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Invited Paper

Spin Trapping of Superoxide and Hydroxyl Radical: Practical Aspects[1]

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RATIONALE OF SPIN TRAPPING

A free radical is by definition a species containing unpaired electrons, and is therefore paramagnetic. This property of paramagnetism forms the basis for the detection of free radicals by electron paramagnetic resonance spectrometry (EPR), whereby the magnetic moment exerted by the unpaired electron is directly detected. A corollary to this property is that only paramagnetic species are detectable by EPR. This high degree of selectivity renders EPR useful in complex biological systems.

A theoretical lower limit of free radical detection by EPR has been discussed by Bolton (1). For detecting radicals in aqueous solutions using existing instruments, the limit of detection is about 10^{-8} M (2). In many instances, in order to resolve hyperfine splitting constants, a practical limit of detection is about 10^{-6} M (2). Thus it is only possible to detect stable free radicals, radicals which accumulate to measurable concentration, or unstable radicals which reach a sufficiently high steady state concentration. There are many examples of such radicals being directly detected in biological systems, including flavin radicals (3), quinone radicals (4), phenothiazine cation radicals (5), aryloxyl radicals (6), and nitroxide radicals (7). Since these studies have been recently reviewed by Mason (8), they will not be discussed further.

Many free radicals of biological interest, however, are highly reactive and never reach a concentration high enough to be detected by EPR. An example of this is the hydroxyl radical, which reacts with itself or with most organic molecules at diffusion controlled rates (9). Its rate of reaction is limited mainly by the frequency which it collides with other species. Thus the direct detection of hydroxyl radical by EPR in a biologic system would be almost impossible.

For short-lived radicals of lesser reactivity compared to hydroxyl radical, there are various means of detection using EPR. A simple method is to slow the rate of disappearance of the radical by rapidly freezing the sample. This has the disadvantage that the radical is no longer in a fluid environment, and the resultant anisotropic effects can obscure the identification of the radical. This technique is further limited by the concentration of the radical present before freezing and by the length of time required to freeze the sample, which is about 5 to 10 ms (1). One can improve the sensitivity of free radical detection in biological samples by lyophilization; this decreases microwave absorption by water and thus increases signal intensity. Artifactual radicals, however, such as that due to ascorbate, are often seen in lyophilized samples exposed to air (2). Continuous flow EPR studies, in conjunction with signal averaging techniques, enabled Yamazaki and Piette to detect the ascorbate semiquinone free radical in the EPR studies of ascorbate oxidase (10). However, such studies are time consuming and require large quantities of enzyme.

In theory, the method of spin trapping can overcome many of these difficulties. This method consists of using a spin trap, i.e., a compound which forms a stable free radical by reacting covalently with an unstable radical. Thus the radical species is

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"trapped" in a "long-lived form," which can be observed at room temperature using conventional EPR equipment. The hyperfine splitting of the adduct provides information which can aid in the identification and quantification of the original radical. Since the stable free radical accumulates, spin trapping is an integrative method of measuring free radicals and is inherently more sensitive than procedures which measure only instantaneous or steady state levels of free radicals.

Nitrones and nitroso compounds are the spin traps most commonly used. With both of these traps, the adduct is a nitroxide free radical, formed by covalent reaction of the original radical with the spin trap. Nuclei having a magnetic moment can interact with the nitroxide unpaired electron, causing further hyperfine splitting. The magnitude and nature of this interaction is dependent upon the nuclear quantum spin number as well as resonance, inductive, and steric effects (11). Unless a conjugated system is present, magnetic nuclei farther than three bond lengths away from the nitroxide, where the unpaired electron is localized, will not cause further resolvable splitting. With nitroso spin traps, the adduct is directly bonded to the nitroxide nitrogen; thus, nitroso spin trap adducts have comparatively large and well-resolved splitting constants. With nitrone spin traps, the trapped radical is bonded to the α-carbon, and magnetic nuclei present in the trapped radical are farther away from the nitroxide nitrogen as compared to nitroso spin traps. Thus, hyperfine splitting due to the original radical is less readily resolved. Spin traps possessing a β-hydrogen, such as 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO),[2] will yield adducts with hyperfine splitting due both to the β-hydrogen and the nitroxide nitrogen. In these spin-trapped adducts the magnitudes of A_N and A_H are very sensitive to the nature of the trapped radical, and this can serve as a means to help identify the trapped species (11–13) (see Fig. 1). These nitrones can trap a large number of different radicals including carbon, hydrogen, oxygen, nitrogen, and halogens (11); whereas nitroso spin traps are more selective and react mainly with carbon and, to a lesser extent, nitrogen and oxygen radicals (11). A series of articles discussing certain aspects of the chemistry of spin traps, their reactivity, and the mechanism of their reaction in organic solvents has been presented by Janzen *et al.* (11–15) and by Evans (16).

This discussion of spin traps will be limited to nitrones since these compounds are the only spin traps currently suitable for detection of hydroxyl and superoxide radicals. Disadvantages of nitroso spin traps in the trapping of hydroxyl radical have been discussed by several authors (15, 17, 18). There has been, however, one report to the contrary (19). The main drawback to the use of nitroso spin traps is the instability of their hydroxyl radical adducts; for example, the hydroxyl adduct of 2-methyl-2-nitrosopropane decomposes to yield t-butyl radicals (17) and disproportionates to give 2-nitro-2-methylpropane (15). In general, alkoxyl and hydroperoxyl radical adducts of nitroso spin traps are unstable at room temperature (20, 21).

CHEMISTRY OF NITRONE SPIN TRAPS

Nitrones are highly reactive compounds, which can participate in a wide variety of reactions other than radical trapping. Indeed, prior to the use of nitrones in spin trapping, their main use was as synthetic intermediates (22). It should be noted that nitroxides can be generated from nitrones by methods other than radical trapping. Thus a familiarity with nitrone chemistry is essential in understanding how artifactual radicals may be generated from nitrones.

Nitrones can be reduced or oxidized into a variety of products (22). Interconversions between nitrones, hydroxylamines, oximes,

[2] Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; TMPO, 2,5,5-trimethyl-1-pyrroline-*N*-oxide; CDMPO, 2-carboxy-5,5,dimethyl-1-pyrroline-*N*-oxide; 2-McPyBN, (2-*N*-methyl pyridinium) *tert*-butyl nitrone; 4-POBN, α-4-pyridyl-1-oxide *N*-*tert*-butyl nitrone; DETAPAC, diethylenetriamine pentaacetic acid; SOD, superoxide dismutase; TEMPO, 2,2,6,6,-tetramethylpiperidinoxy; OXANO, 2-ethyl-2,5,5-trimethyl-3-oxazolidinyloxy; Ac, acetyl; Bu, butyl; Me, methyl; Et, ethyl; DMSO, dimethylsulfoxide; PBN, α-phenyl-*N*-*tert*-butyl nitrone; MDA, malondialdehyde; NtB, nitroso-*tert*-butyl.

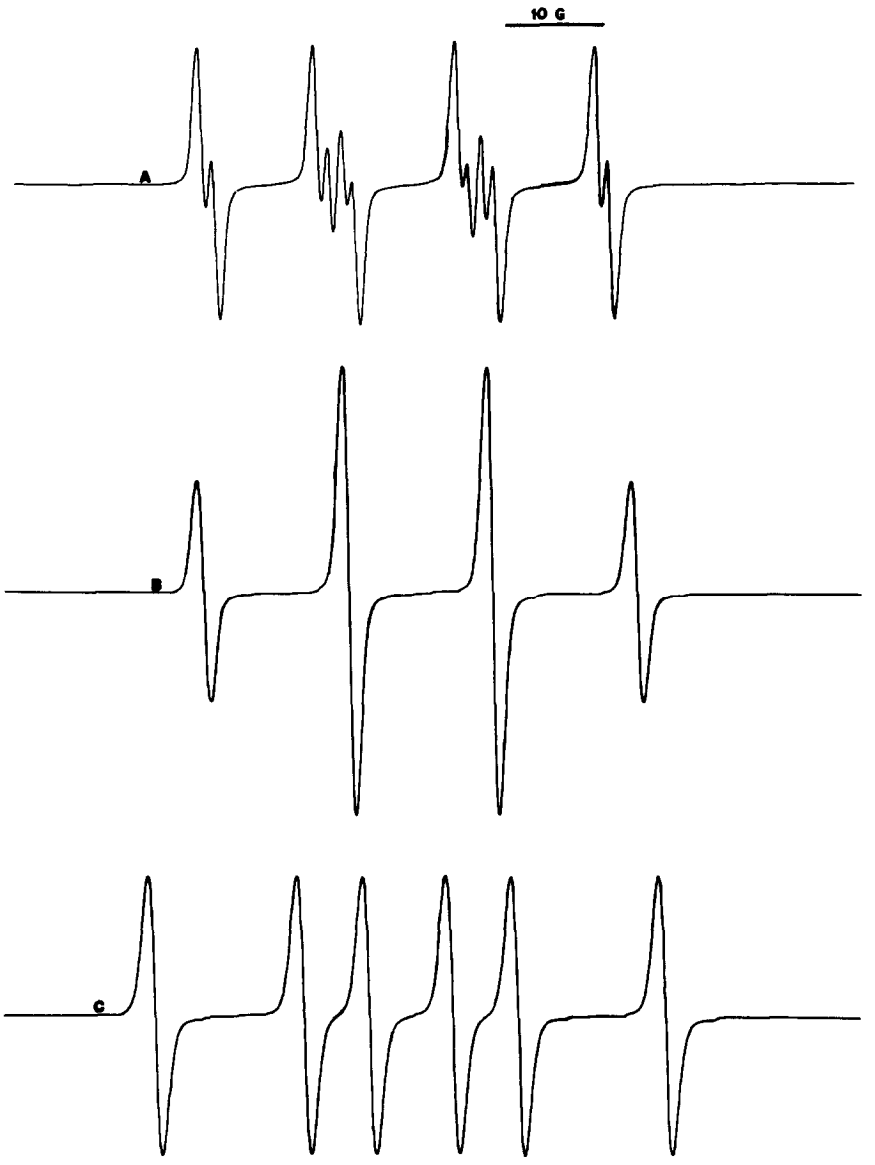
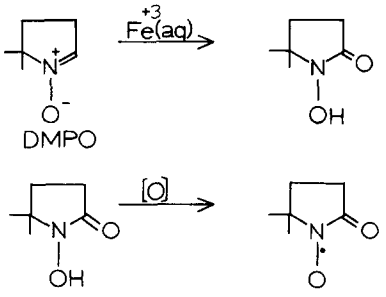


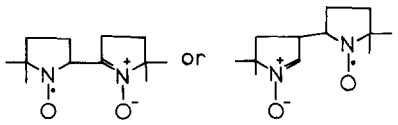
FIG. 1. Computer-simulated spectra (from Ref. (26)). (A) DMPO-OOH, A_N = 14.3 G, A_H^β = 11.7 G, A_H^γ = 1.25 G. (B) DMPO-OH, A_N = 14.87 G, A_H = 14.81 G. (C) Iron–EDTA plus DMPO, A_N = 15.31, A_H = 22.0 G.

imines, hydroxamic acids, nitroxides, and nitroso compounds are possible, depending upon the conditions and the reagents used. Metal ions commonly encountered in biological systems, such as iron and copper, can often carry out or catalyze such reactions. For example, aqueous ferric chloride is known to oxidize DMPO and related nitrones into the corresponding hydroxamic acids (23).



These hydroxamic acids form tight 1:1 complexes with Fe^{3+} and have a visible light absorbance at approximately 540 nm. (For the above Fe^{3+}–hydroxamic acid complex, $\epsilon_{544} = 1075\ M^{-1}\ cm^{-1}$). Oxidation of the hydroxamic acid would produce the corresponding nitroxide as shown above. The production of this nitroxide, DMPOX, has also been reported in a biochemical system containing hematin and cumene hydroperoxide (24). However, in this case, DMPOX arises by spin trapping followed by rearrangement (25)[3] (see Fig. 2). In practice, we find that DMPOX can be produced from DMPO by the action of oxidizing agents such as lead dioxide. Thus, the production of DMPOX from DMPO is undoubtedly a common artifact in many oxidizing systems.

Chelated iron can also produce radicals from nitrones. For example, in phosphate buffer iron–EDTA oxidizes DMPO into a nitroxide, $A_N = 15.3$ $A_H = 22.0$ (26). The spectrum is due to an oxidation product of DMPO itself, as the same signal is observed in Tris buffer containing iron, in the absence of EDTA (27). Only trace amounts of iron are required to generate this spectrum. The iron present in phosphate buffer as an impurity is usually sufficient to produce a detectable signal.[4] We suggest that the spectrum is due to formation of a DMPO dimer.[5]



All of the nitrones we have examined were oxidized by iron–EDTA, producing an EPR signal. The mechanism of this oxidation is under further investigation, and will be presented in greater detail elsewhere.

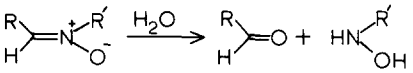
[3] For a detailed discussion of mechanism see Rosen and Rauckman (25).

[4] Phosphate buffer, 50 mM usually contains approximately 1 μM iron. Virtually all laboratory reagents, such as NaOH and KCl, contain significant amounts of iron.

[5] Dimerization of DMPO in the presence of strong base has previously been described (28).

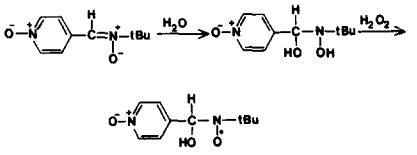
Copper ions can also give rise to artifacts in spin-trapping experiments. For example, the air oxidation of hydroxylamines is greatly accelerated by cupric salts (22). Therefore, we recommend that buffers used in spin-trapping experiments be passed through a Chelex-100 column to remove polyvalent metal ion impurities. The use of DETAPAC, a chelating agent which renders iron incapable of oxidizing DMPO, is also recommended, unless the iron is being used as a reactant in the system (26, 29).

Nitrones are also prone to hydrolysis in aqueous solution, to form an aldehyde and a



hydroxylamine (22). The hydrolysis of nitrones is pH dependent, being more rapid at low pH (22). The susceptibility of nitrones to hydrolysis is also dependent upon their structure. Acyclic nitrones are very susceptible to hydrolysis, while aryl nitrones are less so, and cyclic nitrones are reportedly very resistant to hydrolysis (22). For example, one report claimed that there was little decomposition of an aqueous DMPO solution stored in the dark for 5 months (23), as measured by its uv absorbance. In contrast, the half-life of the aryl nitrone 4-POBN was reported to be 13.8 min at pH 2, although it was stable for 32 h at neutral pH (30).

Hydrolysis of nitrones can also give rise to nitroxides. For example, as Janzen and associates have shown, the addition of water across the double bond of 4-POBN, followed by H_2O_2 oxidation, produces 4-POBN-OH. This is the same species as produced by hydroxyl radical trapping (30).



Thus, hydrolytic reactions can also be a source of artifactual radicals, and may lead to the erroneous assumption that hydroxyl radical is being trapped.

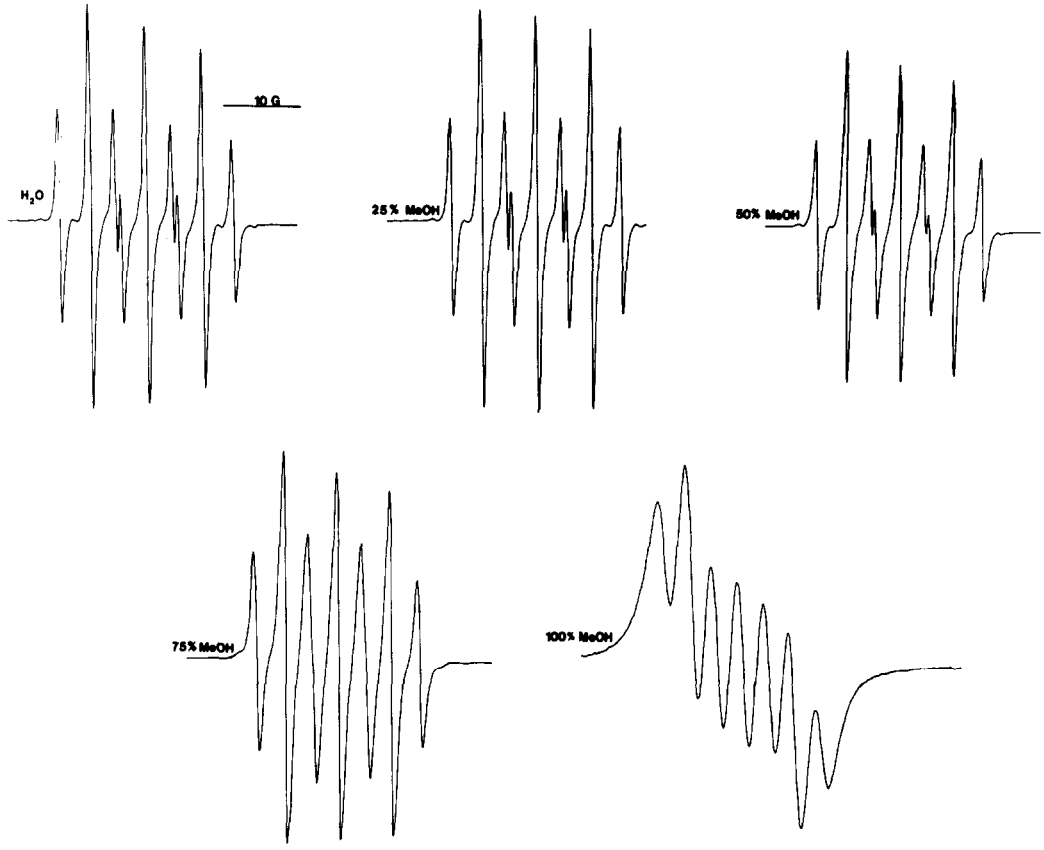
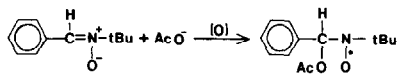
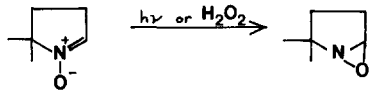


FIG. 2. The spectrum of DMPOX showing solvent effect, from Ref. (25). In water $A_N = 7.2$, $A^{\gamma}_H = A^{\gamma'}_H = 4.1$ G.

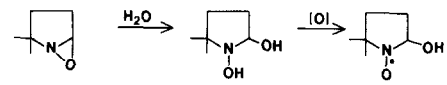
Nitrones are susceptible to nucleophilic attack. This can also lead to the generation of radical products. Evans has reported that acetoxyl ion can add to PBN. Air oxidation of the resultant hydroxylamine produces the corresponding nitroxide (16).



Nitrones undergo photochemical rearrangement to yield the isomeric oxaziranes. The formation of an oxazirane from UV-irradiated DMPO has previously been described (31). It has also been reported that H_2O_2 converts DMPO into the same oxazirane (31).



By analogy to the ring opening of epoxides, hydrolysis of the oxazirane may occur. Oxidation of the resultant hydroxylamine yields DMPO-OH, which is the same nitroxide as formed by hydroxyl radical trapping.



The oxazirane would also be expected to be more susceptible to nucleophilic attack than DMPO. Thus, photochemical systems containing nitrones can potentially give rise to artifactual EPR signals. We have found that DMPO-OH is produced by a hydroxyl radical-independent mechanism in a light–riboflavin–DMPO system (32).

Therefore, it is obvious that the detection of a nitroxide in a spin-trapping experiment is no guarantee that a radical

has been trapped. Further evidence is required. Fortunately, in the case of hydroxyl or superoxide radicals, relatively straightforward procedures exist to determine whether or not these radicals have actually been trapped. These will be discussed in greater detail elsewhere in this paper.

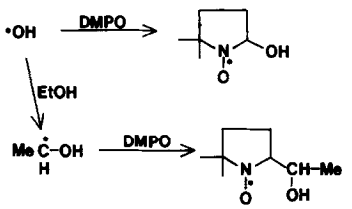
PURIFICATION OF SPIN TRAPS

Nitrones used in spin-trapping experiments should be of the highest purity, and should especially be free from nitroxide or hydroxylamine impurities. Commercially available aryl nitrones such as PBN or 4-POBN are usually of sufficient purity and appear to be stable for a long period of time; however, DMPO and related spin traps, are more susceptible to decomposition by light and oxygen, and thus have shorter shelf lives. Storage of spin traps should always be at −20°C, under nitrogen and away from light.

Commercially available DMPO usually requires further purification. The method of choice, especially for large quantities, is fractional vacuum distillation. DMPO purified by this method is a colorless solid, with a melting point of 25°C. (In contrast, we have found some lots of commercially obtained DMPO are liquid at −20°C). An alternate method of purification is column chromatography using charcoal–Celite, or filtration of an aqueous DMPO solution through charcoal, as described by Buettner and Oberley (33). In place of water, a polar solvent such as methanol can be used, as we have employed in the purification of 2-cyano DMPO (27). Charcoal–Celite behaves as a true reverse phase chromatographic medium, in that polar solvents elute only the nitrone, while nonpolar solvents will elute both nitrone and impurities (27). Elution of DMPO can be conveniently monitored by its uv absorbance (DMPO, $\epsilon_{234} = 7700\ M^{-1}\ cm^{-1}$ (22)).

EXPERIMENTAL CONSIDERATIONS

As discussed in the previous section, the detection of a hydroxynitroxide such as DMPO-OH, *does not necessarily mean that hydroxyl radical has been trapped.* One method of verifying that hydroxyl radical trapping has occurred is to utilize the ability of spin trapping to distinguish between different radical species. For example, hydroxyl radicals react with ethanol to produce α-hydroxyethyl radicals (34). These secondary radicals can then react with the spin trap to produce an adduct with an EPR spectrum distinguishable from that of the hydroxyl adduct as shown.



Thus if the production of DMPO-OH is due to the spin trapping of hydroxyl radicals, the addition of ethanol should both inhibit the production of DMPO-OH and result in the appearance of a new signal due to trapping of the α-hydroxyethyl radical. This is demonstrated in Fig. 3. Splitting constants for hydroxyl and other radical adducts of various spin traps are listed in Table I.

The EPR spectrum of DMPO-OH consists of a characteristic 1:2:2:1 quartet, $A_N = A_H = 14.9$ G. However, this quartet spectrum is not unique to DMPO-OH, since any nitroxide with hyperfine splitting constants such that $A_N = A_H$, will exhibit a quartet spectrum. For example, the EPR spectrum of t-butylhydronitroxide is a 1:2:2:1 quartet with $A_N = A_H = 14.4$ G (17, 18).

Trapping of oxygen-centered radicals other than superoxide can likewise result in spectra similar to DMPO-OOH. For example, the spectrum of the benzyloxy radical adduct of DMPO is similar to that of DMPO-OOH (Fig. 4). Unlike the benzyloxy radical adduct, however, DMPO-OOH is unstable and decomposes into a nonradical species and DMPO-OH (26). The stability of DMPO-OOH is pH dependent (33) and is greater at acid pH. DMPO-OOH can also be converted into DMPO-OH by the action of certain sulfhydryl reductants, such as diethyldithiocarbamate (27). Thus the chemical properties of DMPO-OOH can serve to distinguish it from other species. *Most im-*

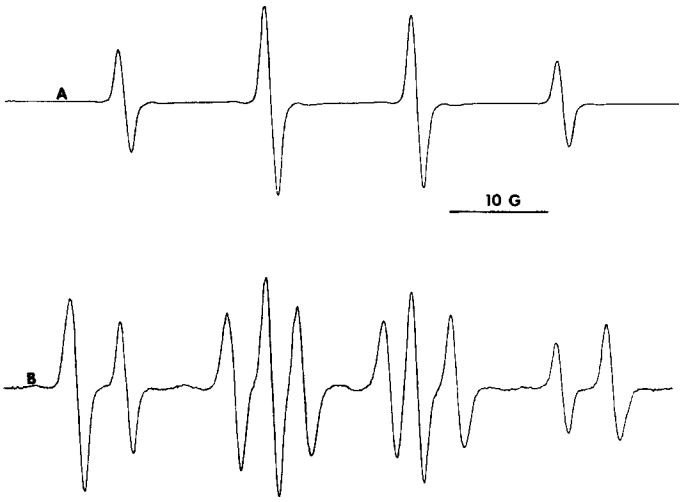


FIG. 3. The effect of ethanol on hydroxyl radical trapping by DMPO, from Ref. (32). (A) Hydroxyl radical adduct generated by uv photolysis of H_2O_2, $A_N = A_H = 14.9$ G. (B) Combination of hydroxyl and α-hydroxyethyl radical adducts generated by uv photolysis of H_2O_2 in the presence of ethanol, $A_N = 15.8$, $A_H = 22.8$ G.

TABLE I

HYPERFINE SPLITTING CONSTANTS FOR AQUEOUS HYDROXYL AND SUPEROXIDE RADICAL ADDUCTS AND THE EFFECTS OF COMPETITIVE INHIBITORS ON HYDROXYL RADICAL TRAPPING[a]

Spin trap	System	Inhibitor	Radical trapped	A_N	A_H^β	A_H^γ	Reference
DMPO	Photolysis of H_2O_2		$O_2^-/HO_2\cdot$	14.3	11.7	1.25	53
DMPO	Photolysis of aqueous chlorophyll		$OH\cdot$	14.9	14.9		70
DMPO	Photolysis of aqueous chlorophyll	HCO_2^-	CO_2^-	15.6	18.7		70
DMPO	Radiolysis of water	CH_3OH	$\cdot CH_2OH$	16.0	22.7		17
DMPO	Fenton reaction	CH_3CH_2OH	$CH_3CH\cdot OH$	15.8	22.8		44
PBN	Photolysis of H_2O_2		$O_2^-/HO_2\cdot$	14.8	2.75		53
PBN	Photolysis of H_2O_2		$OH\cdot$	15.3	2.75		53
PBN	Photolysis of H_2O_2	CH_3OH	$\cdot CH_2OH$	16.2	3.60		58
PBN	Photolysis of H_2O_2	CH_3CH_2OH	$CH_3CH\cdot OH$	16.2	3.34		58
4-POBN	Xanthine–xanthine oxidase		$O_2^-/HO_2\cdot$	14.16	1.75		26
4-POBN	Photolysis of H_2O_2		$OH\cdot$	14.97	1.68	0.34	30
4-POBN	Photolysis of H_2O_2	CH_3CH_2OH	$CH_3CH\cdot OH$	15.56	2.59		27
2-MePyBN	Photolysis of H_2O_2		$OH\cdot$	14.95	3.90		52
3-MePyBN	Photolysis of H_2O_2		$OH\cdot$	14.80	1.42	0.32	52
4-MePyBN	Photolysis of H_2O_2		$OH\cdot$	14.70	1.45	0.38	52
TMPO	Xanthine-xanthine oxidase		O_2^-	15.6			26
	Photolysis of H_2O_2		$OH\cdot$	15.7			27
2-Carboxy DMPO	Photolysis of H_2O_2		$OH\cdot$	14.8			27

[a] Further examples are given in Ref. (58). Splitting constants will vary depending on solvent concentration, when an inhibitor is used.

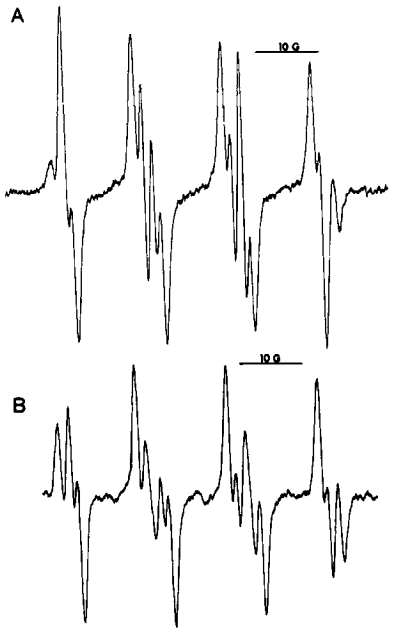


FIG. 4. Similarity between superoxide and benzyloxy radical adducts of DMPO, in aqueous solution. Spectra were recorded with a modulation amplitude of 1 G, time constant 0.3 s, scan time = 4 min. (A) DMPO-OOH produced by reaction of DMPO with superoxide generated by a system containing rat liver microsomes, CN^-, NADPH, and methyl viologen. (B) Spectrum of benzyloxy radical adduct of DMPO, generated by thermal decomposition of benzoyl peroxide in a 1:1 mixture of ethanol and DMPO, which was then dissolved in water.

portantly, the production of DMPO-OOH should be prevented by superoxide dismutase.

A potential problem with spin trapping in biological systems is the reduction, either enzymatically or chemically, of the nitroxide into its hydroxylamine. Since hydroxylamines cannot be detected by EPR, the result will be either an apparent decreased formation of the nitroxide, or the failure to detect any of the nitroxide. Nitroxides can be reduced by various biological systems such as ascorbic acid (35), sulfhydryl compounds (36), the mitochondrial electron transport chain (37), cytochrome *P*-450 (38, 39), an NADPH-dependent nitroxide reductase present in liver cytosol (40), and bacterial electron transport systems (41). The enzymatic reduction of nitroxides by enzyme systems is dependent upon the nitroxide's structure. For example, it was found that phenobarbital-induced rat liver microsomes could reduce TEMPO (2,2,6,6-tetramethylpiperidinoxy) and OXANO (2-ethyl-2,5,5-trimethyl-3-oxazolidinyloxy), whereas only TEMPO was reduced by control or 3-methylcholanthrane-induced rat liver microsomes (42). Both TEMPO and OXANO have identical half-cell reduction potentials (42). Thus even if redox potentials are known, it is difficult to predict whether or not a particular spin-trap adduct will be reduced by an enzyme system. In the studies undertaken to date, biological reduction of the spin-trapped adducts has not appeared to be a problem. Spin trapping has been used in hepatic microsomal systems to detect superoxide (43), hydroxyl radical (44), trichloromethyl (45) or lipid derived radicals (46), and halothane radicals (47). However, none of these studies considered whether or not reduction actually occurred. The situation is further complicated by the finding that superoxide-generating systems, such as xanthine oxidase or mixed function amine oxidase, readily reoxidize hydroxylamines into nitroxides (48). Thus, it is conceivable that redox cycling of spin-trap adducts may occur. The reduction of a nitroso spin trap by microsomes and NADPH has also been described (18).

Most of the nitrone spin traps in current use contain a β-hydrogen. Nitroxides formed from these traps will be susceptible to H · abstraction, or disproportionation, or both. The disproportionation of nitroxides containing a β-hydrogen has been extensively studied (49–51) and involves the intermediate formation of a dimer, which then decomposes. In contrast, nitrones lacking a β-hydrogen, such as TMPO (2,5,5-trimethyl-1-pyrroline-*N*-oxide), form more stable adducts (26). The usefulness of TMPO is limited since one can only obtain A_N values, which do not vary greatly between different trapped radicals. Therefore, using the spin traps currently available, there exists a trade-off between spin-adduct stability (using TMPO) and ease of identification of the trapped radical species (using DMPO).

In general, the hydroxyl radical adducts of aryl nitrones are less stable than the

hydroxyl radical adducts of cyclic nitrones. For example, the half-lives of DMPO-OH and 4-POBN-OH were 2.6 h and 23 s, respectively, at 0.6% hydrogen peroxide and pH 7.4. The decay of 4-POBN-OH was first order (32), indicating that a decay route other than disporportionation had occurred. The low stability of 4-POBN-OH makes it less suitable for hydroxyl radical trapping experiments; however, Janzen has indicated that the analogous *n*-alkyl pyridinium nitrones form spin adducts of greater stability (52). The stability of DMPO-OH is a function of hydrogen peroxide concentration and decreases with increasing hydrogen peroxide concentration (32). Therefore, the stability of the hydroxyl radical adduct is highly dependent upon reaction conditions.

SUPEROXIDE AND HYDROXYL RADICAL TRAPPING: MECHANISTIC ASPECTS

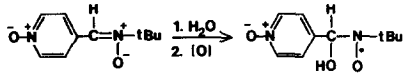
The use of spin trapping to detect superoxide and hydroxyl radicals was first described by Harbour and associates in 1974 (53). These original experiments employed the spin traps PBN and DMPO, and a uv photolysis system. Photolysis of a 1% H_2O_2 solution containing DMPO produced a four-line, 1:2:2:1, spectrum, $A_N = A_H = 15.3$ G, whereas at a higher H_2O_2 concentration (30%) a spectrum with $A_N = 14.3$, $A_H^\beta = 11.7$ and $A_H^\gamma = 1.25$ G was observed. These spectra were attributed to DMPO-OH and DMPO-OOH, formed by trapping hydroxyl and superoxide radicals, respectively. The mechanism described was:

$$H_2O_2 \xrightarrow{h\nu} 2OH\cdot$$

$$OH\cdot + H_2O_2 \rightarrow HO_2^\cdot + H_2O.$$

When photolysis was carried out in 30% H_2O_2, the ratio of the spectra attributed to DMPO-OH and DMPO-OOH varied with DMPO concentration. At high DMPO concentrations, the spectrum of DMPO-OH predominated, whereas at low DMPO concentrations the spectrum of DMPO-OOH predominated. This indicates that hydroxyl is the primary (original) radical formed in the photolysis of H_2O_2, and is consistent with the above mechanism. Other than these concentration effects, no other evidence was presented for the structural assignments of the hydroxyl and superoxide radical adducts.

Janzen and co-workers, using the spin trap 4-POBN, have verified that the hydroxyl radical adduct of a nitrone spin trap is indeed a hydroxy nitroxide (30). The spectrum of the hydroxyl radical adduct of 4-POBN in H_2O shows a hyperfine splitting due to a γ-type hydrogen. In D_2O this splitting was absent, indicating that the γ-hydrogen was exchangeable, and was therefore bonded to oxygen. A spectrum identical to the hydroxyl radical adduct could be generated via acid-catalyzed addition of water to the nitrone double bond, followed by H_2O_2 oxidation. Thus by both isotopic substitution and by independent synthesis, the structure of the hydroxyl radical adduct was shown to be the β-hydroxy nitroxide.



Evidence for the structural assignment of the superoxide adduct is less direct. Our laboratory has shown that the DMPO–superoxide adduct is unstable and spontaneously decays into a nonradical species and DMPO-OH (26). This decomposition was faster in the presence of peroxidase enzymes. Superoxide dismutase could prevent the appearance of the DMPO-superoxide adduct. Additionally, it was found that diethyldithiocarbamate could reduce the DMPO-superoxide adduct into DMPO-OH (27). This is indirect, but strong, evidence that the DMPO-superoxide adduct is a hydroperoxide.

Buettner and Oberley have found that the stability of DMPO-OOH formed in a light–riboflavin–DETAPAC superoxide-generating system is pH dependent. The half-life of DMPO-OOH ranged from 27 s at pH 9 to 91 s at pH 5 (33). It was also reported (29) that the amounts of DMPO-OH and DMPO-OOH produced in a xanthine–xanthine oxidase system were dependent on iron–EDTA concentration, with more DMPO-OH and less DMPO-OOH formed at high iron concentrations. The metal chela-

TABLE II

APPARENT RATE CONSTANTS FOR SUPEROXIDE TRAPPING[a]

Spin trap	Rate constant M^{-1} s^{-1}	Conditions	Reference
TMPO	7	pH 7.8 xanthine–xanthine oxidase, competition with SOD	26
TMPO	1.44	pH 8.1 xanthine–xanthine oxidase, competition with spontaneous dismutation of superoxide	32
DMPO	10	pH 7.8 xanthine–xanthine oxidase, competition with TMPO	26
DMPO	15.7	pH 8.0 light–riboflavin–DETAPAC, competition with SOD	32

[a] Rates were determined by kinetic competition.

tor DETAPAC could prevent the effects of iron. Based on these data, it was suggested that DETAPAC prevents the formation of hydroxyl radical in this system. This has since been confirmed by Halliwell (54), using the hydroxylation of salicylate as a method for detecting hydroxyl radical.

Recent kinetic studies in our laboratory have elucidated certain mechanistic aspects of superoxide trapping. Using kinetic competition methods, we have determined rate constants for superoxide trapping by DMPO and TMPO (26). The results, measured in different superoxide-generating systems, are in reasonable agreement with each other, as shown in Table II (32). The apparent rate constant for $O_2^-/HO_2^\cdot$ trapping by DMPO varied with pH in a nonlinear fashion, and was greater at lower pH (Fig. 5) (32). The data provide a reasonable fit to the equation[6]:

$$K_{app} = \frac{K_{HO_2^\cdot} + (X)K_{O_2^-}}{1 + X}$$

where K_{app} is the apparent rate constant for the reaction of $O_2^-/HO_2^\cdot$ with DMPO, $K_{HO_2^\cdot}$ and $K_{O_2^-}$ are rate constants for reaction of DMPO with $HO_2^\cdot$ and O_2^-, and X is the ratio of the acid dissociation constant of $HO_2^\cdot$ to the hydrogen ion concentration (K_a/[H+]). Using a value of 4.88 (55) for the pK_a of $HO_2^\cdot$, $K_{HO_2^\cdot}$ and $K_{O_2^-}$ are found to be 6.6×10^3 and 10 M^{-1} s^{-1}, respectively. Therefore, according to this model, the reaction with $HO_2^\cdot$ predominates below pH 7.7, whereas the reaction with O_2^- predominates above this pH value. It was also found that the apparent rate constants for $O_2^-/HO_2^\cdot$ trapping by TMPO were similar at both pH 7.8 and 9.3, suggesting that the reaction of TMPO with the anionic form of superoxide (O_2^-) dominates over this pH range.

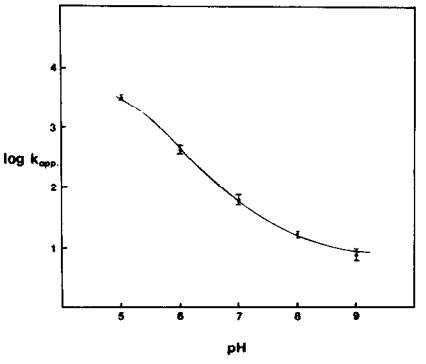


FIG. 5. Apparent rate constant for $O_2^-/HO_2^\cdot$ trapping as a function of pH. Ordinate is expressed in M^{-1} s^{-1}. The solid points are rate constants determined by the relative ability of superoxide dismutase to inhibit DMPO spin trapping in a light–riboflavin–DETAPAC system, as a function of pH. The solid line is theoretical, based on rate constants of 6.6×10^3 and 10 M^{-1} s^{-1} for DMPO trapping of $HO_2^\cdot$ and O_2^-, respectively. From Ref. (32).

[6] This equation can be derived by assuming that the apparent rate constant for superoxide trapping includes the contributions of O_2^- and $HO_2^\cdot$ trapping, i.e., $k_{app} = k_{O_2^-} + k_{HO_2^\cdot}$; and that the relative concentration of O_2^- and $HO_2^\cdot$ are described by the expression $K_a = [H^+][O_2^-]/[HO_2^\cdot]$.

The rate constants for $O_2^{\cdot-}$ trapping are quite low compared to the rate constants of other molecules used to detect $O_2^{\cdot-}$ (56). For example, the rate constants for reaction of $O_2^{\cdot-}$ with cytochrome *c* and tetranitromethane are about 6×10^5 and 2×10^9 M^{-1} s, respectively (57, 56). The low rate constants for $O_2^{\cdot-}$ trapping means that relatively high spin-trap concentrations must be used to trap superoxide before it can decompose via spontaneous dismutation.

Rate constants have also been determined for reaction of hydroxyl radical with nitrone spin traps, using kinetic competition methods. In contrast to the low rate constants found for superoxide trapping, spin traps are efficient detectors of hydroxyl radical, as shown by the data in Table III.

TABLE III

RATE CONSTANTS FOR HYDROXYL RADICAL TRAPPING[a]

Spin trap	Rate constant (M^{-1} s^{-1})
DMPO	3.4×10^9
TMPO	3.8×10^9
4-POBN	1.9×10^9

[a] From Ref. (32).

RECENT STUDIES ON TRAPPING OF SUPEROXIDE AND HYDROXYL RADICALS IN VARIOUS SYSTEMS

The use of nitrone spin traps for detecting superoxide and hydroxyl radicals is becoming increasingly popular, with new studies appearing regularly. Since many of these studies have been reviewed in detail elsewhere (15), the following discussion mainly represents a brief summary of this literature.

Microsomal Systems

Work in Piette's laboratory has been concerned with elucidating reactive intermediates in iron-catalyzed lipid peroxidation. In a study by Saprin and Piette (58), the formation of PBN adducts by a liver microsomal system containing NADPH and iron pyrophosphate was investigated. The authors found that the adducts obtained were dependent upon the "substrate" added to the system. For example, when Tris buffer was used in place of phosphate, a Tris radical adduct was trapped. When ethanol was added to the system, an ethanol radical adduct was trapped. The production of "substrate" radicals was attributed to the initial formation of hydroxyl radical, followed by the reaction of this radical with the substrate to form a substrate-derived radical. This was verified by independent generation of substrate radicals via uv photolysis of aqueous hydrogen peroxide containing these substrates and PBN. The same adducts which were observed in the photolysis system were seen in microsomes. Although PBN-OH production by microsomes was not detected in this study, it was detected in later studies by Lai and Piette, when the incubation conditions were changed (44). The production of DMPO-OH and PBN-OH was attributed to the trapping of hydroxyl radical produced by the Fenton reaction, since catalase (59), but not superoxide dismutase (60), could prevent the formation of DMPO-OH. It was also shown that a system consisting of Fe^{2+}–EDTA, purified NADPH cytochrome *P*-450 reductase, and NADPH could produce DMPO-OH (60). Linolenic acid could inhibit the production of DMPO-OH. The production of DMPO-OH was dependent on pH, Fe^{2+}–EDTA, and KCl concentrations, and was inhibited by *p*-chloromercuribenzoate, but not by metyrapone (59, 60). The inhibition of MDA production by DMPO was attributed to trapping of hydroxyl radical (59). It may alternatively be due to the oxidation of DMPO into its hydroxamic acid. The hydroxamic acid binds iron tightly, thus the inhibition of MDA formation could be due to there being less iron available to catalyze lipid peroxidation.

Floyd and co-workers (61) have detected DMPO-OH and another adduct in a system consisting of nitrosamine carcinogens, and either microsomes or nuclei. DMPO-OH was seen in all instances, but the other adduct varied with the nitrosamine, suggesting that it was due to a nitrosamine-derived radical. Oxygen was necessary for

the production of DMPO-OH, but it decreased the yield of the trapped nitrosamine radical. More controls need to be performed to ascertain whether DMPO-OH production is indeed due to hydroxyl radical trapping in this system.

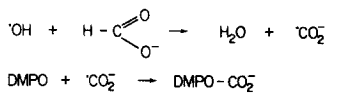
Sealy *et al.* have used DMPO spin trapping to detect superoxide formed during the aerobic microsomal reduction of aromatic nitro compounds (43). This is consistent with previous studies by Mason and Holtzman who have proposed that superoxide is formed during such reactions (62–64).

Photochemical Systems

Harbour and associates have used spin trapping to detect superoxide and hydroxyl radical production in diverse photochemical systems. Harbour and Bolton (65) could detect superoxide production by light-irradiated chloroplasts using DMPO. Methyl viologen increased the production of the superoxide adduct. Based on this, it was suggested that methyl viologen accepts electrons from the primary electron acceptor of photosystem I.

Harbour and Hair have used spin trapping to detect superoxide production by light irradiation of CdS (66, 67) and phthalocyanine (67) pigments in both aqueous and nonaqueous systems. The production of DMPO-OOH by irradiation of CdS was enhanced by EDTA, and by a cationic surfactant, but not an anionic surfactant. Although the effect of EDTA was attributed to its acting as an electron donor, an alternate explanation is the ability of EDTA to render Cu^{2+} impurities ineffective as superoxide dimutases (68). Solvent effects on the hyperfine splitting of the DMPO-OOH adduct spectrum were also studied (67). (These solvent effects are also discussed in detail by Janzen in Refs. (15) and (69).)

A study on the production of hydroxyl radical and its involvement in the destructive photooxidation of chlorophylls has been reported by Harbour and Bolton (70). DMPO-OH was produced during the illumination of both chloroplast suspensions, and detergent-solubilized chlorophyll preparations. The production of hydroxyl radical was verified by the ability of formate to inhibit DMPO-OH production, with the resultant production of the CO_2^- adduct of DMPO, i.e.:



The photodestruction of chlorophyll A could be monitored optically, and was inhibited by DMPO, as expected.

Buettner and Oberley have demonstrated that superoxide can be produced by light irradiation of protoporphyrin, using DMPO as a spin trap. The production of DMPO-OOH was enchanced by prior bubbling with oxygen, and was prevented by superoxide dismutase (71). The relationship between superoxide production in this system and the cutaneous photosensitivity of individuals with protoporphyria was discussed.

An excellent study by Sargent and Gardy investigated the ability of the spin traps PBN, DMPO, and NtB to trap H·, solvated electrons, and hydroxyl radical formed by 3(MeV) electron radiolysis of water (17). Structural assignments of the spectra were verified by isotopic substitution, independent synthesis, computer simulation, or the effects of various competitive inhibitors. It was concluded that "DMPO was an excellent spin trap for such studies." This paper should prove useful both for the spectra shown, and for its methodology.

Hydroxyl Radical Production by Antibiotics

Studies by Buettner and Oberley have used spin trapping in an attempt to demonstrate hydroxyl radical production in Fe^{2+}–bleomycin (72) and Cu^{2+}–tallysomycin (73) systems. In both of these studies the production of DMPO-OH required both the metal ion and the antibiotic. However, these studies did not take into account that DMPO-OH may arise by mechanisms other than hydroxyl radical trapping. Thus the studies are inconclusive, in themselves.

Lown and co-workers (74) have used PBN in an attempt to detect hydroxyl radical produced by a system containing a sodium borohydride-reduced aminoquinone anti-

biotic (either mitomycin B, mitomycin C, or streptonigrin), 0.06 to 0.2 M PBN, and 10% DMSO. The authors could detect a PBN adduct, $A_N = 16.0 A_H = 3.4$ G, whose production was inhibited by either catalase, superoxide dismutase, or EDTA. The identical PBN adduct could be detected in a $Fe^{2+}-H_2O_2$ system containing 0.06 M PBN, and 10% DMSO. The authors assigned this adduct to PBN-OH produced by hydroxyl radical trapping. Although hydroxyl radical is likely produced in their system, their assignment of the PBN adduct is probably incorrect for the following reasons:

(i) Since both the concentration of DMSO (1.4 M) and its rate of reaction with hydroxyl radical (7×10^9 M^{-1} s^{-1} (75)) were much greater than the PBN concentration and rate constant, most of the hydroxyl radical would have reacted with DMSO. Thus the adduct observed is possibly due to a secondary radical derived from DMSO, such as $CH_3\cdot$ (76).

(ii) The direction of the "solvent effect" on the spectrum of PBN-OH due to 10% DMSO, which the authors described, is incorrect. A_N and A_H should be decreased in a less polar solvent, and not increased as described (35). The change in hyperfine splitting is due to a different radical (i.e., from DMSO) being trapped.

(iii) PBN-OH is less stable than alkyl radical adducts of PBN, thus if any PBN-OH were formed, it would likely have decayed during the extended incubation used. (See the discussion in Refs. (44, 58). The spectrum shown by the authors is probably the same as "DMSO signal A" described by Saprin and Piette (58).

Another study has been published describing the detection of PBN-OH and PBN-OOH produced by the interaction of Fe^{2+}–bleomycin with PBN (77). Both Fe^{2+} and oxygen were required to produce an EPR signal. When cobalt was used instead of iron, a weak PBN-OH signal was detected; however, neither Cu^{2+} nor Zn^{2+} were effective in producing an EPR signal. PBN-OOH could only be detected at low Fe^{2+}–bleomycin concentrations; at higher Fe^{2+}–bleomycin concentrations the spectrum of PBN-OH predominated.

Miscellaneous Studies

The first use of spin trapping in an intact living cell has been described by Green and co-workers in a study on superoxide and hydroxyl radical production by stimulated human polymorphonuclear neutrophils (78). Both DMPO-OH and DMPO-OOH could be detected, depending upon the stimulator used. The production of both species was prevented by superoxide dismutase, but not by catalase, and was increased by cyanide or azide. The authors suggested that DMPO-OH production is due to hydroxyl radical trapping, and that the hydroxyl radical is generated by a mechanism which does not involve "free" hydrogen peroxide. Based on our observations concerning spin trapping in the xanthine–xanthine oxidase system (26), an alternate explanation is that DMPO-OH production is due largely to the decomposition of DMPO-OOH formed by superoxide trapping. The effects of cyanide and azide may therefore be due to their inhibition of endogenous superoxide dismutase (79) or superoxide dismutase present as an impurity from erythrocytes. Even small amounts of superoxide dismutase can cause a large inhibition of spin trapping, due to the low rate constant for superoxide trapping by DMPO (26, 32).

A report by Floyd and Wiseman (80) has described the production of DMPO-OH during the autoxidation of 6-hydroxydopamine; SOD, and catalase only partially inhibited DMPO-OH formation. Oxygen was required for DMPO-OH formation. The authors were also able to detect the 6-hydroxydopamine semiquinone radical. The chelators DETAPAC and deferoxamine could inhibit both DMPO-OH and 6-hydroxydopamine semiquinone formation, suggesting the involvement of iron in their formation.

SUMMARY

Reports are regularly appearing in the literature describing spin trapping of superoxide and hydroxyl radicals from various sources. Careful scrutiny of these reports will often reveal that insufficient controls have been run to properly validate the

results. Nitrones are highly reactive compounds which can form nitroxides by mechanisms other than radical trapping. Thus, investigators using spin trapping should be cognizant of the many artifacts which accompany this technique, and take care to validate their results with satisfactory controls. We have described straightforward procedures to determine whether superoxide or hydroxyl radical trapping have occurred, and which can help verify the assignment of the radical adduct.

Nitrones are the only spin traps currently suitable for detection of hydroxyl and superoxide radicals. The various nitrone spin traps in current use each have advantages and disadvantages. In general, the cyclic nitrone traps such as DMPO have greater reactivity with superoxide and hydroxyl radicals, are less readily hydrolyzed, but are more susceptible to oxygen and light, and thus have lesser shelf lives. Aryl nitrones such as 4-POBN or PBN have lesser reactivity with superoxide and hydroxyl radicals, are more readily hydrolyzed, but have greater shelf lives. The stability of DMPO-OH is also greater than that of 4-POBN-OH. Thus in general, DMPO appears to be the most versatile spin trap currently available.

Spin trapping is an inefficient means of detecting superoxide, due to the low rate constants for spin trapping. Spin traps possessing a β-hydrogen will also form unstable superoxide adducts. Spin trapping will, however, undoubtedly prove useful in detecting superoxide under conditions where more conventional methods, such as cytochrome *c* reduction, cannot be used.

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