

$$S + {}^1O_2 \xrightarrow{k_R} \text{products} \quad (5)$$

The steady-state approximation gives, (eq. 6), for the rate of substrate disappearance:

$$-d[S]/dt = K \cdot k_R[S]/\{(k_Q+k_R)[S]+k_d\} \quad (6)$$

where K is the rate of $^1O_2$ production and defined as $K = I_a \cdot \Phi_{isc} \cdot f^1O_2$ (with $I_a$ = rate of absorption of light by the sensitizer in mol. quanta/sec, $\Phi_{isc}$ = sensitizer triplet quantum yield and $f^1O_2$ = yield of $^1O_2$ from triplet sensitizer = 1.0[17-19]).

For $\Delta[S] \ll [S_0]$, the solution of eq. 6 can be approximated as (eq. 7):

$$\{[S]/I_a \Delta t\}^{-1} = \Phi_{isc}^{-1} \{(k_Q+k_R)/k_R + k_d[S_0]^{-1}/k_R\} \quad (7)$$

where $\Delta[S] = [S_0] - [S]$ and $[S_0]$ is the initial substrate concentration. The value of $I_a$ is determined by actinometry using Reinecke's salt[20], and a plot of $\{\Delta[S]/I_a\Delta t\}^{-1}$ vs $[S_0]^{-1}$ for a fixed time period will give a linear plot. The ratio of the slope to the intercept of such a plot is $k_d/(k_Q+k_R)$, and the reciprocal of the intercept is $\Phi_{isc}k_R/(k_Q+k_R)$. Since the $k_d$ values for $^1O_2$ are known in methanol ($k_d = 1.4\times 10^5$ sec$^{-1}$) and chloroform ($k_d = 1.67\times 10^4$ sec$^{-1}$)[18], the values of $(k_Q+k_R)$ can be determined. They are summarized in the table for BR and BR-related substrates. In order to separate $k_Q$ and $k_R$, the values of $\Phi_{isc}$ are required. The value of $\Phi_{isc}$ for RB in methanol is 0.76[19]. Since RB is insoluble in chloroform, RBCE was used, and its $\Phi_{isc}$ value was determined by measuring the rate of 1,3-diphenylisobenzofuran (DPBF), which is known to be a good $^1O_2$ acceptor but not a quencher[17]. By plotting $(\Delta[DPBF]/I_a\Delta t)^{-1}$ vs $[DPBF_0]^{-1}$, the reciprocal of the intercept, $\Phi_{isc}k_R/(k_Q+k_R)$, was found to be 0.36. Since DPBF does not quench $^1O_2$, $k_Q = 0$ and $\Phi_{isc} = 0.36$. By way of comparison, $\Phi_{isc}$ for RBCE in methanol was found to be 0.63 (vs 0.76 for RB in methanol[17]).

*Results and discussion.* The values of $k_R$ and $(k_Q+k_R)$ are presented in the table along with reference values from other work. As noted earlier[17], the values from Nd-YAG laser produced $^1O_2$ are about an order of magnitude lower in some instances. Several important conclusions may be deduced from the data of the table and related data. 1. BR (**1**) and all the other substrates **2-8** quench $^1O_2$ enormously fast, with $k_Q$ approaching the diffusion limit. However, BR is a known $^1O_2$ sensitizer[8,9] with a recently established low-lying triplet at $\sim$37 kcal/mole above the ground state[21]. It thus seems improbable that BR should quench $^1O_2$ (22 kcal/mole above its ground state) by resonance) energy transfer. Rather, a more probable quenching mechanism would involve an electron transfer reaction (eq. 8)[22].

$$BR + {}^1O_2 \rightarrow [BR^{+\cdot} O_2^{-\cdot}] \rightarrow BR + {}^3O_2 \quad (8)$$

This type of mechanism is, in principle, available to all substrates **1-8**; however, the extent to which it participates will depend on their 1-electron oxidation (half-wave potentials). Biliverdin (BV) and probably **7** and **8**, have even lower-lying triplets ($\leq$22 kcal/mole) close to that of $\beta$-carotene[23] and can presumably quench $^1O_2$ effectively by the same (resonance) energy-transfer mechanism ($k_Q \approx 10^{10}$ $M^{-1}$ sec$^{-1}$ for $\beta$-carotene[12]). The $E_T$ values for **6-8** are probably $<$22 kcal/mole since they are not $^1O_2$ sensitizers; whereas, triplet $\beta$-carotene is quenched by $^3O_2$ to give $^1O_2$[24]. 2. The possibility of 2 different $^1O_2$ quenching mechanisms: an electron or charge-transfer mechanism principally involved for **1-5** (eq. 8) and an energy-transfer mechanism principally involved for **6-8** offers an explanation for the greater chemical reactivity ($k_R$) of the former (**1-5**) as compared to the latter (**6-8**). Assume that concerted cycloaddition of $^1O_2$ (1,2- and 1,4-addition) either is not a

significant factor or contributes equally to the $k_R$'s of all of **1–8**. Then, the radical ion pairs [$BR^{+\cdot}$ $O_2^{-\cdot}$] postulated in the quenching mechanism of **1–5** (eq. 8) can account for the observed products either by collapse to give dioxetanes or endoperoxides[25] which decompose to products (eq. 9), or by H· abstraction en route to BV-like products (eq. 10)[6,22]. The comparatively slow rate ($k_R$) of product formation from **6–8** could thus be ascribed to non-involvement of radical ion pairs or to a less favorable partitioning of the radical ion pair in eqs like 9 and 10.

$$[BR^{+\cdot}\ O_2^{-\cdot}] \rightarrow \text{dioxetanes/endoperoxides} \rightarrow \text{products} \quad (9)$$

$$[BR^{+\cdot}\ O_2^{-\cdot}] \rightarrow [BR-H]\cdot\ O_2H \rightarrow BV + H_2O_2 \quad (10)$$

Other evidence for electron transfer reactions with $^1O_2$ may be found in enamine cleavages which appear to go through an electron-transfer or charge-transfer mechanism involving collapse of an ion-radical pair[26]. 3. The values of $k_Q$ for **1–5** are depressed as the $k_R$ values increase, but ($k_Q + k_R$) is reasonably constant (= 1.8–4.3). The values of $k_R$ and $k_Q$ are fairly invariant for **1–3**, and they are solvent independent, with $k_R < k_Q$. Thus, vinyl groups appear to play no special role, and expected conformational changes (on intramolecular H-bonding[27,28]) due to esterification or protic vs aprotic solvent do not have an appreciable effect on the rates. However, aetiobilirubin (**4**) and the related BR model (**5**) both exhibit substantially enhanced $k_R$ values, $k_R \geq k_Q$. The reasons for the $k_R$, $k_Q$ differences are not clear; however, it seems likely that in the absence of propionic acid (ester) groups, **4** probably assumes a nonintramolecularly H-bonded conformation akin to that of **5**[29]. Thus, whereas the ease of formation of an ion-radical pair intermediate (eq. 8) probably differs little for **1–15**, assuming the same half-wave potentials, the data suggest that the relative ease of chemical reaction (eqs 9 and 10) vs quenching (eq. 8) is conformation dependent.

It would appear that BR is able to quench or react competitively fast with any $^1O_2$ produced in vivo during phototherapy. Since it is almost surely the most reactive local substrate in the environment in which $^1O_2$ is produced[30], BR can control its own photodestruction.

1 The authors wish to thank the National Science Foundation (CHE 74-20877) and the National Institute of Child Health (HD 09026) for generous support of this work.

2 D. Bergsma and S.H. Blondheim, eds. Bilirubin Metabolism in the Newborn, II. American Elsevier, New York 1976.
3 D.A. Lightner, Photochem. Photobiol. *26*, 427 (1977).
4 A.F. McDonagh, in: Phototherapy in the Newborn: An Overview, p. 51. Ed. G.B. Odell, R. Schaffer and A.P. Simopoulos. Natl Acad. Sci. U.S., Washington, DC, 1974.
5 D.A. Lightner and A. Cu, Life Sci. *20*, 723 (1977).
6 D.A. Lightner, p. 34 in ref. 4.
7 R. Bonnett and J.C.M. Stewart, J. chem. Soc. Perkin I *1975*, 224.
8 A.F. McDonagh, Biochem. biophys. Res. Commun. *44*, 1306 (1971).
9 R. Bonnett and J.C.M. Stewart, Biochem. J. *130*, 895 (1972).
10 Final Report of the Committee on Phototherapy in the Newborn, Division of Medical Sciences. Natl Acad. Sci. U.S., Washington, DC, 1974.
11 R.E. Behrman, ed. J. Pediatr. *84*, 135 (1974).
12 C.S. Foote, in: Free Radicals in Biological Systems, p. 85. Ed. W. Pryor. Academic Press, New York 1976.
13 C.C. Kuenzle, M.H. Weibel and R.R. Pelloni, Biochem. J. *133*, 357 (1973).
14 D.A. Lightner and D.C. Crandall, Tetrahedron Lett. *1973*, 953.
15 D.A. Lightner, G.B. Quistad and C.S. Pak, Synthesis *1976*, 335.
16 R.M. Boden, Synthesis *1975*, 783.
17 C.S. Foote and T.-Y. Ching, J. Am. chem. Soc. *97*, 6209 (1975).
18 P.B. Merkel and D.R. Kearns, J. Am. chem. Soc. *94*, 7244 (1972).
19 K. Gollnick and G.O. Schenk, Pure appl. Chem. *9*, 507 (1964).
20 E.E. Wegner and A.W. Adamson, J. Am. chem. Soc. *88*, 394 (1966).
21 E.J. Land, Photochem. Photobiol. *24*, 475 (1976).
22 B. Stevens and R.D. Small, Jr, Photochem. Photobiol. *23*, 33 (1976).
23 E.J. Land, unpublished results.
24 D.R. Kearns, Chem. Rev. *71*, 395 (1971).
25 D.A. Lightner and Y.-T. Park, Tetrahedron Lett. *1976*, 2209.
26 C.S. Foote, A.A. Dzakpasu and J.W.-W. Lin, Tetrahedron Lett. *1975*, 1247.
27 P. Manitto and D. Monti, J. chem. Soc. chem. Commun. *1976*, 122, and references therein.
28 R. Bonnett, J.E. Davies and M.B. Hursthouse, Nature *262*, 326 (1976).
29 D.L. Cullen, P.S. Black, E.F. Meyer, D.A. Lightner, G.B. Quistad and C.-S. Pak, Tetrahedron *33*, 477 (1977).
30 D.A. Lightner and R.D. Norris, New Engl. J. Med. *290*, 1260 (1974).
31 I.B.C. Matheson, N.V. Curry and J. Lee, J. Am. chem. Soc. *96*, 3348 (1974).
32 I.B.C. Matheson and M.M. Toledo, Photochem. Photobiol. *25*, 243 (1977).

## 7a-Aza-B-homo[7a,7-d]tetrazole analogues of progesterone and testosterone[1]

H. Singh, K.K. Bhutani, R.K. Malhotra and D. Paul

*Department of Pharmaceutical Sciences, Panjab University, Chandigarh 160014 (India), 16 September 1977*

*Summary.* The tetrazole analogues of progesterone and testosterone, namely, 7a-aza-B-homo-4-pregneno[7a,7-d]tetrazole-3,20-dione (**5**) and 3-oxo-7a-aza-B-homo-4-androsteno[7a,7-d]tetrazol-17β-yl acetate (**8**), have been prepared which are worthy of biological testing.

The steroid hormones analogues possessing fused heterocyclic ring system have been found to be of interest. As an extension of our work on steroidal tetrazoles, we have synthesized 7a-aza-B-homo[7a,7-d]tetrazole analogues of progesterone and testosterone.

Treatment of (25R)-7-oxo-5-spirosten-3β-yl acetate[2], prepared by tert-butyl chromate oxidation of diosgenin acetate, with hydrazoic acid-boron trifluoride in chloroform[3] gave (25R)-7a-aza-B-homo-5-spirosteno[7a,7-d]tetrazol-3β-yl acetate (**1**): $\nu_{max}$ (KBr) 1724 (ester C=O); 1667 (C=C);