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SOLVENT EFFECTS IN THE SPIN TRAPPING OF LIPID OXYL RADICALS

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Studies documenting spin trapping of lipid radicals in defined model systems have shown some surprising solvent effects with the spin trap DMPO. In aqueous reactions comparing the reduction of H_2O_2 and methyl linoleate hydroperoxide (MLOOH) by Fe^{2+}, hydroxyl (HO·) and lipid alkoxyl (LO·) radicals produce identical four-line spectra with line intensities 1:2:2:1. Both types of radicals react with commonly-used HO· scavengers, e.g. with ethanol to produce $\cdot C(CH_3)HOH$ and with dimethylsulfoxide (DMSO) to give $\cdot CH_3$. However, DMSO radicals (either $\cdot CH_3$ or $\cdot OOCH_3$) react further with lipids, and when radicals are trapped in these MLOOH systems, multiple adducts are evident. When acetonitrile is added to the aqueous reaction systems in increasing concentrations, $\cdot CH_2CN$ radicals resulting from HO· attack on acetonitrile are evident, even with trace quantities of that solvent. In contrast, little, if any, reaction of LO· with acetonitrile occurs, even in 100% acetonitrile. A single four-line signal persists in the lipid systems as long as any water is present, although the relative intensity of the two center lines decreases as solvent-induced changes gradually dissociate the nitrogen and β-hydrogen splitting constants. Extraction of the aqueous-phase adducts into ethyl acetate shows clearly that the identical four-line spectra in the H_2O_2 and MLOOH systems arise from different radical species in this study, but the lack of stability of the adducts to phase transfer may limit the use of this technique for routine adduct identification in more complex systems. These results indicate that the four-line 1:2:2:1, $a_N = a_H = 14.9$ G spectrum from DMPO cannot automatically be assigned to the HO· adduct in reaction systems where lipid is present, even when the expected spin adducts from ethanol or DMSO appear confirmatory for HO·. Conclusive distinction between HO· and LO· ultimately will require use of ^{13}C-labelled DMPO or HPLC-MS separation and specific identification of adducts when DMPO is used as the spin trap.

KEY WORDS: DMPO (5,5-dimethyl-l-pyrroline-*N*-oxide), lipids, lipid alkoxyl radicals, hydroxyl radicals, ethanol, dimethylsulfoxide.

INTRODUCTION

Free radicals of lipids are exceedingly difficult to detect directly by electron paramagnetic resonance because of their short lifetimes and oxygen-broadened lines. Thus, the technique of spin-trapping is being used increasingly to convert lipid radical species which are short-lived and/or of low concentration to stable nitroxide radicals (adducts) with spectral hyperfine splittings that reflect the nature and structure of the lipid radicals.

Nitrone spin traps have been most useful because they react less readily with unoxidized lipids than do nitroso compounds, and they trap both alkyl and oxygen-centered radicals of the lipids. However, conclusive identification of specific adducts is not straightforward, especially in complex biological or biochemical systems. Lack

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of appropriate controls, metal contamination, metabolism of traps or adducts *in vivo*, or failure to consider secondary reactions or phase localization of reactants can lead to trapping of artifacts and misassignment of spectra. To document spectral parameters and trapping characteristics of lipid free radicals in complex systems, a variety of spin-traps have been studied to catalog radicals produced during the oxidation of lipids in well-defined model systems.

Some surprising solvent effects have been observed, particularly with DMPO (5,5-dimethyl-l-pyrroline-*N*-oxide). We report here circumstances in which lipid alkoxyl radical adducts of DMPO cannot be distinguished from hydroxyl radical adducts, either spectroscopically or by reactions with commonly-used HO· scavengers.

MATERIALS AND METHODS

High purity methyl linoleate was obtained from Nu-Chek Prep and was oxidized in air to obtain hydroperoxide-enriched lipid, referred to as MLOOH throughout this paper. Previous results have shown that this lipid preparation behaves qualitatively the same as chromatographically purified MLOOH under the conditions of this study. DMPO (Aldrich) was filtered through activated charcoal under argon and protected from light, then stored frozen in concentrated stock solutions until used. Ferrous ammonium sulphate, titanous sulphate (20% in H_2SO_4 reagent), hydrogen peroxide and DMPO solutions were prepared fresh daily in argon-sparged water or water/acetonitrile mixtures. Particular care was taken to prevent ferrous iron oxidation before reactions by holding the iron solutions under argon. 18-Megohm high purity water was used throughout the study.

Reactions were conducted by adding reactants in the following order: water or other diluent (e.g. acetonitrile, ethanol, or dimethylsulfoxide), H_2O_2 or MLOOH, DMPO, and iron or titanium. Reactants were immediately taken up in a syringe and transferred to a quartz EPR flat cell for analyses. Typical transfer times were 15 seconds.

In situ electrolytic reductions of H_2O_2 or MLOOH in the presence of DMPO were conducted in the EPR cavity using a preparative electrolysis flat cell with a platinum gauze cathode within the region of EPR detection and a platinum wire anode outside the detection region; electrolytes were 0.1 M tetraethylammonium perchlorate or KCl for organic and aqueous systems, respectively. Current was applied from a Power Designs power supply, applied voltage was monitored with a Keithley Autoranging DMM voltammeter, and electrolysis current was measured with a microammeter. Actual working potentials could not be measured in this cell, but they were estimated by comparision with applied voltages necessary to reduce substrates (e.g. paraquat) of known reduction potentials. Electrolysis of the spin trap alone verified that the applied voltages employed with MLOOH were within the useful potential windows previously reported for DMPO.[1]

EPR spectra were obtained with a Varian E-12 spectrometer, using a TE_{102} microwave reflection cavity with field modulation at 100 kHz. Spectra were recorded as first derivatives by signal averaging 100-gauss magnetic field scans of 2048-channel resolution at a data acquisition rate of 60 Hz (34.13 sec/scan). With an EPR time constant of 0.03 sec, spectrometer output was passed through a Krohn-Hite Model 3750R active filter operating in the low-pass mode, with a cut-off at 30 Hz. A Masscomp 5500 computer provided the voltage ramp for spectrometer field scans as well as recording,

signal averaging, storage, processing, and analysis of data. Field or frequency drift on consecutive scans was offset by using a marker signal from the spectrometer's field-frequency lock to align each scan to the same reference channel before adding it to the data buffer used for signal averaging. Microwave frequency was measured with a frequency counter; magnetic field scanning and *g*-value determinations were calibrated using Fremy's salt (peroxylamine disulphonate).[2] Splitting constants were calculated from computer-generated second derivatives of the spectra, after optimizing signal-to-noise ratios, and were verified by computer simulations.

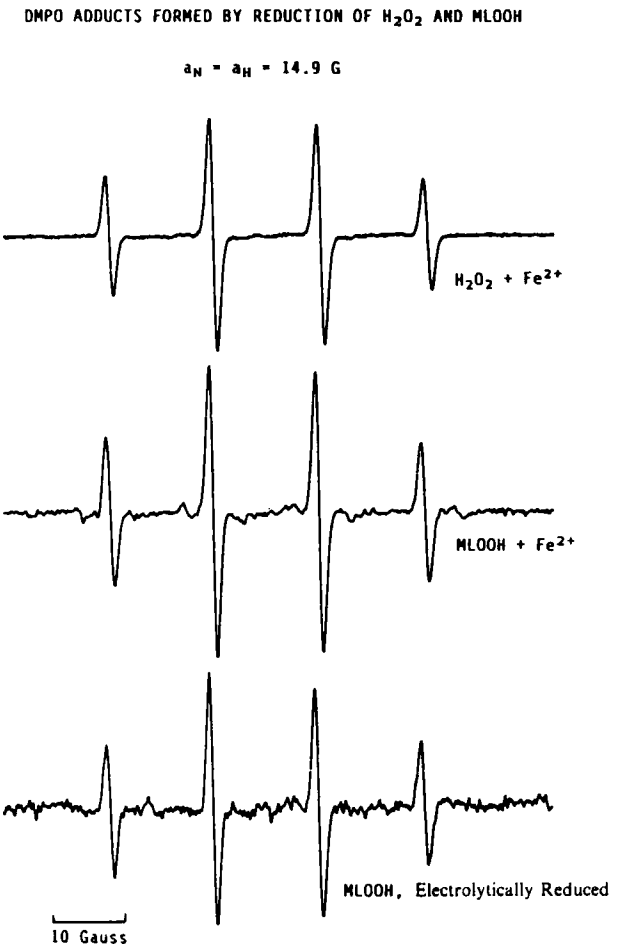

FIGURE 1 Spectra of DMPO adducts formed by reduction of H_2O_2 and MLOOH in water. Top trace: reduction of H_2O_2 by Fe^{2+} (ammonium sulphate) [Ti^{3+} or reductive electrolysis give identical spectra]. Middle trace: reduction of MLOOH by Fe^{2+}. Bottom trace: electrolytic reduction of MLOOH. Splitting constants for all spectra: $a_N = a_H = 14.9$ G; relative line intensities: 1:2:2:1. Instrumental conditions: 0.5 G modulation, 10 mW power, 2000 gain (top), 5000 gain (middle), 10000 gain (bottom). Reactant concentrations: (top) 30 mM DMPO, 55 mM H_2O_2, 0.016 mM Fe^{2+}; (middle) 30 mM DMPO, 80 mM MLOOH, 10^{-4} M Fe^{2+}; (bottom) 30 mM DMPO, 30 mM MLOOH, 0.01% Triton-X 100, 0.1 M KCl.

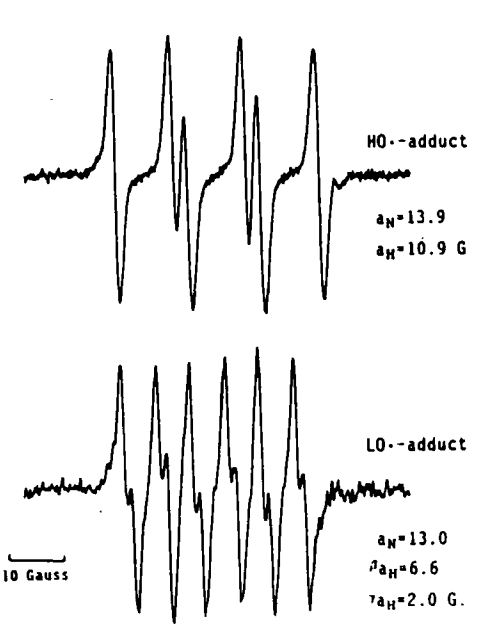

FIGURE 2 Spectra of DMPO adducts from aqueous phase Ti^{3+} reduction of H_2O_2 (top spectrum) and Fe^{2+} reduction of MLOOH (bottom spectrum) extracted into ethyl acetate. Top spectrum: Reactant concentrations: 30 mM DMPO, 230 mM H_2O_2, 0.09 mM $TiSO_4$; instrumental conditions: 1 G modulation, 10000 gain, 10 mW power. Bottom spectrum: Reactant concentrations: 30 mM DMPO, 80 mM MLOOH, 0.1 mM Fe^{2+}; instrumental conditions: 0.5 G modulation, 10000 gain: 20 mW power.

RESULTS

Reduction of H_2O_2 by Fe^{2+}, by Ti^{3+}, or by electrolysis *in situ* in the presence of DMPO in argon-sparged water yielded four-line spectra with 1:2:2:1 relative line intensities and splitting constants (a_N = 14.9, a_H = 14.9 G) typical of the HO· adduct of DMPO (Figure 1). Reduction of MLOOH (solubilized by triton-X 100) by Fe^{2+} under the same conditions resulted in spectra identical to those produced by HO· adducts, as shown in the middle trace of Figure 1. Since reductive electrolysis of MLOOH also produced this spectrum (lower trace, Figure 1), no signals were detected in deaerated control systems of Fe^{2+} and DMPO without MLOOH or H_2O_2, and only very weak signals were produced in aerated controls, the adducts were not dependent on the presence of iron, nor did they result from autoxidation of iron. Either the HO· and the lipid-derived adducts were different and their spectra were fortuitously identical in water, or both spectra resulted from HO· adducts, which could conceivably (though not likely) be formed in the lipid system by homolytic reduction of the hydroperoxides or by degradation of the lipid alkoxyl radical adduct.

The kinetics of formation of the two adducts differed substantially. An intense HO· adduct formed immediately in the H_2O_2 system, then decayed rapidly. This decay could be slowed by decreasing the Fe^{2+} or Ti^{3+} concentrations from the usual 0.03–0.12 mM. In contrast, the adduct spectrum in the MLOOH system formed

slowly and increased with time; it was much more sensitive to oxygen, broadening and decreasing in intensity when oxygen was present. These characteristics support the production of two distinct adducts in these systems, but in themselves are not conclusive evidence.

Several additional approaches were used to determine the origins of the adducts in the H_2O_2 and MLOOH systems. First, adducts were generated as usual, spectra were verified by EPR, and then adducts were extracted into ethyl acetate, as prescribed by Trudell.[3] The spectra of the corresponding adducts in ethyl acetate are shown in Figure 2. The line pattern and splitting constants (a_N = 13.9, a_H = 10.9 G) of the HO· adduct are comparable to those previously reported.[3] However, the spectra of the lipid-generated adducts were distinctly different, with splitting constants a_N = 13.0, $^{\beta}a_H$ = 6.6, $^{\gamma}a_H$ = 2.0 G. If, in fact, the same adducts that were responsible for the spectra in water were extracted into ethyl acetate, then it clearly seems that in water the HO· and lipid adducts of DMPO are spectrally identical but chemically different.

With regard to extraction into ethyl acetate or other organic solvents, it should be noted that neither adduct was very stable to the phase transfer. The MLOOH-generated adduct was markedly destabilized by the transfer, especially in the presence

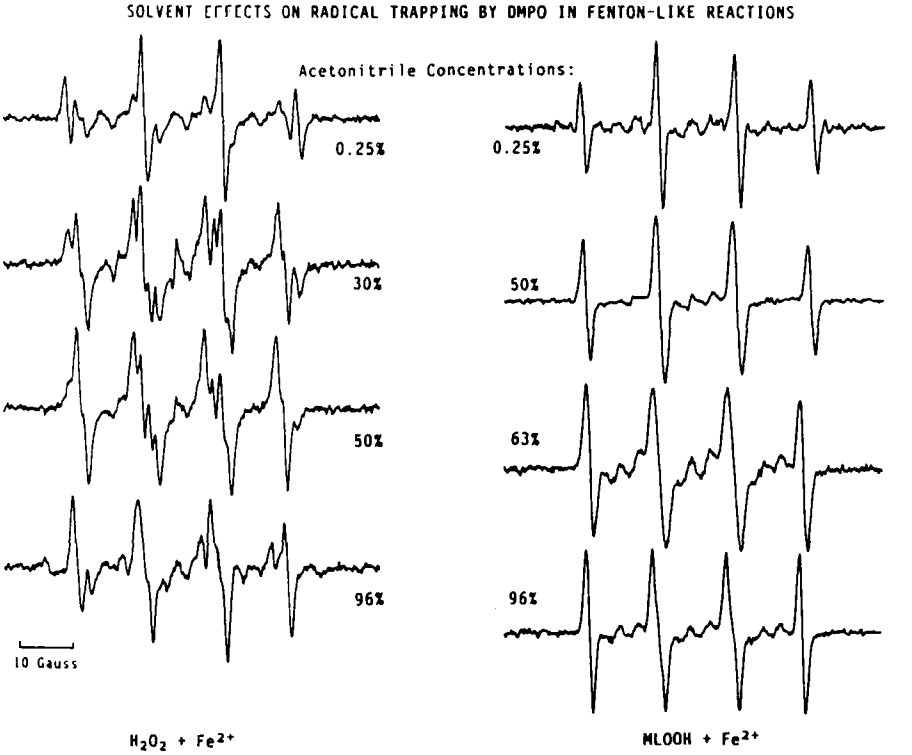

FIGURE 3 Effects of varying acetonitrile concentrations on radical trapping and spectral characteristics in Fenton-like reactions of H_2O_2 and MLOOH. Instrumental conditions for all spectra: 0.5 G modulation, 10 mW power, gains varying from 6300 to 12500. H_2O_2 systems: 22–30 mM DMPO, 40 mM H_2O_2, 0.025–0.12 mM Fe^{2+}, in acetonitrile as indicated. MLOOH systems: 22–30 mM DMPO, 50 mM MLOOH, 0.12 mM Fe^{2+}, in acetonitrile as indicated.

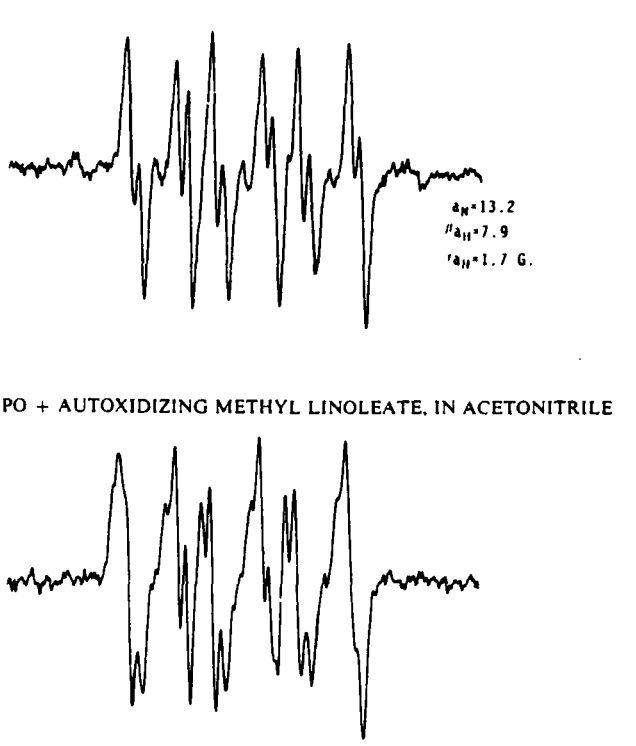

FIGURE 4 Lipid radical adducts of DMPO formed in acetonitrile. (Top): DMPO adduct formed by electrolytic reduction of MLOOH in acetonitrile. Reactant concentrations: 100 mM DMPO, 50 mM MLOOH, 0.1 M tetraethylammonium perchlorate. Instrumental conditions: 0.32 G modulation, 10 mW power, 10000 gain. (Bottom): DMPO adduct formed when oxidizing methyl linoleate was dissolved directly in aprotic acetonitrile in the presence of DMPO. Lack of a hydrogen donor in this case prevented MLOOH formation, thereby stabilizing MLO· and MLOO·. Reactant concentrations: 100 mM DMPO, 70 mM oxidizing methyl linoleate. Instrumental conditions: 0.32 G modulation, 10 mW power, 10000 gain. Splitting constants for major component same as for top sepctrum.

of oxygen. Also, because of the oxygen solubility in organic solvents, careful degassing or argon-sparging of the ethyl acetate was necessary to obtain spectra.

To further examine whether solvent effects alone accounted for the identical HO· and lipid spectra, the HO· and lipid adducts were studied in acetonitrile/water mixtures. This system was selected because acetonitrile has one of the lowest rate constants for reaction of HO· with organic solvents,[4] and because it both solubilizes lipids and mixes with water, providing a single-phase system. As can be seen in Figure 3, HO· and lipid radicals behaved differently in mixed solvent systems. With even traces of acetonitrile present, the six-line spectrum of the $\cdot CH_2CN$ adduct, resulting from attack of HO· on acetonitrile, was evident in the H_2O_2 system; and this adduct increased with the proportion of acetonitrile. However, no such secondary radical was evident in the MLOOH systems, even at greater than 96% acetonitrile. Instead, the splittings decreased in their degeneracy as the percentage of acetonitrile increased, until line intensities were approximately equal and distortions of the lineshapes of the two center lines indicated that unresolved splittings were clearly present.

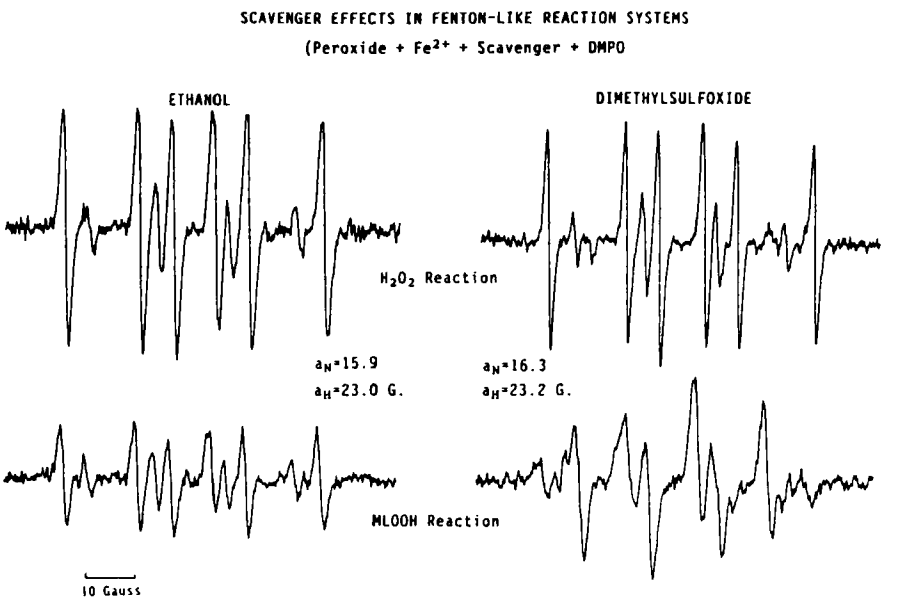

FIGURE 5 Effects of HO· scavengers on radicals trapped by DMPO during Fe^{2+} reductions of H_2O_2 and MLOOH. Left spectra: Both HO· and lipid radicals (presumably LO·) attack ethanol to form ·C(CH_3)HOH radicals, which give the 6-line spectra dominant in these traces. For both spectra, splitting constants are a_N = 15.9, a_H = 23.0 G; instrumental conditions 1 G modulation, 10000 gain, 20 mW power. Right spectra: HO· attacks DMSO to give ·CH_3 radicals and the dominant 6-line spectrum (top trace), but a complex multicomponent spectrum, consisting of ·CH_3 and additional radicals, is produced in the MLOOH system. Weaker 4-line spectra of HO·/LO· radical adducts also are evident in all reactions. Splitting constants: a_N = 16.3, a_H = 23.2 G (H_2O_2, top); unresolvable for MLOOH system (bottom). Instrumental conditions: (H_2O_2, top) 0.5 G modulation, 10000 gain, 20 mW power; (MLOOH, bottom) 1 G modulation, 12500 gain, 20 mW power. Reactant concentrations: H_2O_2 reaction (32 mM H_2O_2, 30% EtOH or 16% DMSO, 33 mM DMPO, 0.03 mM ferrous ammonium sulphate); MLOOH reaction (50 mM MLOOH, 30% EtOH or 0.033% DMSO, 33 mM DMPO, 0.06 mM ferrous ammonium sulphate).

Since iron salts are not soluble in neat acetonitrile, the spectral characteristics of the lipid adduct in 100% acetonitrile were determined by electrolytic reduction of MLOOH in an EPR electrolysis cell. Figure 4 shows the spectrum obtained at an applied voltage of −0.80 V, corresponding to a reduction potential of approximately −0.22 V vs. the normal hydrogen electrode (NHE). This was the lowest applied voltage at which a strong, clearly resolvable spectrum could be detected, although weaker, poorly resolved spectra were observed at applied potentials as high as −0.50 V (approximately −0.15 V vs NHE). The spectrum is comparable to that obtained in ethyl acetate, with differences in splitting constants arising from the different solvents: a_N = 13.2, $^{\beta}a_H$ = 7.9, and $^{\gamma}a_H$ = 1.7 G, values typical of alkoxyl radical adducts of DMPO.[5-7]

A composite spectrum whose major component was identical to that formed by reductive electrolysis was formed when DMPO was used to trap radicals of oxidizing neat methyl linoleate in aprotic acetonitrile (Figure 4, lower spectrum), although an additional unresolvable adduct was also present in lower quantities. Splitting constants for the dominant adduct in acetonitrile were a_N = 13.2, $^{\beta}a_H$ = 7.9, and $^{\gamma}a_H$ = 1.7 G. No radicals could be trapped directly in oxidizing methyl linoleate if,

instead of acetonitrile, the lipid was added to a protic organic solvent or to water where a ready supply of abstractable protons probably converted LO· and LOO· to LOH and LOOH, respectively.

That the same spectra are generated by several distinctly different reactions, all of which would be expected to produce alkoxyl radicals, certainly supports a tentative assignment of MLO· adducts for the spectra in organic solvents (Figures 2 and 4). Therefore, if the adduct formed in water was indeed extracted without change into ethyl acetate in these studies, the four-line "HO·-like" adduct spectrum of DMPO in water also clearly seems to arise from methyl linoleate alkoxyl radical adducts of DMPO.

Reaction with various scavengers has been commonly used as a test for HO·.[7-11] Thus, the effects of ethanol and dimethylsulfoxide on the H_2O_2 and MLOOH reactions were investigated to gain information about the reactivity of the radicals formed in the H_2O_2 and MLOOH systems, and to explore various means of experimentally distinguishing between HO· and lipid alkoxyl radicals. Typical $\cdot C(CH_3)HOH$ and $\cdot CH_3$ adducts from HO· attack on ethanol and DMSO, respectively, were evident in the H_2O_2 systems (Figure 5). The $\cdot C(CH_3)HOH$ from ethanol was also produced using MLOOH, although the total signal intensity was quenched more than with H_2O_2. However, concentrations of DMSO which produced an adduct in the presence of H_2O_2 prevented adduct formation completely with MLOOH. The signal shown in Figure 5 was obtained only with very low DMSO concentrations and with limitation of oxygen concentrations. Thus, EPR spectral characterisitcs of these two scavengers cannot be used as a conclusive test for HO· in biological or biochemical systems since MLO· and HO· produce similar spectra.

When DMPO is used to trap oxygen-centered radicals in multiphasic biochemical systems, it is important to be able to distinguish between lipid radicals in aqueous environments and HO· in lipid environments. Hence, the DMPO–HO· adduct formed in water by reduction of H_2O_2 was extracted into oleic acid in the same manner as noted above for ethyl acetate. Results indicate that in lipid as well as aqueous phases, HO· and lipid oxyl radicals form spectrally indistinguishable adducts with DMPO. The HO· spectrum shown in Figure 6 (top) is identical to the spectrum of the radical trapped in oxidizing oleic acid (Figure 6, bottom), where no HO· were present. Thus, even in lipid environments of membranes it may not be possible to distinguish HO· and lipid alkoxyl radicals by the EPR spectra of their respective DMPO adducts.

DISCUSSION

One of the problems in using nitrone spin traps has been establishing the identity of individual adducts conclusively, a task which is often difficult in complex systems when the variations in splitting constants for different radicals are very small.[5] This problem is compounded in multiphasic systems, because solvent effects on adduct splitting constants may be greater than the intrinsic differences between the specific radicals forming the adducts.

DMPO has been a very popular spin trap for use in biological systems, particularly for oxygen radical studies, for several reasons. Unlike nitroso compounds or phenyl t-butylnitrone (PBN) and related spin traps, DMPO reacts rapidly with oxygen-centered radicals to produce reasonably stable adducts which have distinctively

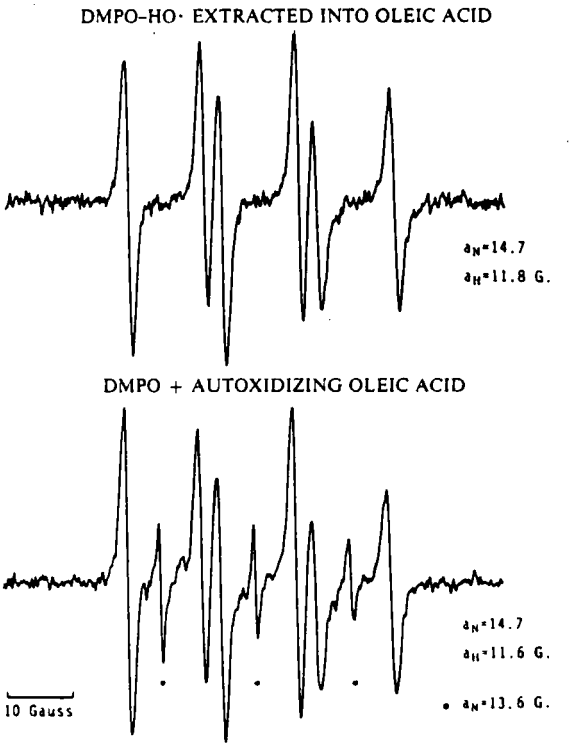

FIGURE 6 Indistinguishable signals produced from DMPO-HO· formed in water via reduction of H_2O_2 by Fe^{2+}, then extracted into oleic acid (top spectrum) and from lipid peroxyl or alkoxyl radicals trapped by DMPO in oxidizing oleic acid. Top spectrum: Instrumental conditions 0.05 G modulation, 10000 gain, 10 mW power; reactant concentrations (original reaction before extraction) 30 mM DMPO, 230 mM H_2O_2, 0.9 mM $TiSO_4$. Bottom spectrum: Instrumental conditions 0.5 G modulation, 10000 gain, 5 mW power; reactant concentrations 100 mM DMPO in oleic acid in which oxidation was initiated several hours previously by 10^{-7} M Fe^{3+} partitioned into the lipid phase.

different line patterns and/or splittings for HO·, alkoxyl, peroxyl, and carbon-centered radicals.[12] Indeed, DMPO has been the spin trap of choice for detection of HO· because of the unique four-line signal of the HO· adduct, with line intensity ratios 1:2:2:1. Furthermore, experience in this laboratory has shown that DMPO, although not impervious to formation of artifactual spectra, is not as prone to production of artifacts in oxidizing systems as are PBN and its analogues.

The appearance of the unique "HO·" signal with DMPO has commonly been taken as proof that HO· are produced in a wide range of experimental situations; and production of characteristic secondary radicals from various scavengers has been considered verification that the four-line signal originates from authentic HO· rather than from decomposition of the HOO· DMPO adduct or other adducts.[8,10] However, the data reported here indicate that these assumptions do not hold in reaction systems containing lipids. Whether in aqueous phase or lipid phase, HO· and lipid alkoxyl radical adducts of DMPO may be impossible to distinguish by EPR analyses alone.

EPR spectra produced in aqueous model systems containing methyl linoleate hydroperoxides being reduced to MLO· in the presence of DMPO were identical to

the typical "HO· spectra" produced by reduction of H_2O_2 to HO·. These spectra were clearly shown to arise from lipid species rather than HO· adducts produced either in homogeneous decomposition of MLOOH or by rearrangement or decomposition of lipid adducts. Until more definitive data is obtained, it can only be surmised that an 18-carbon lipid produces an adduct spectrum identical to that of HO· in water because hydrophobic forces cause a close alignment of the two segments of the lipid and repulsion of this complex from the DMPO molecule so that no interaction between the β-hydrogen and the lipid hydrocarbon chain results.

There are circumstances in the lipid phase as well when the HO· and lipid oxyl radical adduct spectra are spectroscopically indistinguishable. For example, HO· adducts which form in the aqueous phase and migrate into the lipid phase give the same spectra as radicals trapped in autoxidizing lipid, with no HO· present. Thus, with the appearance of either adduct in a membrane or other multiphase reaction system containing lipids, one would not be able to determine by EPR spectra alone whether the four-line signal was produced by HO· in the aqueous phase or by LO· with some access to water, e.g. at a membrane surface or phase interface. Similarly, with the appearance of the six-line signal, one would not be able to distinguish conclusively between lipid radicals formed in the lipid phase and HO· formed in the aqueous phase and subsequently partitioned into the lipid phase.

The issue of localization is complicated still further by the fact that HO· formed in lipid phases and trapped there by DMPO give spectra which differ from those of aqueous phase adducts and of aqueous adducts extracted into lipid. The resulting spectra and chemistry depend critically on the nature of the lipid: specifically, its proticity and degree of unsaturation.[13] This poses a particularly troublesome problem for research seeking to identify oxygen radical species associated with various pathologies, since amphiphilic nitrone spin traps, including DMPO, have been shown to assemble with lipid micelles and vesicles such that the nitrone functionality resides in the interfacial region, and radicals are trapped both in the hydrophobic domain of the micelle or vesicle and in the aqueous exterior domain.[14]

Nor are scavenger effects specific for HO·. Both HO· and MLO· reacted with ethanol to produce characteristic secondary $\cdot C(CH_3)HOH$ radicals; and the possibility that other alkoxyl radicals may react similarly with ethanol also should be considered.

HO· reacted with DMSO to give $\cdot CH_3$ radicals, but the reaction of LO· with DMSO was more complex. A small amount of $\cdot CH_3$ adduct *was* evident in the spectrum, as were several other radicals. It has been shown by Raleigh[15,16] that $\cdot CH_3$ rapidly forms $\cdot OOCH_3$ in the presence of oxygen, and that the latter radical reacts with lipids. Results of the present study support the reactivities of LO· with DMSO and of $\cdot CH_3$ and/or $\cdot OOCH_3$ with lipids. High concentrations of DMSO (more than a few percent) prevented the production of a detectable EPR spectrum completely. At very low concentrations of DMSO, $\cdot CH_3$ and/or $\cdot OOCH_3$ appeared to react with lipids to form secondary radicals of yet unknown identity.

How, then, in practical terms, may HO· and LO· be differentiated conclusively, particularly in complex systems such as those just described? Selective use of organic solvents may provide one means of discriminating which radicals have produced the four-line spectrum. For example, the rate constant for the reaction of HO· with acetonitrile is about $10^6 M^{-1} sec^{-1}$.[4] That MLO· did not react with acetonitrile probably reflects a reaction rate constant which is substantially lower than this and also lower than that for DMPO trapping of MLO·. It may be possible to use this reactivity difference to advantage if appropriate consideration is given to proticity and solvation changes this solvent may induce.

A second approach to differentiating HO· and LO· radicals may utilize extraction of DMPO adducts from aqueous phases into organic solvents such as ethyl acetate, where the four lines separate into more line with different splitting patterns. However, this kind of extraction requires deoxygenation and may not be feasible with all reaction systems. Even under favorable circumstances, HO· and MLO· adducts in this study were somewhat unstable to the phase transfer. Thus, this approach may not always be successful (i.e. produce detectable spectra), especially in systems more complicated than the ones used in this study. Use of this technique will also require further verification that the radicals extracted are the same ones originally formed in the aqueous phase.

Clearly, analyses supplementary to EPR are necessary for both conclusive identification and localization of adducts. One potential alternative for providing this information is use of ^{13}C-labelled spin traps to afford more definitive EPR spectral differentiation of adducts; also, ^{13}C chemical shifts in the NMR spectra of these traps can be used to gauge the polarity of the environment experienced by a spin trap in heterogeneous systems.[17] Ultimately, HPLC separation[18-21] and independent chemical analyses of adducts may be necessary to identify conclusively the radical adducts of DMPO and other spin traps. Preliminary studies using this technique have verified the production of different adducts in the H_2O_2 and MLOOH reaction systems, but further work is needed before the chromatographic patterns of DMPO and its adducts produced during the reduction of H_2O_2 and MLOOH are fully understood.

Acknowledgements

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