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Spin trapping of free radicals during hepatic microsomal lipid peroxidation

(superoxide/lipid peroxyl radicals/hydroxyl radicals)

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ABSTRACT **We have used spin-trapping techniques to identify radical species formed during the NADPH-stimulated peroxidation of rat hepatic microsomes. Using 5,5-dimethyl-1-pyrroline-1-oxide, we have confirmed the presence of substantial quantities of superoxide but found evidence for the formation of only small quantities of hydroxyl radical. Use of the spin traps *N-tert*-butyl-α-4-pyridylnitrone-1-oxide and 2-methyl-2-nitroso-1-propanol have allowed us to determine that lipid peroxyl radicals are the predominant "lipid-type" radical found in peroxidizing microsomes under aerobic conditions. These data suggest that microsomal lipid dienyl radicals react with molecular oxygen at near diffusion-controlled rates.**

Peroxidation of polyunsaturated membrane lipids is a naturally occurring free radical chain reaction that has been implicated as a mechanism of tissue damage (1). Extensive research has been conducted in an effort to identify the primary free radical that initiates endogenous NADPH-stimulated lipid peroxidation. Convincing evidence exists that both superoxide and hydroxyl radical are involved in the initiation step. However, there is some controversy concerning the role and relative importance of these species. Fong *et al.* (2) and others (3, 4) have proposed that an ADP-Fe^{3+} catalyzed Haber–Weiss reaction, requiring hydrogen peroxide and superoxide, produces hydroxyl radical which is the actual initiating species. The importance of superoxide in such a sequence has been further demonstrated by Svingen *et al.* (5) who showed that superoxide dismutase prevents the NADPH-stimulated lipid peroxidation.

The direct detection of radical initiators and intermediates involved in lipid peroxidation is theoretically possible with electron paramagnetic resonance (EPR) spectrometry; however, most of these radical species are so reactive that they never reach the steady-state levels required for direct detection. For example, the hydroxyl radical reacts with most organic molecules at diffusion-controlled rates (6). In theory, spin-trapping techniques can overcome this problem. This method consists of using a spin trap, a compound that forms a stable or semistable nitroxide radical by reacting covalently with an unstable free radical such as the hydroxyl radical. In this way, the unstable radical is "trapped" as a long-lived species that can be observed at room temperature by using conventional EPR equipment. The hyperfine splitting constants of the adduct provide information that can aid in the identification and quantification of the original radical. Because the relatively stable nitroxide free radical accumulates, spin trapping is an integrative method for measuring free radicals and is inherently more sensitive than procedures that detect only instantaneous or steady-state levels of free radicals (for current reviews, see refs. 7 and 8). It has been reported (9–11) that, in microsomes, only hydroxyl radical can be spin trapped even though other radical species, such as superoxide and lipid hydroperoxyl radicals, have been shown to be generated during lipid peroxidation (1).

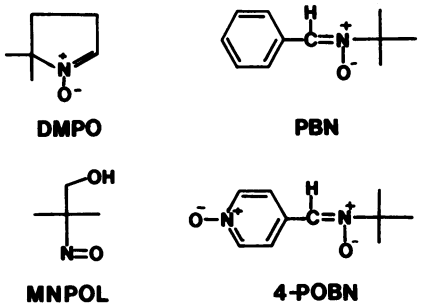

FIG. 1. Structures of the spin traps.

Here we describe experiments showing that, in the presence of rat liver microsomes and NADPH, superoxide, hydroxyl, and lipid peroxyl radicals (LOO·) are spin trapped; we could not find any evidence for the existence of a carbon-centered dienyl lipid radical (L·).

MATERIALS AND METHODS

General Comments. Hemin, *N,N*-bis(2-[bis(carboxymethyl)amino]ethyl)glycine (DETAPAC), soybean lipoxygenase, and NADPH were purchased from Sigma. Linoleic acid was obtained from Nu Chek Prep (Elysian, MN) and was >99% pure. 2-Methyl-2-nitropropane was purchased from Fluka/Tridom. Chelex-100 was obtained from Bio-Rad. ESR spectra were recorded by using a Varian Associates model E-9 spectrometer. The spin traps 2-methyl-2-nitroso-1-propanol (MNPOL) and 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) were prepared according to the methods outlined by DeGroot *et al.* (12) and Bonnett *et al.* (13). The spin trap *N-tert*-butyl-α-4-pyridylnitrone-1-oxide (4-POBN) was a gift from William Yamanashi (Department of Ophthalmology, Emory University, Atlanta, GA). Because the rate of spin trapping of some free radicals is slow [e.g., the rate of spin trapping superoxide by DMPO is 10 $M^{-1}sec^{-1}$ (8)], high concentrations of the probes are essential. For this reason, we chose to use the water-soluble spin traps DMPO, 4-POBN, and MNPOL (Fig. 1). Unless otherwise indicated, all buffers used were passed through a Chelex-

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Abbreviations: EPR, electron paramagnetic resonance; LOO·, lipid peroxyl radical; L·, dienyl lipid radical; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO-OH, 5-hydroxy-2,2-dimethyl-1-pyrrolidinyloxyl; DMPO-OOH, 5-hydroperoxy-2,2-dimethyl-1-pyrrolidinyloxyl; 4-POBN, *N-tert*-butyl-α-pyridylnitrone-1-oxide; 4-POBN-OH, *tert*-butyl(hydroxy)-4-pyridylmethyl nitroxide; 4-POBN-OOH, *tert*-butyl(hydroperoxy)-4-pyridylmethyl nitroxide; MNPOL, 2-methyl-2-nitroso-1-propanol; MNPOL-H, 2-methylpropyl-1-nitroxide; DETAPAC, *N,N*-bis(2-[bis(carboxymethyl)amino]ethyl)glycine.

100 column as described by Poyer and McCay (14) in order to remove divalent metal impurities. Unless indicated otherwise, DETAPAC (1 mM) was included in the buffers to prevent hydroxyl radical formation; iron–DETAPAC is unable to catalyze hydroxyl radical formation from hydrogen peroxide plus superoxide (15, 16).

Spin trapping of free radicals generated during NADPH-stimulated lipid peroxidation was undertaken as follows. In a typical experiment, the reaction medium contained 0.1 M spin trap, 250 μM NADPH, 0.1 ml of rat liver microsomal suspension [prepared as described (17)], and sufficient buffer (0.1 M phosphate/1 mM DETAPAC adjusted to pH 7.4) to bring the final volume to 0.5 ml. The following necessary controls were performed and no discernable EPR signals were detected: all spin traps were incubated with rat hepatic microsomes without NADPH and with NADPH, but without rat liver microsomes.

The spin trapping of L· by MNPOL as described by DeGroot *et al.* (12) was undertaken by using the action of lipoxygenase on linoleic acid. The effect of 4-POBN on lipid oxidation by lipoxygenase was determined by measuring the rates of oxygen uptake at 20°C with a Clarke type electrode. Identical rates of oxygen uptake (0.14 mmol/min) were obtained in the absence and presence of 4-POBN (0.1 M) from a solution of linoleic acid (sodium salt, 2.1 mM) and soybean lipoxygenase (30 μg/ml, Sigma type I) in 0.2 M Chelex-treated sodium borate buffer at pH 9.0 containing 1 mM DETAPAC.

The assignment of the MNPOL-H spectrum was verified by the chemical reduction of MNPOL with sodium borohydride. A solution of 0.1 M MNPOL in pH 9.0 sodium borate buffer was carefully added to a freshly prepared solution of 0.025 M sodium borohydride in water at room temperature. The EPR spectrum obtained was identifical to that observed when MNPOL was added to the rat liver microsome/NADPH system. MNPOL in buffer solution exhibited no EPR signal under the conditions utilized to observe the spin adducts.

RESULTS

The incubation of DMPO with rat hepatic microsomes and NADPH resulted in the formation of paramagnetic species (Fig. 2). The spectrum illustrated in Fig. 2*A* is a combination of 5-hydroperoxy-2,2-dimethyl-1-pyrrolidinyloxyl (DMPO-OOH) and, to a lesser extent, 5-hydroxy-2,2-dimethyl-1-pyrrolidinyloxyl (DMPO-OH). This assignment was based on previously published spectra of these species (18, 19). The appearance of both spin adducts in this peroxidizing mixture suggests the presence of both superoxide and hydroxyl radical; however, we have earlier reported (19) that DMPO-OOH rapidly decomposes into DMPO-OH. Ethanol (0.95%) was added to the incubation mixture to determine whether hydroxyl radical is formed in this peroxidizing enzyme system. It has been established that the hydroxyl radical reacts with ethanol to produce the α-hydroxyethyl radical while superoxide does not undergo such hydrogen atom abstraction (20). This secondary radical can react with DMPO to produce a spin adduct with an EPR spectrum distinguishable from that of the hydroxyl radical adduct (Fig. 2*B*). Thus, if the production of DMPO-OH is due to the spin trapping of hydroxyl radical, the addition of ethanol should inhibit the production of DMPO-OH and result in the appearance of a new signal due to the spin trapping of the α-hydroxyethyl radical. When DMPO was incubated with rat liver microsomes, 0.95% ethanol, and NADPH, a small but finite quantity of α-hydroxyethyl radical was spin trapped (Fig. 2*C*). The two outermost lines in the spectrum are due to the DMPO-α-hydroxyethyl radical species. It can be surmised from the relative magnitude of these lines that only a small quantity of hydroxyl radical is formed in this peroxidizing microsomal mixture. Thus, it appears that most of the observed DMPO-OH spectrum is due to decomposition of DMPO-OOH.

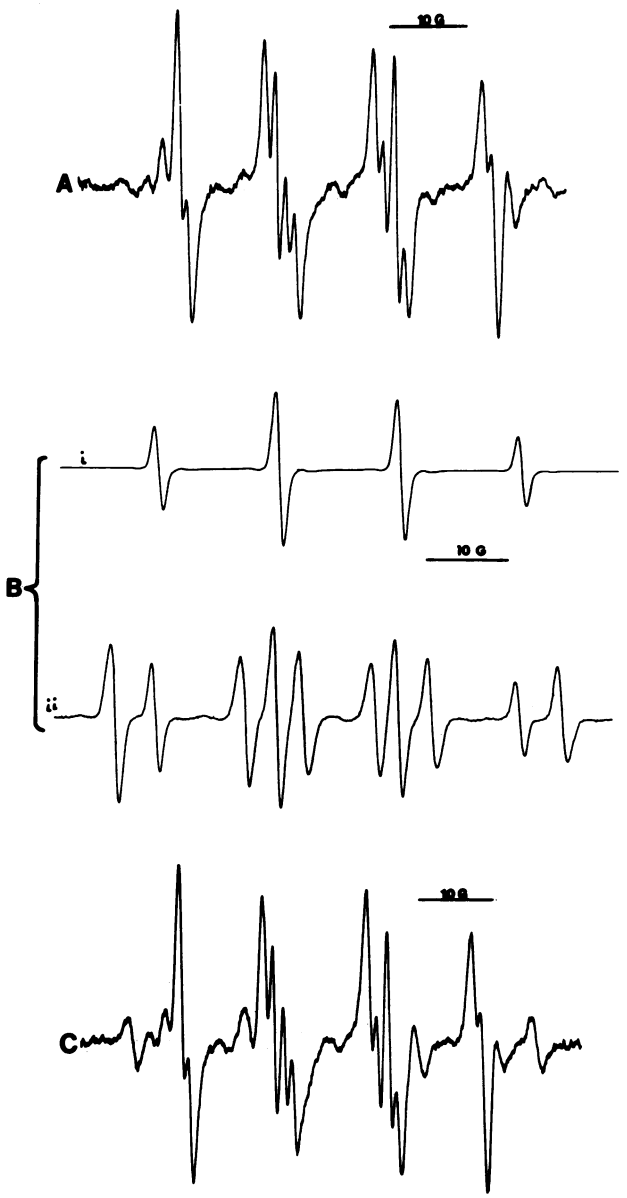


FIG. 2. (*A*) EPR of DMPO-OOH produced by the reaction of DMPO with rat hepatic microsomes and NADPH at pH 7.4 containing 1 mM DETAPAC. A_N = 14.3 G; A_H = 11.7 G; and A_H^γ = 1.25 G. (*B*) Effect of ethanol on hydroxyl radical trapping by DMPO. (*i*) Hydroxyl radical adduct generated by UV photolysis of H_2O_2. $A_H = A_N$ = 14.9 G. (*ii*) Combination of hydroxyl and α-hydroxyethyl radical adducts generated by UV photolysis of H_2O_2 in the presence of ethanol. A_N = 15.8 G; A_H = 22.8 G. (*C*) EPR spectrum of DMPO-OOH and the α-hydroxyethyl radical adduct produced by the reaction of DMPO and ethanol with rat liver microsomes and NADPH at pH 7.4 with 1 mM DETAPAC. A_N = 14.3 G, A_H = 11.7 G, and A_H^γ = 1.25 G for DMPO-OOH; A_N = 15.8 G and A_H = 22.8 G for the α-hydroxyethyl DMPO spin-trapped adduct.

The inability of DMPO to spin trap any other detectable free radicals under these conditions does not imply that other radical species such as L· or LOO· are not produced in the peroxidizing mixture. It is possible that conformational restrictions prevent reaction of DMPO with these free radicals or that these spin adducts are not sufficiently stable to be observed by conventional EPR spectrometry. To test this hypothesis, we treated DMPO or 4-POBN, another spin trap, with linoleic acid and lipoxygenase in which L· are known to be produced (12). Al-

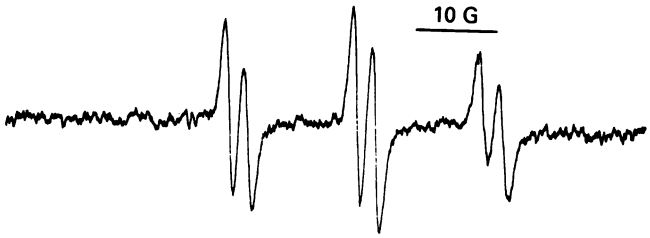


FIG. 3. EPR spectrum of 4-POBN-OOL obtained by the action of lipoxygenase on linoleic acid in the presence of 4-POBN at pH 9.0. A_N = 15.8 G; and A_H = 2.6 G.

though 4-POBN was able to spin trap a free radical (Fig. 3) which we have assigned to be the nitroxide 4-POBN-OOL (see *Discussion*), there was no evidence for the existence of a spin-trapped adduct of DMPO.

The assignment of the spectrum in Fig. 5 to 4-POBN-OOL was made with the aid of another spin trap, MNPOL, and previous observations by Wargon and Williams (21). These investigators (21) showed that, although nitroso compounds are capable of spin trapping both carbon- and oxygen-centered radicals, the oxygen-centered radical (e.g., MNPOL-OOL) decomposes too rapidly at ambient temperature to be observed by EPR spectrometry. Thus, only carbon-centered radicals are detected by this spin trap. Upon addition of MNPOL to the lipoxygenase/linoleic acid system, linoleic acid free radical (L·) was spin trapped. This species, however, could not be detected until 20 min after the components were mixed (Fig. 4). As discussed earlier, when 4-POBN was substituted for MNPOL in the above reaction mixture, an EPR spectrum was observed immediately (Fig. 3). These results suggest that in the linoleic acid/lipoxygenase system the linoleic acid free radical (L·) is initially formed but its reaction with molecular oxygen is faster than its reaction with MNPOL. Within 15 min, the reaction mixture became anaerobic as determined by monitoring oxygen consumption with a Clarke type electrode. Thus, MNPOL-L is the only product observed by EPR spectrometry. However, when 4-POBN was used in place of MNPOL, no L· radical was spin trapped. We arrive at this conclusion on the basis of several observations. First, the EPR spectrum of 4-POBN-OOL was obtained soon after the reaction began. Second, the rate of formation of the spin-trapped adduct 4-POBN-OOL approached zero during the time when the reaction approximated anaerobiosis. Finally, when the mixture was bubbled with nitrogen prior to the addition of lipoxygenase, no EPR spectrum appeared in the presence of 4-POBN. Although we were surprised at this observation, it is possible that steric hindrance may play a major role in preventing the spin trapping of L· by 4-POBN. Based on these experiments with lipoxygenase and linoleic acid, we conclude that the radical that is spin trapped by 4-POBN during the NADPH-stimulated hepatic microsomal lipid peroxidation is a lipid peroxyl radical, LOO·.

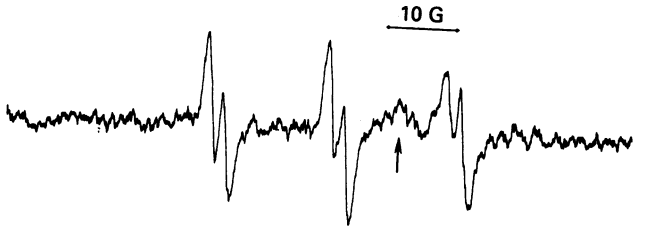


FIG. 4. EPR spectrum of MNPOL-L obtained by treating MNPOL with the linoleic acid/lipoxygenase system. The spectrum was observed 20 min after the reaction was begun. A_N = 16.6 G; and A_H = 2.1 G. The broad peak (arrow) is due to an impurity in the EPR cell.

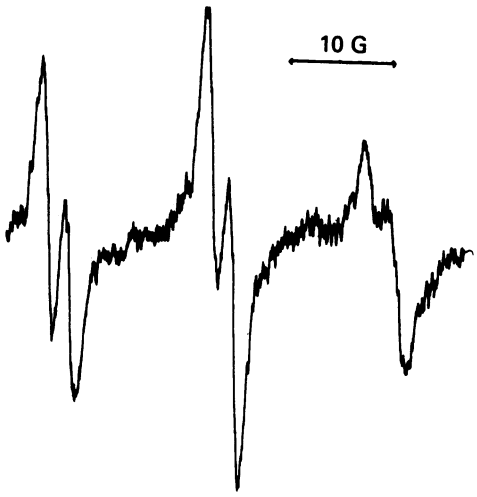


FIG. 5. EPR spectrum of 4-POBN-OOL obtained by allowing 4-POBN to react with rat liver microsomes and NADPH at pH 7.4 with 1 mM DETAPAC. A_N = 15.8 G; and A_H = 2.6 G.

To determine whether L· or LOO· can be spin trapped during NADPH-stimulated lipid peroxidation of rat hepatic microsomes, 4-POBN or MNPOL was added to the microsomal incubation mixture. In the case of 4-POBN, we had previously reported (19) that 4-POBN-OOH and 4-POBN-OH are quite unstable and decompose rapidly during the prolonged scan time required to obtain the EPR spectrum shown in Fig. 5. The species spin trapped in this illustration is identical to the nitroxide obtained from the aerobic reaction of lipoxygenase on linoleic acid. Thus, we conclude that, in the presence of 4-POBN, LOO· can be spin trapped during NADPH-dependent peroxidation of microsomes.

The incubation of the spin trap MNPOL with rat liver microsomes and NADPH gave only a six-line spectrum which we believe is the hydrogen spin-trapped adduct MNPOL-H (Fig. 6). Proof that this is the correct assignment was obtained by carefully titrating MNPOL with sodium borohydride in the presence of oxygen. As discussed earlier, unsaturated LOO· can be spin trapped by MNPOL but such radicals decompose at a rate that does not allow them to reach sufficiently high steady-state concentrations to be observed by conventional EPR spectrometry. Because the microsome/NADPH reaction mixture never achieved anaerobiosis during the time course of the spin

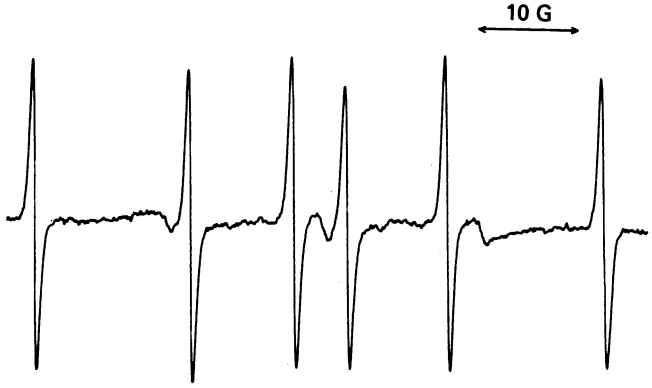


FIG. 6. EPR spectrum of MNPOL-H obtained by allowing MNPOL to react with rat liver microsomes and NADPH. The identical EPR spectrum was obtained by carefully titrating MNPOL with sodium borohydride and oxygen. A_N = 15.7 G; and A_H = 26.2 G. There is also a second species exhibiting a three-line spectrum with A_N = 16 G. This species is most likely due to the dimerization of MNPOL.

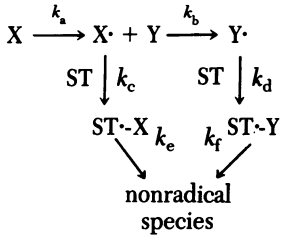
trapping experiments, we were unable to observe any lipid radical potentially spin trapped by MNPOL. This result confirms our experiments with the linoleic acid/lipoxygenase model system in which we could only spin trap the unsaturated lipid radical (L·) after anerobiosis was achieved.

DISCUSSION

The identification of biologically generated free radicals is difficult, especially if one considers the number and complexity of the primary and secondary radicals formed. Reactive primary radicals have the opportunity to give rise to secondary radicals. This immediately results in a depressed level of primary radicals and leads to the occurrence of what is often an overwhelming level of interfering radical species. An example of this phenomenon is the enzymatic generation of superoxide. Dismutation can lead to hydrogen peroxide formation. If ferrous ion is present, hydroxyl radical can be produced.

A further complication may occur in the presence of unsaturated phospholipids. Allylic hydrogen abstraction by hydroxyl radical would produce L· which can react with molecular oxygen to give the corresponding LOO·.

Thus, the production of superoxide can easily result in the production of at least four distinct radical species: O_{2}·, HO·, L·, and LOO·. The ability of a technique such as spin trapping to identify any of these radicals depends upon several factors including: the rate of primary free radical production (k_a), the rate of secondary radical formation (k_b), the rate of spin trapping of the different radical species (k_c,k_d), and the rate of spin-trapped adduct decomposition (k_e,k_f). If we let X· be the free radical derived from X, this free radical can either form a spin-trapped adduct ST·-X or react with another species Y giving the secondary free radical Y·. Again, spin trapping of this species leads to the spin-trapped adduct ST·-Y. Finally, decomposition of the spin-trapped adducts ST·-X and ST·-Y can give EPR invisible nonradical products.



Thus, in order to observe only the primary spin-trapped adduct ST·-X, $k_c \gg k_b$ and $k_c > k_e$. However, if $k_b \geq k_c$ and k_d and $k_c \gg k_f$ and k_e, two different spin-trapped adducts can be noted. In biological systems, various secondary radicals can be generated and spin trapped. Frequently, as described herein, the rates of spin adduct formation, k_c and k_d, as well as the rates of spin adduct decomposition, k_e and k_f, vary markedly for the various spin traps used. In fact, it is the disparate decomposition rates (k_e,k_f) that make the identification of these biologically generated free radicals possible. However, few studies to date have recognized the inherent complexity of the spin-trapping technique in biological systems. Nevertheless, the spin trapping of certain radicals in the biological milieu has been achieved, either by good fortune or by cognizance of the relevant rate constants.

Further complications arise when the spin adduct itself rearranges to give a new radical species. This phenomenon is best illustrated by the spin trapping of superoxide by DMPO. Although observable by EPR spectrometry, the ensuing nitroxide, DMPO-OOH, is unstable and rapidly decomposes into the more stable DMPO-OH and a nonradical species (19).

In microsomal preparations, in which there are several different enzyme systems capable of generating both superoxide and hydrogen peroxide as well as numerous polyunsaturated fatty acid lipids to act as sources of secondary radical formation, the identification of these free radicals by means of spin-trapping techniques should be practically impossible. However, we have found that the differential reactivity of various spin traps as well as the varying rate of spin adduct decomposition can be used to identify various radical species produced by peroxidizing hepatic microsomes. When using DMPO in this biological system, we can only identify the presence of superoxide and hydroxyl. Apparently, geometric restrictions by the cyclic spin trap DMPO prevent the spin trapping of bulky free radicals like L· and its corresponding LOO·. However, when 4-POBN is substituted for DMPO in the peroxidizing microsomal mixture, only LOO· is spin trapped. The spin probe MNPOL allows us to demonstrate that the concentration of L· in these aerobically peroxidizing microsomes is apparently very low. The evidence suggests that, if L· is generated, it reacts with oxygen at a more rapid rate than it does with MNPOL. Because MNPOL is structurally similar to nitroso-*tert*-butane, which has been reported to react with carbon-centered radicals with a rate constant of approximately 10^7 $M^{-1}sec^{-1}$ (22, 23), we think that the reaction of oxygen with L· is likely to have a rate constant of the order of 10^9 $M^{-1}sec^{-1}$.

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