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Physicochemical Modeling of the Role of Free Radicals in Photodynamic Therapy

V. Quantification of Spin-Trapping Kinetics

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A new kinetic approach is suggested and experimentally supported for quantification of the spin-trapping procedure. Accordingly, the concentration of the spin adduct formed in the interaction between the spin-trap DMPO and various free radicals (cyanopropylperoxy, cumylperoxy, phenylethylperoxy, and hydroperoxy radicals) generated by the initiated oxidation of the parent molecules is followed by kinetic ESR spectrometry. The initial sections of the corresponding kinetics are linear indicating that during this period the consumption of the adduct is negligible and thus the rate of accumulation (W_A) approximates the rate of formation (W_f): $W_A \sim W_f = k_{ST}[\mathrm{Rad}^\bullet][\mathrm{DMPO}]$, supported also by the fact that the rate of initiation of oxidation equals W_A at high [DMPO]. In addition, the circulatory experimental apparatus enables calculation of the rate of molecular decomposition of the adduct by stopping circulation (W_f becomes negligible) and following the decrease of the ESR signal. Corresponding rate constants are summarized. © 2000 Academic Press

It has been suggested earlier (1) that free radicals generated in live tissues (native free radicals, NFR) might play important role in photosensitization. Experimental evidence according to which the total concentrations of NFR in tumor cells of mice measured by ESR technique decreased in the presence of excited triplet sensitizer molecules (^{3}PS*) (2) supported this assumption similarly as *in vitro* measurements with macrophages where induced chemiluminescence (CL) generated by radicals diminished in the presence of ^{3}PS* (3).

Unfortunately neither of these methods did enable to determine the actual type of radicals the interactions of which yielded results mentioned above. To overcome this difficulty and perform kinetic calculations, however, requires detailed information concerning the elementary reaction with emphasis to the species Rad$^\bullet$.

$$\mathrm{Rad}^\bullet + {}^3\mathrm{PS}^* \rightarrow \mathrm{products} \qquad [1]$$

Since NFR-s are, as a rule, very active species distinction between them needs sophisticated experimental procedures among which spin trapping seems to be the most suitable transforming short lived NFR-s into relatively long-lived radicals by their interactions with spin-rapping molecules (ST):

$$\mathrm{Rad}^\bullet + \mathrm{ST} \rightarrow \mathrm{RadST}^\bullet \qquad [2]$$

discussed in details by Janzen and co-workers (4) and called “spin counting” and later by Griller and Ingold (5) called “free radical clock.” Since then different products formed in [2] and called spin adduct (A$^\bullet$) were analyzed by ESR spectroscopy and the spectra of structures obtained were summarized (6).

Numerous results have been published recently on the application of spin trapping in biology (see e.g., papers by Samuni *et al.* (7); Slater and co-workers (8); Buettner and co-workers (9); Kohno *et al.* (10) as well as the good review by Thornalley (11)).

Nevertheless, determinations of quantitative parameters such as concentrations, formation and consumption rates of NFR-s are essentially hindered by the fact that the (biologically) most appropriate ST-s yield adducts which, though more stable than the trapped radicals, undergo consumption and thus the numerical evaluation meets serious problems.

In principle, if the spin trap is consumed exclusively in its interaction with the NFR-s, the rate of accumu-

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lation of the adduct ($W_A = d[A^\bullet]/dt$) can be expressed as

$$W_A = W_f - W_c, \quad [3]$$

where W_f and W_c correspond to the rate of formation and rate of consumption of the radical adducts, respectively (11), while the former one can be expressed as

$$W_f = k_{ST}[Rad^\bullet][ST]. \quad [4]$$

According to literature data (11) it is assumed that the rate of the decay of the adduct corresponds to

$$W_c \approx W_{dec} + W_{rad} \quad [5]$$

where the first member ($W_{dec} = [A^\bullet]\{k_m + k_b [A^\bullet]\}$ refers to the mono and/or bimolecular decomposition of the adduct, while the second one ($W_{rad} = k_{rad} [A^\bullet][Rad^\bullet]$) to the radical induced consumption. Since the analytical solutions of Eq. [3] have not been performed (as a rule only values $[A^\bullet]$ and $d[A^\bullet]/dt$ are followed experimentally), competitive methods are applied (11–13).

An interesting approach by measuring the rates of the mono and/or bimolecular decomposition of the spin adduct using rapid oxygen depletion has been applied by Kotake *et al.* (14), while using rate constants of radical termination for the determination of the rate constant of spin trapping has been also proposed [15].

In the present paper we intend to introduce a new kinetic approach enabling the quantitative treatment of results obtained in spin trapping experiments and applied for four (structurally) different oxygen centered radical. The spin trap is considered kinetically a specific inhibitor the product of which formed during inhibition undergoes decomposition.

MATERIALS AND METHODS

Materials. Cumene, ethylbenzene, cyclohexanol, chlorobenzene, and acetonitrile were commercial products (Fluka) and used after distillation. The initiator azo-bis-isobutyronitrile (AIBN) was obtained from Fluka and used without further purification. The spin trap 5,5-dimethyl-1-pyroline-*N*-oxide (DMPO) purchased from Sigma and 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) obtained from Aldrich Chemical Co. were used without purification.

Oxidation runs (thermal initiation). Liquid phase oxidation of the initiator and initiated oxidation of cumene, ethylbenzene, and cyclohexanol were used for generation of the corresponding peroxy radicals (cyanopropylperoxy, cumylperoxy, α-phenylethylperoxy and hydroperoxy radicals) (16–19).

Hydrocarbons and alcohol were used as 10% solutions in chlorobenzene. The concentration of AIBN was 0.1 M in all cases. Experiments were carried out at 60°C in a standard thermostated glass apparatus with gas inlet under oxygen bubbling and an additional mixing during reaction (16).

Solutions of DMPO in parent solvents were injected with a syringe through a sampling valve after the mixture had been equilibrated thermally. The concentrations of DMPO added to the mixture were varied from 1×10^{-4} to 1.2×10^{-1} M.

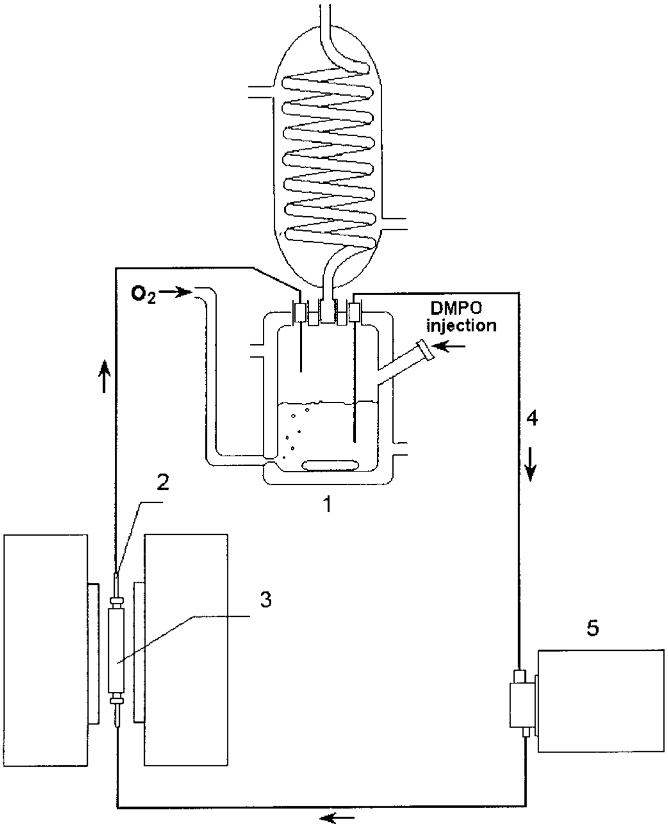

FIG. 1. Circulatory system. 1, oxidation vessel; 2, flow cell; 3, ESR cavity; 4, connecting tubes; 5, HPLC pump.

Oxidation runs (photochemical initiation). For comparison, oxidation of AIBN (0.1 M) was completed also by its photolysis in the presence of oxygen with 7.7×10^{-5} M < [DMPO] < 1.21×10^{-2} M at 25°C and 350 nm. The rate of initiation being $(2.3 \pm 0.3) \times 10^{-5}$ M^{-1} s^{-1} was calculated from the initial slope of the formation of the adduct vs time curve at high [DMPO]. In this run the start of the reaction was the time switching on the light.

Circulatory system. To separate the rates of formation and consumption of the adduct (see Eq. [3]) a circulatory system shown schematically in Fig. 1 has been devised.

The reaction mixture from the standard glass oxidation vessel 1 has been circulated continuously via the flow cell 2 of an internal diameter 2 mm placed into the cavity of the ESR spectrometer 3 by an HPLC pump 5 (L-6000A Merck–Hitachi), the rate of circulation being 6 ml/min. The ratio of the total volumes of the circulating reaction mixture to the one in the oxidation vessel was 1.25.

ESR measurements. ESR measurements were carried out with a Magnettech model CU4 modular computer controlled spectrometer. The ESR spectra of the spin adducts were registered at the following conditions: microwave power: 18 mW; modulation frequency: 100 kHz; modulation amplitude 1.175 G; scan time 1 min.

The strong overmodulation (9.4 G) was used for kinetic measurements to avoid the variation in line width and the effect of field instability. To follow continuously both the formation and consumption of spin adducts, the high field peak was chosen in each case, magnetic field fixed on the top of this line and the changes of signal amplitude in time followed automatically. The registration of the

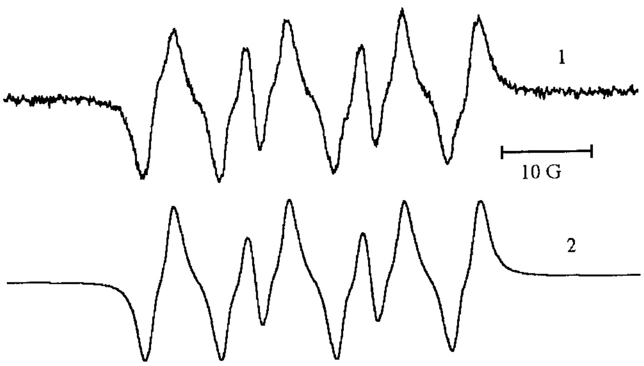

FIG. 2. ESR spectrum of adduct formed during oxidation of cumene initiated by AIBN. 1, experimental; 2, simulated.

signal intensity was started 1 min before the addition of DMPO to the mixtures.

After the kinetic experiments in the presence of varying concentrations of DMPO were completed, in separate runs the circulation was stopped at different adduct concentrations (in some cases at their maximal values) by switching off the pump and the signal followed in time. Intensity values observed from the fifth min after stopping circulation were used for calculation of the rate parameters of the molecular decomposition of the adducts.

Concentrations of the spin adducts were determined by double integration of the ESR spectra of spin adducts normalized to the signal from Mn/MgO and of TEMPO solutions ($3 \times 10^{-6} \div 6 \times 10^{-4}$ M) as an integration standard using the computing "Program EPR" developed by Rockenbauer and Korecz at the Technical University of Budapest (21). All spectra were obtained with the same instrumental setting and identical conditions (solvent, temperature, rate of oxygen bubbling, etc.).

UV measurements of DMPO consumption. In separate runs consumption of the spin trap was monitored against time in acetonitrile solutions (5×10^{-3} M < [DMPO] < 1×10^{-2} M) both in the presence (0.1 M) and in the absence of AIBN by a HP 8452 A spectrophotometer (molar absorption coefficient at 244 nm: 8.3×10^3 M^{-1} cm^{-1}).

RESULTS

Kinetics of DMPO Consumption

It has been established that DMPO did not undergo decomposition or oxidation at 60°C. In the presence of AIBN, the rates of consumption of DMPO were equal the initiation rates within experimental error (16, 18). Consequently, DMPO is consumed exclusively in its interaction with free radicals generated in the system.

ESR Spectra

In the absence of free radicals the spin trapping system is ESR-silent, while similar but slightly distinct ESR spectra are obtained in their presence. A typical ESR spectrum registered during the oxidation of cumene is shown in Fig. 2 consisting of a sextet due to the nitrogen and β-hydrogen splittings.

Since spectra in each system studied belong to a single type of radical they were assigned to DMPO-peroxy radical adducts, DMPO-OOR, and their hfcc values are summarized in Table 1, changing only slightly with the source of the parent radical and/or the solvent properties in reasonably good agreement with values reported for peroxy radical adducts (6, 20).

TABLE 1

ESR Parameters of DMPO-OOR-Type Spin Adducts

Radical-generating system	a_N, G	a^H_β, G	a^H_γ, G
AIBN/O$_2$	12.7	8.2	1.1
AIBN/cumene/O$_2$	12.8	8.4	1.7
AIBN/ethylbenzene/O$_2$	12.8	7.9	1.7
AIBN/cyclohexanol/O$_2$	13.0	8.1	1.2

In the course of the oxidation of cumene and ethylbenzene initiated by AIBN simultaneously two types of peroxy radicals are generated: cyanopropylperoxy radicals ($rO_2^\bullet$) as well as the corresponding chain carrier peroxy radicals ($RO_2^\bullet$). The latter in the case of cyclohexanol are $HO_2^\bullet$ radicals. Due to the relatively large rate constants of the chain transfer between $rO_2^\bullet$ and the parent hydrocarbon or alcohol (23, 24) it can be assumed that apart from high concentrations of DMPO the $RO_2^\bullet$ + DMPO interaction predominates over the $rO_2^\bullet$ + DMPO reaction, affected mainly by solvents. The excellent agreement between experimental spectra and simulated ones supports this assumption.

Accumulation Kinetics of Spin Adducts

Maximum curves of the accumulation of spin adducts vs time during the oxidation of cumene in the presence of various amounts of DMPO are shown in Fig. 3. Similar results were obtained for the other systems.

The initial sections of the accumulation, being strictly linear and their slope proportional to the initial concentrations of DMPO, are given in Fig. 4.

Figure 4 indicates that during the early period of the accumulation consumption of the adduct is negligible. The initial rates of the accumulation of $A^\bullet$ in all the

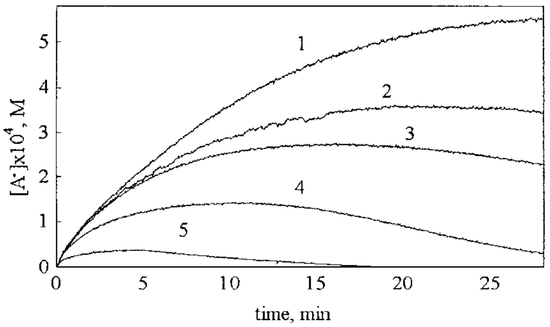

FIG. 3. Accumulation of spin adducts vs time in the course of oxidation of cumene. [DMPO]: 1, 0.12; 2, 1.4×10^{-2}; 3, 1.0×10^{-2}; 4, 5.0×10^{-3}; 5, 3.0×10^{-3} M.

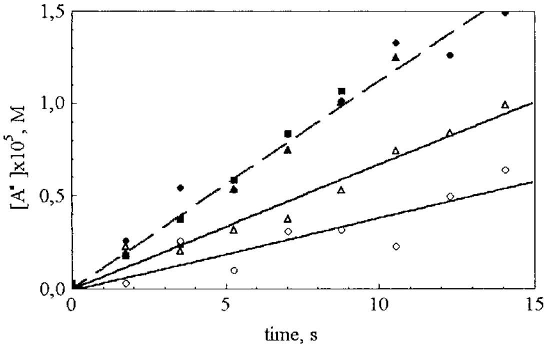

FIG. 4. The same as Fig. 3, limited to the initial sections of accumulation. [DMPO]: 0.12 (●); 1.4×10^{-2} (▲); 5.0×10^{-3} (■); 3.0×10^{-3} (△); 1.0×10^{-3} (○) M. The slope of the dashed line is equal to W_i.

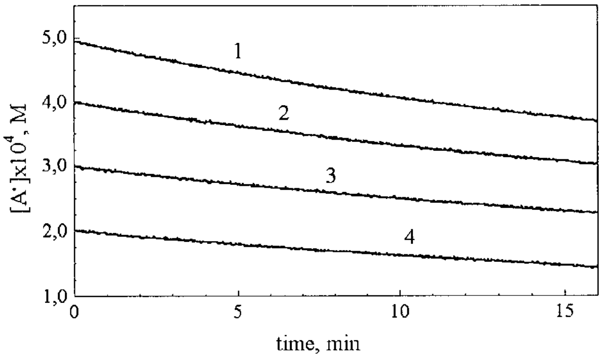

FIG. 6. Decay kinetics of spin adduct formed in oxidation of cumene. [DMPO]: 1, 0.12; 2, 6.6×10^{-2}; 3, 3.4×10^{-2}; 4, 1.2×10^{-2} M. $T = 25°C$.

systems under study vs the concentrations of DMPO given in Fig. 5 show saturation being equal the rate of initiation.

Molecular Decomposition of the Spin Adducts

Decrease in the concentration of the spin adducts was registered from the fifth min after circulation has been stopped. The corresponding curves are represented by Fig. 6 obtained for the oxidation of cumene in the presence of DMPO.

Due to the experimental arrangement it can be assumed that during the period of measurements without circulation, apart from an initial short time interval, concentrations of free radicals are negligible.

DISCUSSION

Rate Constants of Spin Trapping

According to the experimental results the initial sections of the accumulation of the spin adducts can be described instead of Eq. [3] by the expression [6]:

$$(\mathrm{d}[\mathrm{ST}]/\mathrm{d}t)_{t\to 0} = (W_\mathrm{A})_{t\to 0} \approx W_\mathrm{f} \qquad [6]$$

which is the rate of consumption of the ST can be represented by the rate of the formation of the adduct.

With respect to the chain carrier peroxy radicals generated by oxidation and taking into account their bimolecular combination processes, for this time period:

$$\left(\frac{\mathrm{d}[\mathrm{RO}_2^\bullet]}{\mathrm{d}t}\right)_{t\to \mathrm{o}} = W_\mathrm{i} - k_\mathrm{ST}[\mathrm{RO}_2^\bullet][\mathrm{DMPO}] - k_\mathrm{t}[\mathrm{RO}_2^\bullet]^2 = 0, \qquad [7]$$

where w_i and k_t are the rate of initiation and rate constant of termination, respectively.

As derived by Denisov *et al.* (2), Eqs. [4], [6], and [7] yield

$$\frac{x}{\sqrt{1-x}} = \frac{k_\mathrm{ST}}{\sqrt{W_\mathrm{i} k_\mathrm{t}}}[\mathrm{DMPO}] \qquad [8]$$

where

$$x \equiv \{\mathrm{d}[\mathrm{A}^\bullet]/\mathrm{d}t\}_{t\to 0}/W_\mathrm{i}. \qquad [9]$$

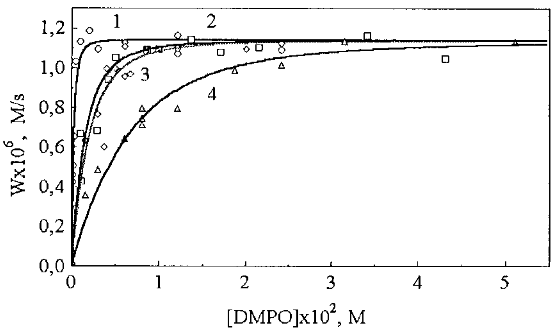

FIG. 5. Initial rates of formation of spin adduct vs [DMPO] at 60°C. 1, AIBN/O_2; 2, AIBN/ethylbenzene/O_2; 3, AIBN/cumene/O_2; 4, AIBN/cyclohexanol/O_2.

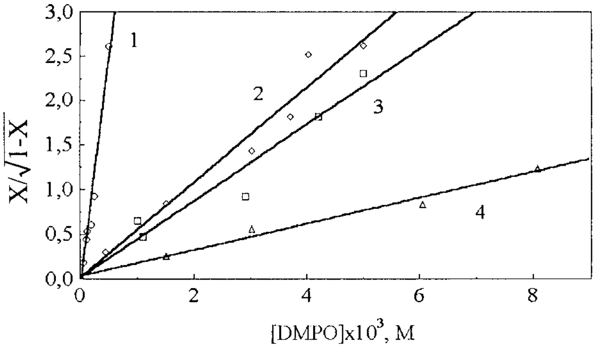

FIG. 7. Initial rates of adduct formation in coordinates of Eq. (8). Radicals generated: 1, cyanopropylperoxy; 2, α-phenylethylperoxy; 3, cumylperoxy; 4, hydroperoxy radicals.

TABLE 2

Rate Constants of Spin Trapping with DMPO (k_{ST}) and Chain Termination (k_t) for Different Peroxy Radicals

Radical-generating system	Peroxy radical	$k_{ST} \times 10^{-3}$ ($M^{-1}\ s^{-1}$)	Ref	k_t ($M^{-1}\ s^{-1}$)	Ref	Solvent
AIBN/O_2 thermal	$(CH_3)_2C(CN)OO^\bullet$	1.5 ± 0.05		1×10^5	(19)	Chlorobenzene
AIBN/O_2 photochem	$(CH_3)_2C(CN)OO^\bullet$	1.1 ± 0.5		1×10^5	(19)	Chlorobenzene
AIBN/cumene/O_2	$C_6H_5C(CH_3)_2OO^\bullet$	0.2 ± 0.09		1.2×10^5	(26)	Chlorobenzene
AIBN/ethylbenzene/O_2	$C_6H_5CH0(CH_3)O$	2.32 ± 0.10		1.9×10^7	(17)	Chlorobenzene
AIBN/cyclohexanol/O_2	$HO_2^\bullet$	3.44 ± 0.16		6.3×10^8	(20)	Chlorobenzene
Hypoxanthine/xantineoxidase	$HO_2^\bullet$	6.6	(16)		—	Water, pH 5
Hypoxanthine/xantineoxidase	$O_2^{\bullet -}$	0.010–0.017	(7, 11, 16)		—	Water, pH 7–8

Consequently, if W_i and k_t are known the rate constant of the spin trapping can be calculated using Eq. [8]. The corresponding curves are given in Fig. 7 while absolute values of k_{ST} for four different peroxy radicals together with k_t-s taken from literature data (16–19, 24) are summarized in Table 2.

Rate Constants of the Molecular Decomposition of the Spin Adducts

Evaluation of data shown in Fig. 6 as well as obtained for the other systems indicates that both mono and bimolecular decomposition takes place during the consumption of $A^\bullet$ in the absence of free radicals.

The corresponding k_m and k_b values are summarized in Table 3, and it can be established that they are practically independent on the radical scavenged by DMPO. It should be emphasized, however, that these rate constants are only approximate (and minimal) ones since the decomposition of the adduct might yield also ESR-active species which decrease the original fall observed resulting in possible experimental error.

On the Radical-Induced Decomposition of the Adduct

According to Eq. [5] in addition to the mono and/or bimolecular decomposition of the adduct a radical induced consumption can not be ruled out. Unfortunately, the present experimental arrangement does not enable the direct determination of the latter.

Although a possible approach might be the calculation of the difference between ($W_f - W_A$), representing the total consumption, and the molecular decomposition of $A^\bullet$, this approximation seems very inaccurate due to error propagation. A more sophisticated experimental procedure for this purpose is envisaged in the future.

TABLE 3

Rate Constants of Decomposition of Various Spin Adducts

Radical-generating system	Solvent	$k_m \times 10^4$ (s^{-1})	k_b ($M^{-1}\ s^{-1}$)
AIBN/O_2	Chlorobenzene	2.0 ± 0.4	0.3 ± 0.1
AIBN/cumene/O_2	Chlorobenzene	2.4 ± 0.3	0.3 ± 0.2
AIBN/ethylbenzene/O_2	Chlorobenzene	3.0 ± 0.2	0.2 ± 0.1
AIBN/cyclohexanol/O_2	Chlorobenzene	3.7 ± 0.3	0.5 ± 0.1

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