Decay of the Hydroperoxyl Spin Adduct of 5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-Oxide: an EPR Kinetic Study

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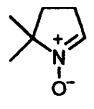
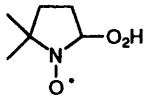
The decay kinetics of the hydroperoxyl spin adduct of both 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), a new β-phosphorylated cyclic nitrone and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were studied in various media by EPR spectroscopy. In organic solvents, both first- and second-order processes were shown to contribute to the decay of the two spin adducts. However, in aqueous solution the DMPO–hydroperoxyl spin adduct decay was pure first-order. The half-lives of the two spin adducts were determined in every medium tested and the DEPMPO–hydroperoxyl spin adduct was shown to be significantly more persistent (from five times in organic solvents to 30 times in a pH 5.6 buffer) than the DMPO–hydroperoxyl spin adduct.

The use of nitrones to trap free radicals in biological milieu becomes increasingly important with special interest in the spin trapping of oxygen-centred radicals.[1] The commercially available 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) **1**,[2] one of the



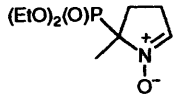
DMPO

1



DMPO–OOH

2



DEPMPO

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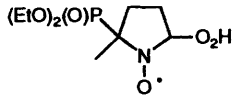

DEPMPO–OOH

4

most popular spin traps in biological milieu, has been shown to trap hydroxyl and hydroperoxyl radicals,[3] thereby yielding EPR-detectable aminoxyl radicals, the so-called DMPO–hydroxyl (DMPO–OH) and DMPO–hydroperoxyl (DMPO–O_2H) **2** spin adducts, respectively. However, trapping of hydroperoxyl with DMPO is not without its limitations[4] and one of the major problems encountered in these studies is the short life-time of DMPO–O_2H, especially in aqueous or biological media (the half-life of DMPO–O_2H in buffer is reported to be *ca.* 80 s at pH 6 and only *ca.* 35 s at pH 8).[4,5] In addition, the DMPO–O_2H decomposition was found to produce the aminoxyl DMPO–OH[4,6] and this may be a source of misinterpretation in spin trapping experiments. Various DMPO-type nitrone spin traps have been elaborated,[7] but they all presented the same or even more severe limitations than DMPO.

Recently, we described the two-step synthesis of a new β-phosphorylated cyclic nitrone analogous to DMPO, the 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO)

† Inverted spin trapping of oxygen-centred radicals with DEPMPO is unlikely since the redox potentials (*i.e.* E_p^{ox} = 1.9 and E_p^{red} = −2.1 V *vs.* SCE in acetonitrile) are very close to those reported for DMPO.[9]

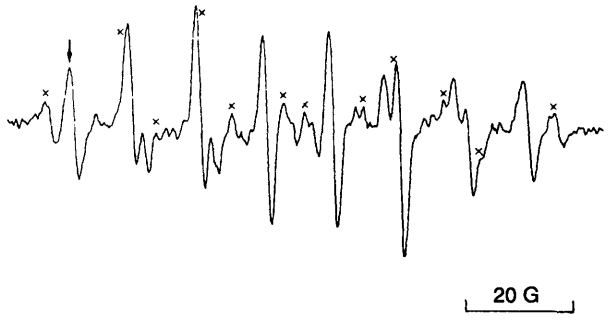

Fig. 1 EPR signal obtained by irradiating a solution containing riboflavin, DTPA and 0.1 mol dm^{-3} DEPMPO in 0.1 mol dm^{-3} phosphate buffer at pH 7: major signal corresponds to the hydroperoxyl spin adduct DEPMPO–O_2H [a_N = 13.17, $a_{\beta-H}$ = 10.97, $a_{\gamma-H}$ = 0.91 (1 H) and 0.40 (6 H) and a_P = 50.08 G]; minor signal (×) corresponds to a carbon-centred radical spin adduct of DEPMPO (a_N = 14.89, a_H = 20.94 and a_P = 47.52 G). An arrow indicates the line chosen to record the hydroperoxyl spin adduct decay at a fixed magnetic field.

3[8] and showed that this compound efficiently trapped various free radicals including hydroperoxyl.† We described in detail the EPR spectrum of the DEPMPO–hydroperoxyl spin adduct (DEPMPO–O_2H) **4** and showed that, in buffered solutions, the addition of hydroperoxyl to **3** seemed to be almost stereospecific, only one diastereoisomer of DEPMPO–O_2H being detected by EPR spectroscopy. We also noticed that this signal could be observed for several minutes at pH 7 although the rate of spin trapping of the hydroperoxyl radical with DEPMPO was found to be close to the one reported for DMPO. In order to explain these results and to compare the performances of the two nitrones DMPO and DEPMPO, we undertook a kinetic study of the decay of their hydroperoxyl spin adducts in organic media and in phosphate buffers.

Results

In the various media studied, the hydroperoxyl spin adducts DEPMPO–O_2H and DMPO–O_2H were always obtained by irradiating a riboflavin solution in the presence of an electron donor diethylenetriaminepentaacetic acid (DTPA) and a nitrone (DEPMPO or DMPO), as described in the Experimental section. A typical EPR spectrum obtained at pH 7 with DEPMPO is shown in Fig. 1. With such a hydroperoxyl generating system, the concomitant formation of a carbon-centred radical spin adduct occurred with either DEPMPO or

Table 1 Kinetic parameters[a] for the decay of DEPMPO–O_2H and of DMPO–O_2H in various media

	DEPMPO–O_2H			DMPO–O_2H		
	$k_a/10^3$ s^{-1}	$k_b[SA]_0/10^4$ s^{-1}	k_b/dm^3 mol^{-1} s^{-1}	$k_a/10^3$ s^{-1}	$k_b[SA]_0/10^4$ s^{-1}	k_b/dm^3 mol^{-1} s^{-1}
DMSO[b]	0.26	0.2	1.3	1.5	3.7	16.7
DMF[c]	0.34	0.8	2.7	1.4	1.6	15.8
Buffer[d] pH 5.6	0.38	8.1	18.5	8	—	—
Buffer[d] pH 7	0.9	6.1	16.9	14	—	—
Buffer[d] pH 8.2	1.1	5.7	17.4	17	—	—

[a] In each case, the standard deviation between experimental and calculated points is always in the range of the noise amplitude. [b] Dimethyl sulfoxide. [c] Dimethylformamide. [d] 0.1 mol dm^{-3} phosphate buffer.

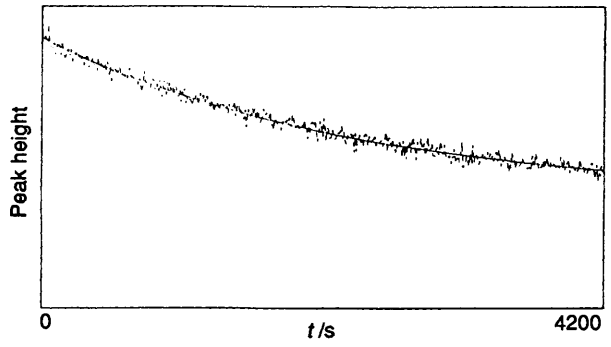

Fig. 2 Decay of DEPMPO–O_2H in DMF. The experimental curve is represented by the dotted line. The hydroperoxyl spin adduct was generated by a light–riboflavin–DTPA system in the presence of 0.1 mol dm^{-3} nitrone. The signal decay was monitored at a fixed field corresponding to the first low field line of the DEPMPO–O_2H EPR spectrum. The calculated curve is represented by the continuous line and was obtained from computer integration of eqn. (1) and with the kinetic parameters given in Table 1.

DMPO, as mentioned in the literature.[3] In buffered solutions, the EPR spectrum of the carbon-centred radical exhibited the following hyperfine splitting constants: a_N = 15.3 and a_H = 21.1 G with DMPO and a_N = 14.89, a_H = 20.94 and a_P = 47.52 G with DEPMPO.

In the timescale of our experiments, the generation of hydroperoxyl and the resulting spin trapping could be considered to cease when illumination was cut. Then, after removing the light, the decay of the spin adducts was followed by monitoring the height of the first low field peak of the EPR spectrum of DEPMPO–O_2H (see Fig. 1) or of DMPO–O_2H. The kinetic decay curves were recorded in two orgnic solvents, *i.e.* dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) and also in 0.1 mol dm^{-3} phosphate buffer at pH 5.6, 7.0 and 8.2.

Modelling of the decay curves showed that both first- and second-order processes (which could be a disproportionation of the spin adduct[1,4,10]) contribute to the decay of the spin adducts, and the resulting rate of decay is given by eqn. (1) in

$$-d[SA]/dt = k_a[SA] + k_b[SA]^2 \quad (1)$$

which [SA] represents the spin-adduct concentration and k_a and k_b are the rate constants of the first- and of the second-order processes, respectively. Actually, the intensity of the EPR peak monitored may be related to the spin-adduct concentration by a scale factor which is obtained by double integration of this peak compared with that of a 10^{-5} mol dm^{-3} TEMPO (2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl) solution used as standard sample. Thus, k_a and the product $k_b[SA]_0$, which are independent of this scale factor, were first determined using a computer program which fitted the curves calculated from eqn. (1) to the experimental curves. Then, after the determination of the initial concentration of spin adduct $[SA]_0$ in every case studied, the second-order kinetic constant k_b was calculated. Experimental curves for the decay of both DEPMPO–O_2H and DMPO–O_2H in the various media tested have all been modelled using this method and the values obtained in each case for the kinetic parameters are shown in Table 1.

Table 2 Half-lives ($t_{1/2}$) calculated for DEPMPO–O_2H and for DMPO–O_2H in various media as the neperian logarithm of two divided by k_a and from the values indicated in Table 1 for the constant k_a

	$t_{1/2}$/s	
	DEPMPO–O_2H	DMPO–O_2H
DMSO[a]	2673	460
DMF[b]	2040	505
Buffer[c] pH 5.6	1824	87
Buffer[c] pH 7	780	50
Buffer[c] pH 8.2	630	41

[a] Dimethyl sulfoxide. [b] Dimethylformamide. [c] 0.1 mol dm^{-3} phosphate buffer.

The decay of DMPO–O_2H generated under the conditions previously described was pure first-order in aqueous media (see Table 1). Actually, irrespective of the medium tested, the decay of DEPMPO–O_2H or of DMPO–O_2H always became pure first-order after a few minutes (at the most after 10 min). Thus, the first-order reaction appeared to be the most important of the two processes contributing to the spin-adduct decay, particularly in buffered solutions, and the half-life values ($t_{1/2}$) reported in Table 2 have been evaluated considering only the first-order process. With such an approximation, the half-lives of DEPMPO–O_2H and of DMPO–O_2H are slightly overestimated; however they give a good indication of the behaviour of these spin adducts with time.

Discussion

The short half-life of DMPO–O_2H at physiological pH is one of the most important limitations to the use of DMPO in biological media. In order to appreciate the perspectives which DEPMPO could open in this field, it was then of prime importance to compare the half-lives of DEPMPO–O_2H and of DMPO–O_2H generated under the same conditions in various environments.

First, we focussed on the decay of these spin adducts in anhydrous DMSO and DMF. The values obtained for the kinetic parameters (see Table 1) indicated that the decay of the two aminoxyl radicals always corresponded to a combination of first- and second-order processes. To illustrate these results, we have shown in Fig. 2 both the experimental curve obtained for DEPMPO–O_2H in DMF and the curve calculated from computer integration of eqn. (1). The good fit between the two curves confirmed the validity of the kinetic model proposed for the decay in organic media. It appeared then that the two aminoxyl radicals showed similar behaviour in these solvents,

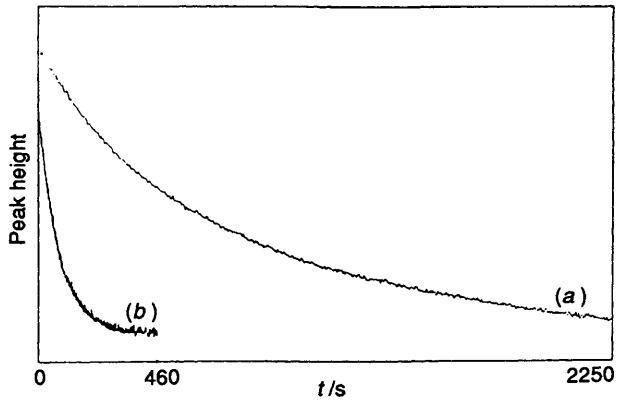

Fig. 3 Experimental decay of (*a*) DEPMPO–O_2H and of (*b*) DMPO–O_2H in a pH 7 phosphate buffer. The hydroperoxyl spin adducts were generated by a light–riboflavin–DTPA system in the presence of 0.1 mol dm^{-3} nitrone. The signal decay was monitored at a fixed field corresponding to the first low field line of either DEPMPO–O_2H or DMPO–O_2H EPR spectrum.

although DEPMPO–O_2H was more persistent than DMPO–O_2H: the half-lives determined from the first-order process were in the range of 35 to 45 min for the former and always lower than 9 min for the latter, while the second order kinetic constant k_b was from six to almost 13 times higher for the DMPO–O_2H decay.

We then studied the decay of the two hydroperoxyl spin adducts in buffered solutions at pH 5.6, 7.0 and 8.2. The values calculated for the kinetic parameters, which are shown in Table 1, demonstrated that the first-order decay is strongly pH-dependent and is slower at acid pH, especially in the case of DEPMPO–O_2H; these results confirm those previously described about the decay of DMPO–O_2H.[4,5] On the contrary, the constant k_b does not seem to vary with pH. The most interesting result was that, in buffered solutions, DEPMPO–O_2H disappeared much more slowly than DMPO–O_2H: DEPMPO–O_2H decay was 15 times slower than the DMPO–O_2H one at neutral or basic pH and about 30 times slower at acid pH. Fig. 3, which shows the experimental curves obtained with the two aminoxyl radicals in a pH 7 buffer, clearly illustrates the difference in the persistence of DEPMPO–O_2H and DMPO–O_2H. As can be seen from the values indicated in Table 1, there was no need to introduce a second-order process to fit the decay of DMPO–O_2H in different buffers. In these media, DMPO–O_2H decomposed so rapidly that its concentration was never high enough for the bimolecular self-reaction of this aminoxyl to be important (in our experiments, DMPO–O_2H concentration in buffer was always lower than 7×10^{-6} mol dm^{-3}). The concentration reached by DEPMPO–O_2H in the same environments was significantly higher (more than six times higher in our experiments) and this explains the difference in the decay of the two spin adducts in buffered solutions. On the contrary, in organic solvents, DMPO–O_2H reached a higher concentration and a second-order reaction was then shown to contribute to its decay. Nevertheless, in every case, after a few minutes and according to the low concentration of the two spin adducts, their decay became pure first-order.

We can reasonably assume that the observed first-order decay corresponds to pseudo-first-order processes. Among the different pathways of decay, we believe that the main process could be the reduction by the reduced riboflavin of the spin adduct to the corresponding EPR-silent hydroxylamines. Actually we observed that when the irradiation was maintained after the consumption of all the molecular oxygen dissolved in the solution, both DEPMPO–O_2H and DMPO–O_2H decomposed much more rapidly. In this case, the concentration of reduced riboflavin rose suddenly, thereby increasing the rate of reduction of the aminoxyl. In addition, we also noticed that DEPMPO–O_2H decomposed more rapidly when the DEPMPO concentration was raised: in a pH 7 buffer, the k_a value was *ca.* 1.5 times higher with 0.2 mol dm^{-3} DEPMPO than with 0.1 mol dm^{-3} DEPMPO. Presently, we have not yet elucidated the mechanism of a possible reaction between the nitrone and the aminoxyl; however, we determined that, at pH 7.0, the more intense EPR spectrum of DEPMPO–O_2H was obtained when the DEPMPO concentration was decreased to 0.05 mol dm^{-3}. On the contrary, when using DMPO, the nitrone concentration must be kept to at least 0.1 mol dm^{-3} to obtain an EPR spectrum of DMPO–O_2H exhibiting a correct signal-to-noise ratio. The apparent first-order process occurring in the decay of the two hydroperoxyl spin adducts could then involve at least two pseudo-first-order reactions. The mechanism of this decay seems then to be rather complex, depending on the concentration of most of the species present in the media and is now under further investigation.

Conclusions

The values (Table 2) determined for the half-lives of the two aminoxyl radicals in the various media tested clearly illustrate the advantage of using DEPMPO in hydroperoxyl spin-trapping experiments. Actually, DEPMPO–O_2H disappears about five times more slowly than DMPO–O_2H in organic media and from 15 to 30 times more slowly in aqueous solutions. The optimal concentration of nitrone necessary to obtain hydroperoxyl spin adducts' EPR spectra exhibiting a correct signal-to-noise ratio in buffers was also shown to be two times lower when for DEPMPO.

Another important result is that the DEPMPO–O_2H decay does not yield any paramagnetic species: in our experiments, we never observed the formation of the aminoxyl DEPMPO–OH. On the contrary, DMPO–O_2H is reported to decompose partially into DMPO–OH.[6,7] So it seems that hydroperoxyl spin trapping with DEPMPO is not as prone to artifacts as with DMPO.

Finally, note that the exceptional persistence of such a hydroperoxyl spin adduct possessing a β-hydrogen, which is known to be prone to dismutation, makes the DEPMPO suitable for unambiguous detection of hydroperoxyl not only *in vitro*, but also *in vivo*: the first experiments performed in this field showed that it was possible to detect the formation of hydroperoxyl during the reperfusion of ischemic isolated rat hearts.[8]

All these data suggest that DEPMPO offers a very promising alternative to DMPO in spin trapping experiments in every kind of media, allowing the detection of small levels of radicals. It appears that the diethoxyphosphoryl group plays an important role in the stabilization of the aminoxyl obtained by trapping hydroperoxyl. However, this role has not yet been elucidated and is still under investigation. The synthesis of a series of nitrones bearing various kinds of phosphorylated groups in different positions relative to the nitrone function is now in progress in our laboratory; we hope that complete kinetic studies of the decay of the hydroperoxyl spin adduct of these nitrones will allow us to understand better the exact role played by the phosphoryl group in the mechanism of this decay.

Experimental

Materials.—5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), diethylenetriaminepentaacetic acid [$(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)CH_2CH_2N(CH_2CO_2H)$] (DTPA), TEMPO and riboflavin were purchased from SIGMA Chemical Company; 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was synthesized and purified as described previously.[8] All

buffers were stirred for six hours in the presence of a chelating iminodiacetic acid resin, provided by SIGMA Chemical Company, in order to remove trace metal impurities. Organic solvents were of the highest grade of purity commercially available, twice distilled and dried. EPR assays were carried out at 20 °C in a flat cell by using a computer controlled Varian E-9 EPR spectrometer.

Kinetics.—The standard light–riboflavin–DTPA system used in our experiments to generate hydroperoxyl contained 4 mmol dm^{-3} DTPA, 0.1 mmol dm^{-3} riboflavin and 0.1 mol dm^{-3} DMPO or DEPMPO in either 0.1 mol dm^{-3} phosphate buffer (pH ranging from 5.6 to 8.2) or organic solvent (dimethyl sulfoxide or dimethylformamide). Oxygen was bubbled into the reaction mixture, then the medium was transferred into an EPR flat cell and irradiated directly in the cavity using a tungsten filament 100 W lamp as the visible light source. The increase in the low field peak was followed and the light was shut off when this height reached a maximum. With such a system, the hydroperoxyl adduct formation occurred solely during irradiation. Then, the decay of the hydroperoxyl adduct was monitored by recording the EPR signal at the fixed field corresponding to the first low field peak of the spectrum of either DMPO–O_2H or DEPMPO–O_2H. A complete spectrum was also systematically recorded at the end of the kinetic study in order to verify the stability of the magnetic field. Computer modelling of the kinetic curves was achieved using the program DAPHNIS elaborated in our laboratory.[11] In this routine, the signal amplitude at time t_n was calculated from the signal amplitude at time t_{n-1} and using the rate equation, *e.g.* eqn. (1). The standard least-squares method was then applied to fit the experimental curves, yielding the kinetic parameters indicated in Table 1. This program allowed the simulated curves to be calculated point by point by integrating the rate equation. In every medium tested, initial concentrations of DMPO–O_2H or of DEPMPO–O_2H, *i.e.* $[SA]_0$, were determined using a TEMPO solution as a standard sample. When the intensity of the hydroperoxyl spin adduct EPR signal was maximal, a complete spectrum was recorded before shutting out the light. Double integration of a pure line of DMPO–O_2H or DEPMPO–O_2H spectrum compared with that of a 10^{-5} mol dm^{-3} TEMPO sample allowed the absolute radical concentration to be obtained.

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