Spin-trapping Study of Free Radical Penetration into Liposomal Membranes

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Despite the recognized role of the hydroxyl radical in human pathology, experimental data regarding the membrane penetration of this highly reactive species is lacking. We have attempted to study this question utilizing the EPR spin-trapping technique with two new hydrophobic analogues of 5,5-dimethyl-1-pyrroline 1-oxide (DMPO),† 2,2-dimethyl-4-phenyl-2*H*-imidazole 1-oxide (DMPIO, **1**) and its 2-methyl-2-nonyl analogue (MNPIO, **2**). EPR spectra were obtained for these spin-traps with $HO^{\bullet}$, $CH_3^{\bullet}$, $HOCH_2^{\bullet}$, $CH_3(OH)CH^{\bullet}$ and $CH_3CH_2CH_2(OH)CH^{\bullet}$ radicals in phosphate buffer, in sodium dodecyl sulfate (SDS) micelles and, for DMPIO only, in dimyristoylphosphatidylcholine liposomes. The data suggest that these radicals do not penetrate into the lipid phase. In the case of the mildly lipophilic DMPIO, the spin-adduct signals observed result either from a rapid exchange of spin-adduct between the lipid and water phases or, more likely, from the fact that the aminoxyl group of the spin-adduct is located at the interface and, hence, available for interaction with radicals and paramagnetic ions. The highly lipophilic MNPIO, on the other hand, resides deep in the lipid bilayer of liposomes and hence no spin-trapping is observed.

It is now generally accepted that oxy-radicals are responsible for many pathological processes in living systems and are important causative agents of ageing and of several human diseases, including cancer, multiple sclerosis, Parkinson's disease, autoimmune disease, and senile dementia. The mechanism of action involves lipid peroxidation, destruction of proteins, sugars, amino acids, phospholipids and nucleic acids as well as oxidative damage to vitamins, hormones and enzymes.[1,2] Perhaps the best explored oxy-radicals are superoxide anion ($O_2^{\bullet -}$) and its derivative, the highly reactive hydroxyl radical ($HO^{\bullet}$).[3] Superoxide is generated in numerous dark biological processes, such as the xanthine oxidase conversion of xanthine into uric acid[4] and the autoxidation of many NAD(P)H-dependent enzymes and metal-containing proteins.[2] The main biological source of hydroxyl radical, on the other hand, is the metal-catalysed reduction of H_2O_2 [dubbed the Fenton reaction, eqn. (1)], which is often superoxide-driven [eqn. (2)].[3]

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)} + HO^{\bullet} + HO^{-} \quad (1)$$

$$M^{(n+1)} + O_2^{\bullet -} \longrightarrow M^{n+} + O_2 \quad (2)$$

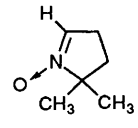
Hydrogen peroxide is produced, in turn, by the spontaneous or superoxide dismutase catalysed disproportionation of $O_2^{\bullet -}$ in aqueous media [eqn. (3)].[3]

$$2O_2^{\bullet -} + 2H_2O \longrightarrow H_2O_2 + O_2 + 2HO^{-} \quad (3)$$

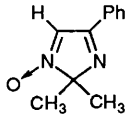
$O_2^{\bullet -}$ and H_2O_2 are reported to cross both biological[5] and model membranes.[6,7] Although the rate of $O_2^{\bullet -}$ transfer across these membranes is reported to be low (permeability coefficient of 2×10^{-6} cm s^{-1}),[7] the process can nevertheless play an important biological role. This is because such a mechanism enables the generation of the highly reactive hydroxyl radical in various cell compartments. Despite the recognized role of the hydroxyl radical in human pathology, there is, to our knowledge, no comparable experimental data regarding the membrane penetration of this highly reactive species.

† IUPAC-recommended name: 2,2-dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide.

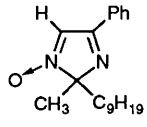
In the work described below, we have been able to shed some light on this question utilizing the EPR spin-trapping technique[8] with two new hydrophobic analogues of the well studied 5,5-dimethyl-1-pyrroline 1-oxide (DMPO),[8,9] namely 2,2-dimethyl-4-phenyl-2*H*-imidazole 1-oxide (DMPIO, **1**) and its 2-methyl-2-nonyl analogue (MNPIO, **2**). We hoped that, despite the polar *N*-oxide head-group, the hydrophobic substituents would drive these spin-traps well into the lipid phase of micelles and liposomes and away from the lipid–water boundary. If this were indeed the case, then spin-trapping could be effectively used to glean information regarding the penetration of radicals into membranes.



DMPO



1 (DMPIO)



2 (MNPIO)

Results and Discussion

In order to obtain some quantitative measure of the relative lipophilicity of DMPO, DMPIO and MNPIO, we measured the distribution coefficients of these spin-traps in an equivolume mixture of octanol and water. These values were found to be 0.09, 8.5 and $> 10^3$, respectively, and indeed correspond to the expected order of increasing lipophilicity of these compounds based on their substitution pattern.

We then wished to verify that these spin-traps could indeed be incorporated into the bilayer of dimyristoylphosphatidylcholine liposomes and did not simply self-associate as micelles. This was accomplished by taking ^1H NMR spectra of liposomes containing either DMPIO or MNPIO (see the Experimental section). In contrast with the distinct absorptions visible in the ^1H NMR spectrum of the liposomal solutions, no coherent spectra could be observed for sonicated buffer solutions of these spin traps. These results indicate that indeed the spin-traps are intercalated in the liposome bilayer; furthermore, in the absence of lipid, the spin-traps precipitate out of solution and, hence, cannot be detected by means of NMR spectroscopy.

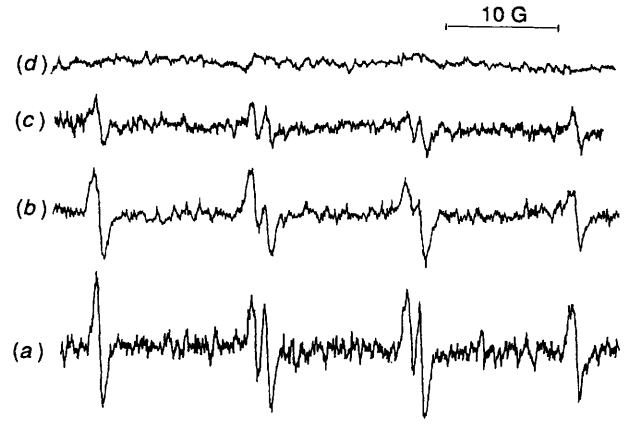


Fig. 1 EPR spectrum of DMPIO–HO• adduct obtained from Fenton reagent [Fe^{+2} (10^{-3} mol dm^{-3}), EDTA (2×10^{-3} mol dm^{-3}), H_2O_2 (*ca.* 0.2%)] under various conditions: (*a*) DMPIO (0.1 mol dm^{-3}) dissolved in phosphate buffer (0.1 mol dm^{-3}, pH 7.8); (*b*) DMPIO (33×10^{-3} mol dm^{-3}) in SDS micelles prepared in ultrapure water (Elgastat UHQ); (*c*) DMPIO (0.1 mol dm^{-3}) in dimyristoylphosphatidylcholine liposomes; (*d*) DMPIO (0.1 mol dm^{-3}) in dimyristoylphosphatidylcholine liposomes after addition of 20 μl of $K_3Cr(C_2O_4)_3 \cdot 3H_2O$ (1 mol dm^{-3}). Recording parameters: microwave power 20 mW; scan range 100 G; time constant 0.3 G; scan time 4 min; modulation amplitude 0.8 G for (*a*) and (*c*), 1.0 G for (*b*), and 2.0 G for (*d*); gain 1.6×10^4 for (*a*), 10^4 for (*b*) and (*c*), and 1.25×10^4 for (*d*).

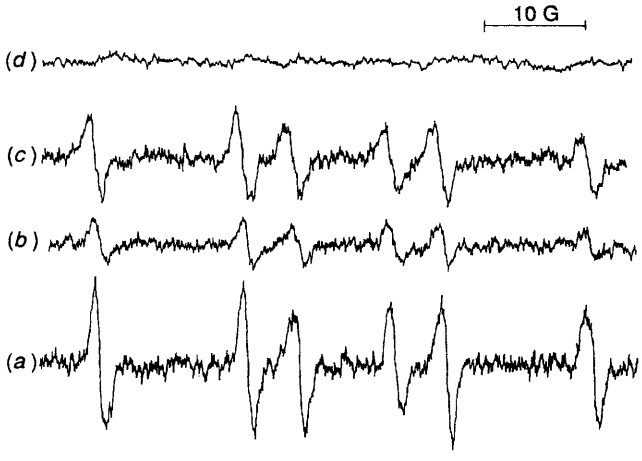


Fig. 2 EPR spectrum of DMPIO–$CH_3(OH)HC$• adduct obtained from Fenton reagent [Fe^{+2} (10^{-3} mol dm^{-3}), EDTA (2×10^{-3} mol dm^{-3}), H_2O_2 (*ca.* 0.2%)] in the presence of ethanol under various conditions: (*a*) DMPIO (0.1 mol dm^{-3}) dissolved in phosphate buffer (0.1 mol dm^{-3}, pH 7.8); (*b*) DMPIO (5.3×10^{-2} mol dm^{-3}) in SDS micelles prepared in ultrapure water (Elgastat UHQ); (*c*) DMPIO (0.1 mol dm^{-3}) in dimyristoyl phosphatidylcholine liposomes; (*d*) DMPIO (0.1 mol dm^{-3}) in dimyristoylphosphatidylcholine liposomes after addition of 20 μl of $K_3Cr(C_2O_4)_3 \cdot 3H_2O$ (1 mol dm^{-3}). Recording parameters: microwave power 20 mW; scan range 100 G; time constant 0.3 G; scan time 4 min; modulation amplitude 0.8 G; gain 1.6×10^4 for (*a*), 10^4 for (*b*), and 1.25×10^4 for (*c*) and (*d*).

We then looked for a convenient method of generating a range of oxy-radicals under various conditions. Fortunately, the literature already documents that the Fenton system, a convenient source of HO• radicals,[10] can easily be modified to give a variety of other reactive radicals depending on the solvent system and/or additive used. Thus, CH_3• is obtained in the presence of DMSO,[11] while $HOCH_2$•, $CH_3(OH)CH$• and $CH_3CH_2CH_2(OH)CH$• radicals are generated when methanol, ethanol and *n*-butanol are added, respectively.[12] In addition to the chemical Fenton source, hydroxyl radicals could be generated enzymatically *via* the Haber–Weiss reaction[13] from xanthine oxidase and xanthine in the presence of Fe^{+2}–EDTA. As shown in Figs. 1 and 2 and Table 1, we have obtained and characterized EPR spectra for the spin-adducts of DMPIO and MNPIO with HO•, CH_3•, $HOCH_2$•, $CH_3(OH)CH$• and $CH_3CH_2CH_2(OH)CH$• radicals in aqueous, alcoholic, micelle and—in the case of DMPIO only—liposomal media. These spectra remained essentially unchanged during the course of the EPR experiment (*ca.* 1 h). Despite repeated efforts under various reaction conditions and with an assortment of radicals, no spin-trapped adduct could be observed with MNPIO in liposomes.

Table 1 EPR parameters for DMPIO and MNPIO under various conditions

Trap	Radical	Medium	a_N/G	a_H^β/G
DMPIO	HO•	Buffer[a]	13.75	14.75
		Micelles[b]	13.75	14.75
		Liposomes[c]	13.75	14.9
	$CH_3(OH)CH$•	Buffer[a]	14.75	19.5
		Micelles[b]	14.5	19.5
		Liposomes[c]	14.5	19.5
	CH_3•	1:1 H_2O–DMSO	15.0[d]	20.1
	$HOCH_2$•	CH_3OH	14.25	20.0
	$CH_3CH_2CH_2(OH)CH$•	Buffer[a]	14.5	20.25
MNPIO	HO•	Micelles[b]	13.75	14.75
	$CH_3(OH)CH$•	CH_3CH_2OH	14.25	19.75
	$HOCH_2$•	CH_3OH	14.5	20.0

[a] Phosphate buffer 0.1 mol dm^{-3}, pH 7.8. [b] SDS micelles. [c] Dimyristoylphosphatidylcholine liposomes. [d] The *g* factor for the DMPIO–CH_3• spin-adduct is 2.0058.

EPR hyperfine coupling constants are known to be sensitive to the nature of the reaction solvent.[14] Nevertheless, the data for DMPIO in Table 1 indicate that the hyperfine coupling constants remain essentially the same irrespective of whether the spin-trap is dispersed in pure buffer, micelles or liposomes. Assuming that the lipophilic DMPIO resides in the lipid phase, this result is particularly surprising in the case of liposomes, which are stable structures below their transition temperature. As a result, we began to suspect that, perhaps, DMPIO was not completely immersed in the lipid phase. In order to determine exactly where the spin-adduct was situated within the liposome, we repeated the EPR experiment after the addition of potassium ferricyanide or chromium oxalate—spin broadening agents *which do not penetrate into membranes.*[15] In both cases, full broadening of the EPR signals resulted—whether the radical species generated was HO•, or the more lipophilic $CH_3(OH)$-CH• radical (see Figs. 1 and 2).

It should be noted that these broadening results might, *a priori*, be accounted for by invoking a rapid exchange of spin-adduct between the lipid and water phases. In such a case, however, increasing the lipid concentration should drive the equilibrium toward the lipid phase; yet, complete broadening was still observed even when the lipid concentration was doubled (from 100 to 200 mg cm^{-3}). We, therefore, think it more likely that, while the aromatic tail of DMPIO is anchored in the lipid bilayer, its aminoxyl head-group juts out into the water–lipid interface. As a result, the lipophilic DMPIO is available for interaction with radicals and paramagnetic ions in the aqueous phase. Similar suggestions have already been made by the groups of Janzen[16] and Walter[17] in analogous cases.

As noted above, in the case of the extremely lipophilic MNPIO (distribution coefficient octanol–water > 10^3), we experienced some difficulty in trapping radicals in general and did not obtain any spin-trapped radical when the reaction medium was liposomal. Since we do not expect any chemical reactivity difference between DMPIO and MNPIO, the

difference in spin-trapping ability between these two spin-traps must be due to the much greater hydrophobicity of MNPIO. Its lipophilic substituents carry the aminoxyl head-group deep into the lipid bilayer, thereby rendering MNPIO totally unavailable for trapping radicals from the aqueous phase. The absence of any signal in the case of liposomes—even with lipophilic radicals—suggests that *oxy-radicals, including* HO$^{\bullet}$, *do not penetrate into the lipid bilayer.*

Experimental

DMPO, dimyristoylphosphatidylcholine, sodium dodecyl sulfate (SDS), xanthine and xanthine oxidase were obtained from Sigma. The coloured impurity present in the commercial DMPO was removed by filtration with neutral decolourizing charcoal.[18] The resulting aqueous solution was frozen until use, to slow the process of thermal signal growth (autoxidation).[18] Spin-traps DMPIO and MNPIO (**1** and **2**, respectively) were synthesized at The Institute of Organic Chemistry, Novosibirsk, Russia.[19] $FeSO_4 \cdot 7H_2O$ (Merck), potassium ferricyanide (Riedel–deHaen), potassium chromium(III) oxalate trihydrate (Aldrich), and EDTA (Fluka) were used as supplied. All glassware was rinsed first with acid (conc. HCl) to remove all traces of detergents and finally with Elgastat UHQ purified water. Ultrapure water was used in the preparation of a 0.1 mol dm^{-3} phosphate buffer (pH 7.8) solution. The latter was utilized in turn to prepare all aqueous solutions, unless otherwise specified.

Electron spin resonance spectra were measured on a Varian E-12 EPR spectrometer operating at X-band frequency with 100 kHz magnetic field modulation at room temperature in a 200 μl flat cell. Probe sonication was performed with an MSE titanium probe ultrasonic disintegrator model MK2 at 20 kHz output frequency. At the beginning of each working day fresh solutions of 2×10^{-3} mol dm^{-3} EDTA in buffer, 1×10^{-3} mol dm^{-3} $FeSO_4$ and H_2O_2 (0.2%) in water were prepared. For the enzymatic generation of HO$^{\bullet}$, solutions of 1×10^{-3} mol dm^{-3} xanthine and 0.077 μ cm^{-3} xanthine oxidase were prepared. The exact reaction conditions are recorded in the captions to Figs. 1 and 2.

In a typical preparation of liposome, 50 mg and (10–50) $\times 10^{-3}$ mol of the appropriate spin-trap were dissolved in chloroform and transferred to a round-bottomed flask. The solvent was removed by rotary evaporation leaving a uniformly thin layer of lipid on the walls of the flask. The flask was then charged with 0.5 cm^3 of 0.1 mol dm^{-3} phosphate buffer. The lipid film was dispersed by vigorously agitating the flask contents on a vortex for 10 min to obtain multi-lamellar liposomes. These were sonicated with a titanium probe to give unilamellar liposomes. We found that samples for EPR detection gave the best results when mixed in the following order: spin-trap, alcohol (when added), EDTA, Fe^{+2} and H_2O_2.

^{1}H NMR (200 MHz) spectra were obtained on Bruker AC 200 Fourier transform spectrometer, in phosphate buffer (see above) with 10% D_2O which served as the internal standard. The spectral data of DMPIO (**1**) and MNPIO (**2**) intercalated into the bilayer of dimyristoylphosphatidylcholine liposomes are as follows. **1**, δ 8.20 (s, 1 H, vinyl, H 2), 7.90 (d, J = 6 Hz, 2 H, *ortho*), 7.50 (m, 3 H, *meta* and *para*), 1.55 (s, 6 H, C 5-methyl); **2**: 8.35–8.15 (br s, 1 H, vinyl, H 2), 8.1–7.9 (br m, 2 H, *ortho*), 7.6–7.2 (br m, 3 H, *meta* and *para*), 1.54 (br s, 3 H, C 5-methyl), 1.3–1.1 (br m, 19 H, nonyl). It should be noted that the dimyristoyl groups in these spectra absorb as broad multiplets at 2.5–2.0 ppm (4 H, methyl α to carbonyl), 1.6–1.0 (24 H, methylenes) and 1.0–0.7 (6 H, terminal CH_3).

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