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PHYSICO-CHEMICAL MODELING OF THE ROLE OF FREE RADICALS IN PHOTODYNAMIC THERAPY . III. INTERACTIONS OF STABLE FREE RADICALS WITH EXCITED PHOTOSENSITIZERS STUDIED BY KINETIC ESR SPECTROSCOPY

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The loss of paramagnetism of the stable free radical 4,4'-di-*tert.*-butyl-diphenyl-1-nitroxy during illumination of the photosensitizer *meso*-tetrahydroxyphenyl chlorin by light above 620 nm in the absence of oxygen has been followed by kinetic ESR spectroscopy. Addition of the spin trap α-(4-pyridyl-1-oxide)-N-*tert.*-butyl-nitrone reduces the initial rate of the disappearanc of the free radical enabling to separate triplet-doublet interactions from processes between radicals stemming from the triplet sensitizer and the stable free radical. Kinetic treatment of the mechanism suggested yielded the rate constant of the triplet-doublet interaction proceeding *via* electron transfer being about 6% of the rate constant of the overall interaction including both energy transfer *and* electron transfer.

It has been suggested by us earlier [1] that in the course of photosensitization in biology parallel to the Type I and Type II mechanisms, according to which radicals formed from the excited sensitizer molecules or singlet oxygen generated in the interaction between triplet sensitizer and ground state oxygen *mediate* the photodynamic effect, the direct interaction between triplet sensitizer and (doublet) free radicals formed in the live tissue might considerably contribute to the overall photodynamic effect.

The existence of such type of interactions have been proved beyond doubts [2-4] and our measurements using laser flash photolysis technique have shown [5] that stable free radicals both in the presence and absence of oxygen decrease the lifetime of triplet sensitizers applied in biology. Unfortunately, this method, though appropriate for measuring quantitatively the direct effect exerted by the interaction on the *triplet sensitizer,* does not yield information with respect to the transformation of the *free radicals* added to the system.

Moan [6] and Shopova *et al.* [7] studied the decrease of the concentration of stable free radicals in the presence of excited sensitizer molecules by ESR method and assumed that it proceeds by the Type I. mechanism, that is, radicals formed from the triplet sensitizer interact with radicals added to the system.

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Not excluding the contribution of the Type I. mechanism, we intend to show by kinetic ESR spectroscopy that at the same time the direct interaction between triplet sensitizer molecules and doublet free radicals plays also a decisive role in the transformation of the latter. In order to separate the two pathways : Type I. mechanism and triplet - doublet interaction (called MTO mechanism [1]), in one series of experiments, parallel to the stable free radicals, also spin traps have been added to the system.

MATERIALS AND METHODS

meso-tetrahydroxyphenyl-chlorin (m-THPC) was supplied by Scotia Pharmaceutical Ltd. with the kind support of Professor H. van den Bergh. Aldrich 2,2,6,6- tetramethyl-1-piperidinyloxy (TEMPO) was used without further purification. 4,4'-di-*tert.*-butyl-diphenyl-1-nitroxy (TBPNO) was synthesized by Dr L. Sümegi (purity ~ 99.9 %). SIGMA α-(4-pyridyl-1-oxide)-N-*tert.*-butyl-nitrone and Acros Chimica N.V. dimethylsulphoxide have been used without further purification as spin trap (ST) and solvent, respectively.

Preparation of samples for ESR measurement

Reaction mixtures were placed into glass capillary tubes (20 μl each), thoroughly deoxygenated by repeated "freeze-pump-thaw" cycles and before sealed were filled with purified nitrogen.

Kinetic ESR spectroscopy

A JEOL JES-FE3X X-band ESR spectrometer has been used with 100 kHz field modulation and equipped with an on-line computer system. Experiments were carried out at 298 K. The spectrum of TBPNO has shown a well resolved hyperfine structure given in Fig.1. In order to follow continously the disappearance of the stable free radical, the magnetic field was fixed at the top of the largest line and the signal amplitude registered. Strong overmodulation was used (5G) to avoid the variation in line width and to minimize the effect of field instability.

Calibration curve of signal amplitude *vs* radical concentration is given in Fig.2. Illumination has been carried out by a PTL PENTA 250 W lamp in the cavity of the ESR spectrometer at wavelength range 610-740 nm.

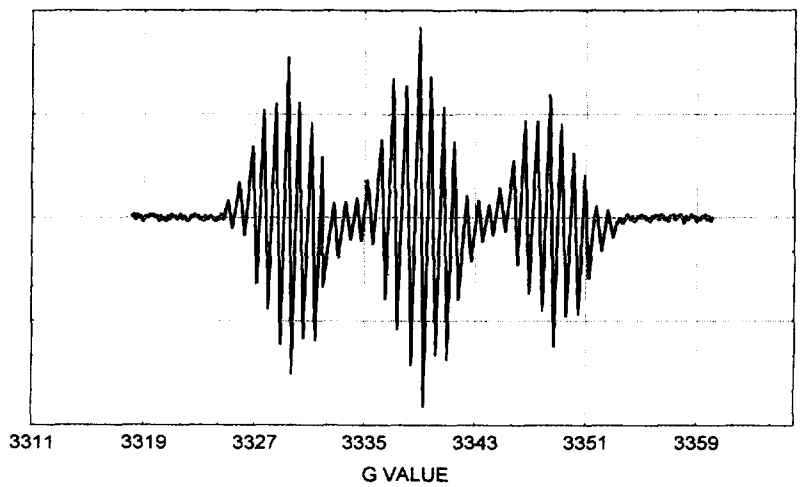

Fig.1. ESR spectrum of the stable free radical TBPNO.

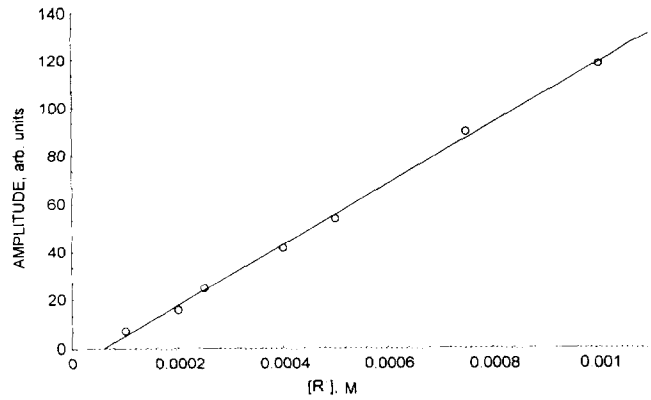

Fig.2. ESR amplitude *vs* concentration of the stable free radical TBPNO used as calibration curve.

RESULTS

Though preliminary experimental runs were performed with the stable free radical TEMPO, it turned out that due to its sublimation *in vacuo* its initial concentration after deoxygenation was irreproducible and consequently later on we have been using TBPNO. Furthermore, it has been shown that neither TBPNO nor the spin trap (including their simultaneous presence) undergo changes observable by ESR during illumination in the *absence* of photosensitizer.

In order to carry out also chemical analysis, it seemed expedient to use sensitizer molecules with well defined structure instead of applying commercially available hematoporphyrin derivatives. Therefore m-THPC has been used being a prospective sensitizer for Photodynamic Therapy (PDT) as supported by results of H. van den Bergh and coworkers [8].

Following systems have been investigated kinetically :

System A: sensitizer (S) + stable free radical (R$^{\bullet}$) + solvent + hν + ——

System B: sensitizer (S) + stable free radical (R$^{\bullet}$) + solvent + hν + spin trap (ST)

System C: sensitizer (S) —— + solvent + hν + spin trap (ST)

The initial concentration of the photosensitizer was in all systems : $[\text{m-THPC}]_0$ = = 10^{-4} mol l^{-1}. The solvent was dimethylsulphoxide (M) and [M] = 14.1 mol l^{-1}.

With respect to System A , the decrease in the concentration of R$^{\bullet}$ against time was followed at six different initial concentrations of the stable free radicals and is illustrated by Fig. 3. (curve *a*) at one initial concentration of TBPNO.

Since the decrease represented by curve *a* in Fig.3. can be due to interactions of R$^{\bullet}$ with free radicals (S$^{\bullet}$) formed from the triplet excited sensitizer (^{3}S*) *and / or* directly with ^{3}S*, System B was used to separate these two possibilities, in which case the same parameter has been followed in the presence of ST

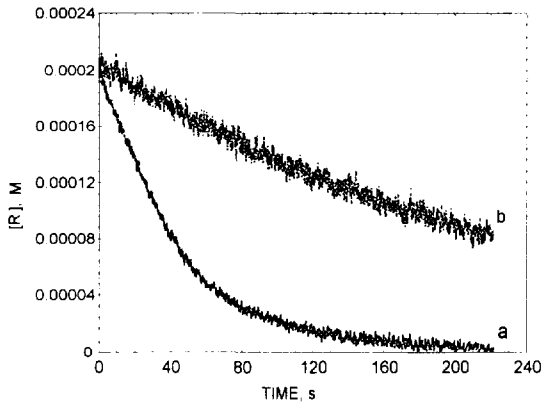

Fig.3. Kinetics of the consumption of the stable free radical TBPNO in the course of illumination of photosensitizer m-THPC measured by kinetic ESR spectroscopy. $[TBPNO]_0 = 2 \times 10^{-4}$ mol l^{-1} .
a : in the absence of spin trap; *b* : in the presence of spin trap.

at varying concentrations of R' . A sample of the experimental runs is also shown in Fig.3. (curve *b*). As it can be seen from Fig.3., the corresponding decrease becomes much slower in the presence of ST.

In order to support the assumption that this slow-down is the result of the *elimination* of S' by ST, experiments were performed in System C and Fig.4. represents the ESR spectrum of the radicals trapped by ST in the absence of TBPNO indicating that free radicals formed in the course of illumination are actually trapped.

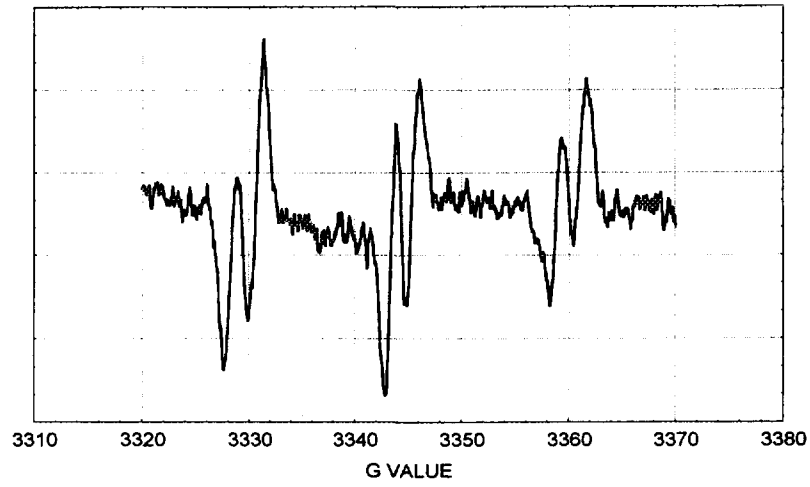

Fig.4. Trapped radical formed during illumination of photosensitizer m-THPC
Center field : 3350 ± 50 Gs; Modulation : 2 Gs ; Sweep time : 8 min.;
Time constant : 1 s. ; Gain : 7900 ; Spin trap : α-(4-pyridyl-1-oxide-N-*-tert.*-butyl-nitrone.
Reaction time : 4 min.; At t = 0 time no ESR signal observed.

DISCUSSION

With respect to *System A* following mechanism is suggested :

$$S + h\nu \xrightarrow{\phi I_{abs}} {}^1S^* \xrightarrow{ISC} {}^3S^* \qquad (1)$$

$${}^3S^* \xrightarrow{k_2} S \qquad (2)$$

$${}^3S^* + M \xrightarrow{k_3} S + M \qquad (3)$$

$${}^3S^* + M \xrightarrow{k_4} S^{\cdot} + \text{product} \qquad (4)$$

$$S^{\cdot} + M \xrightarrow{k_5} S + \text{product} \qquad (5)$$

$$R^{\cdot} + {}^3S^* \xrightarrow{k_6} \text{products} \qquad (6)$$

$$R^{\cdot} + {}^3S^* \xrightarrow{k_7} R^{\cdot} + S \qquad (7)$$

$$S^{\cdot} + {}^3S^* \xrightarrow{k_8} S^{\cdot} + S \qquad (8)$$

$$R^{\cdot} + S^{\cdot} \xrightarrow{k_9} \text{products} \qquad (9)$$

where processes (1) - (3) correspond to the formation and deactivation of the triplet excited sensitizer; (4) and (5) are the formation and consumption of sensitizer radical or radical ion, ($S^{\cdot}$); process (6) represents the triplet - doublet interaction. Processes (7) and (8) lead to the deactivation of the triplet by radicals present in the system and finally process (9) is a radical - radical reaction.

In the case of *System B* this mechanism changes in the following manner :

Instead of process (5) we have a fast reaction of $S^{\cdot}$ with the spin trap, (ST) leading to trapped radicals, $STS^{\cdot}$: process (5') and consequently processes (8) and (9) proceed with the participation of ($STS^{\cdot}$), instead of with $S^{\cdot}$: processes (10) and (11).

$$S^{\cdot} + ST \xrightarrow{k_{5'}} STS^{\cdot} \qquad (5')$$

$$STS^{\cdot} + R^{\cdot} \xrightarrow{k_{10}} \text{products} \qquad (10)$$

$$STS^{\cdot} + {}^3S^* \xrightarrow{k_{11}} \text{products} \qquad (11)$$

In the case of *System C* the mechanism becomes simplified :

It consists only of six elementary processes, (1) - (4) ; (5') and (11).

The corresponding differential equations based on the mechanism suggested for System A yield the expression (12) with respect to the initial rates of the consumption of the stable free radicals , if assuming that $w_8 \sim 0$ at $t \to 0$,

$$(-\frac{d[R^{\cdot}]}{dt})_{A,t\to 0} \equiv (W_{R^{\cdot}})_{A,0} = \frac{w_1}{\alpha+\beta[R^{\cdot}]}\,(k_6 + \frac{\gamma}{\delta+[R^{\cdot}]})\,[R^{\cdot}] \qquad (12)$$

while the same expression in the case of *System B* is represented by (13) assuming that $w_{10} \sim 0$ and $w_{11} \sim 0$ at $t \to 0$:

$$(-\frac{d[R^{\cdot}]}{dt})_{B,t\to 0} \equiv (W_{R^{\cdot}})_{B,0} = k_6 \frac{w_1}{\alpha+\beta[R^{\cdot}]}[R^{\cdot}] \qquad (13)$$

and equation (14) refers to *System C* neglecting $w_{11} \sim 0$ at $t \to 0$:

$$(\frac{d[STS^{\cdot}]}{dt})_{C,t\to 0} \equiv (W_{STS^{\cdot}})_{C,0} = \frac{\gamma w_1}{\alpha} \qquad (14)$$

where

$$\alpha \equiv k_2 + (k_3 + k_4)[M]\ ; \qquad \beta \equiv k_6 + k_7\ ;$$

$$\gamma \equiv k_4[M]\ ; \qquad \delta \equiv \frac{k_5}{k_9}[M]$$

From (12) and (13) by rearrangement we obtain :

$$\frac{(W_{R^{\cdot}})_{B,0}}{(W_{R^{\cdot}})_{A,0} - (W_{R^{\cdot}})_{B,0}} \equiv F = k_6\frac{\delta}{\gamma} + \frac{k_6}{\gamma}\,[R^{\cdot}] \qquad (15)$$

Fig.5. shows the ratio of the initial rates, (F), against the concentration of the stable free radicals introduced initially into the system.

Fig.5. enables us to calculate k_6/γ and δ being 5.8×10^2 l mol^{-1} and 9.2×10^{-5} mol l^{-1} , respectively. According to literature data [9 - 11] the value of

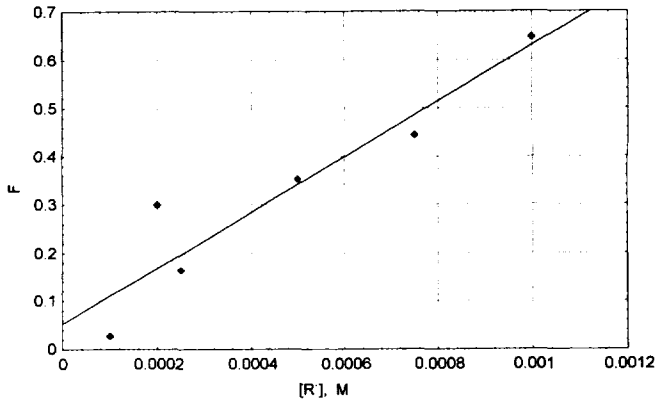

Fig.5. Ratios of intial rates (F) *vs* initial concentration of TBPNO, [R$^{\cdot}$].

$k_3 + k_4$, that is, the overall deactivation of the triplet sensitizer (by energy transfer *and* electron transfer) is in the range (2 - 6) x 10^4 l mol-1 s-1 . Therefore $k_4 \leq 10^4$ l mol^{-1} s^{-1}. By using for $k_4 \sim 10^4$ l mol-1 s-1, we obtain :

$$k_6 = 8 \times 10^7 \text{ l mol-1 s-1} \qquad (16)$$

Since our direct measurements by laser flash photolysis [5] (using the same stable free radical) yielded $k_6 + k_7 = 1.35 \times 10^9$ l mol-1 s-1, this means that about 6 % of the overall deactivation proceeds *via* electron transfer.

It should be noted, however, that the decrease in the initial rate of the consumption of R' in the presence of ST is not *necessarily* due exclusively to the elimination of S' by ST , since the deactivation of $^3S^*$ by ST might also contribute to this effect. Consequently, the difference $(w_{R'})_{A,0} - (w_{R'})_{B,0}$ in equ. (15) corresponds to a maximal value and thus equ. (16) refers to a minimal value of k_6 being possibly somewhat higher.

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REFERENCES

1. Gál D : *Biochem. Biophys. Res.Commun.* **186;** 1032-1036; (1992)
2. Hiratsuka H., S.Rajadurai, P.K.Das, G.L.Hug, R.W.Fessenden : *Chem. Phys Letters* **137;** 255-260; (1987)
3. Seret A., E.Gandin, A.van de Vorst : *J. Photochem.* **38;** 145-155; (1987)
4. A.Kawai, T.Okutsu, K.Obi : *J. Phys. Chem.* **95,** 9130-9134, (1991)
5. Vidóczy T., S.Elzeinzam, D.Gál : *Biochem. Biophys. Res. Commun.* **189;** 1548-1552; (1992)
6. Moan J. : *Acta Chem. Scand.* **B 34;** 519-521; (1980)
7. Shopova M., Ganchev T. : *J. Photochem. Photobiol. B:Biol.* **2;** 365-371; (1988)
8. Savary J.F., Monnier P., Wagnieres G.,Braichotte D., Fontolliet Ch. van den Bergh H. : in "Photodynamic Therapy of Cancer",Jori G., Moan J., Star W. M., Editors , *Proc. SPIE,* pp. 330-340, (1994)
9. Craw M., Redmond R., Truscott T.G. : *J. Chem. Soc. Faraday Trans. I.* **80;** 2293-2299
10. Gorman A.A., Rodgers M.A.J. : *J. Photochem. Photobiol. B. Biol.* **14;** 159-176 (1992)
11. Pottier R., Truscott T.G. : *Int. J. Radiat. Biol. :* **50;** 421-452; (1986)