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Forum Review

Detection of Reactive Oxygen and Nitrogen Species by EPR Spin Trapping

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ABSTRACT

Electron paramagnetic resonance spin trapping has become an indispensable tool for the specific detection of reactive oxygen free radicals in biological systems. In this review we describe some of the advantages as well as some experimental considerations of this technique and how it can be applied to biological systems to measure oxidative stress. *Antioxid. Redox Signal.* 6, 619–629.

INTRODUCTION

THE HEIGHTENED INTEREST in reactive oxygen (ROS) or nitrogen (RNS) species results from the knowledge accumulated over the past decade regarding their role as critical mediators in various physiological oxidative processes and disease mechanisms (4, 26, 101). Of these species, the oxygen free radicals (OFRs), superoxide and hydroxyl radical, as well as their secondary radicals are of particular biomedical importance. Figure 1 shows how superoxide radical $O_2^{\bullet -}$ production can be mediated by various sources in biological systems, and then transformed to other highly reactive species that lead to cellular injury. Although low concentrations of these species play an important role as modulators of cellular function, signaling, and immune response, high levels of these species can be detrimental to cellular function and viability. Over the last 15 years, it has also been appreciated that the free radical gas nitric oxide (NO) is a critical regulator of a broad range of biological functions. Studies over this time have lead to the understanding of the critical functional role of NO in neurotransmission, immune response, and the regulation of vascular tone. Its ability to modulate $O_2^{\bullet -}$ production has also led to the understanding that NO in high levels is toxic and can result in stroke, hypertension, vascular dysfunction, atherosclerosis, or inactivation of mitochondrial respiratory processes (26). Figure 2 shows the reactivity of NO with various radicals and how this leads to the formation of RNS.

Radicals can be generated from a variety of biochemical and enzymatic pathways. Common pathways of superoxide generation include the enzymes xanthine oxidase, NADPH oxidase, and the mitochondrial electron transport chain (101). These pathways can also result in protein oxidation and radical generation. For example, mitochondrial cytochrome *c* oxidase has been shown to react with H_2O_2 resulting in the formation of protein-centered radicals (8), and organic peroxides in the presence of NADPH or NADH produce carbonyloxyl ($CO_2^{\bullet -}$) and hydroxyl radicals ($^{\bullet}OH$) (25). NO is synthesized by inducible NO synthase (NOS) (or NOS2) from L-arginine (L-Arg) and O_2. Xia *et al.* (94) demonstrated a shift of inducible NOS enzyme function to $O_2^{\bullet -}$ generation following depletion of cytosolic L-Arg.

Superoxide radical production in cells has been quantitatively measured from polymorphonuclear leukocytes after activation with a variety of stimulators, including phorbol esters or opsonized zymosan (68). Endothelial cells subjected to anoxia and reoxygenation generate $O_2^{\bullet -}$-derived $^{\bullet}OH$ (102). Oxygen radical generation has also been measured from mitchondria both in isolated form and in cells (27). In organs such as the heart, superoxide ($O_2^{\bullet -}$)-derived $\cdot OH$, $^{\bullet}R$, and $ROO\cdot$ radicals are generated following ischemia and reperfusion (103). More recently, superoxide has been measured in vessels that have shown to be formed by a vascular oxidase and to have an important role in regulating vascular tension (74, 75).

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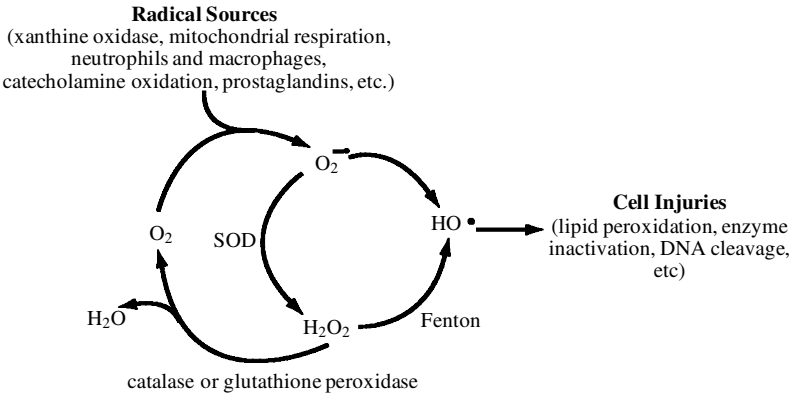


FIG. 1. Chemistry of ROS.

Radicals have also been studied in simple systems such as the anticancer drug adriamycin, which has been shown to produced ROS *via* O_2 reduction catalyzed by Fe^{3+} (23, 99). Photolysis and radiolysis also produce radicals. For example, ionizing gamma radiolysis of Chinese hamster V79 cells and human erythrocytes yields $^{\bullet}OH$ radicals (69), NO and C-centered radicals are produced from *N*-nitrosoamines (30) or $^{\bullet}OH$ from H_2O_2 by ultraviolet (UV) irradiation (33). Riboflavin with visible light irradiation in the presence of O_2 has been applied to study the kinetics of $O_2^{\bullet-}$ trapping by various nitrones (86). Ultrasound was reported to yield hydrogen atom (51) or phenyl radical (41) adducts of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) or *N-tert*-butyl-α-phenyl nitrone (PBN) in aqueous medium, and alkyl radicals in organic solvents (52).

It has therefore become clear that it is of critical importance to have direct or indirect techniques capable of quantifying free radicals in systems ranging from chemical to enzymatic reactions, and cellular to *in vivo* systems, in order to understand the mechanisms and processes underlying oxidative damage in biological systems. While a number of methods have been developed to measure ROS or RNS, these are often times limited by specificity and sensitivity. This review will focus on the application of electron paramagnetic resonance (EPR) spectroscopy using spin trapping techniques in the detection of transient free radicals. The advantages and the limitations of this approach over other commonly used methodologies of radical detection will be discussed. It is our goal to introduce to beginners, or review for experienced free radical researchers, the vast areas in which EPR spin trapping can be applied, along with some of its shortcomings, and ongoing developments and advances.

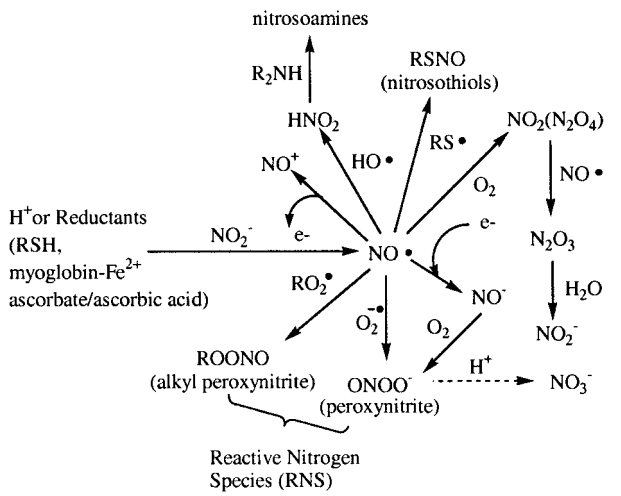


FIG. 2. Reactivity of NO and its derivatives with various radicals.

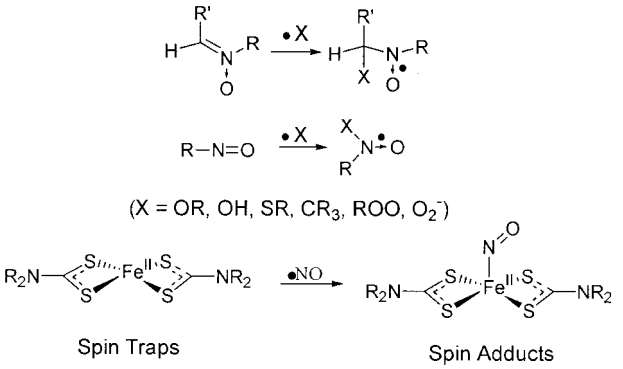
What is EPR spin trapping?

EPR (or electron spin resonance [ESR]) spectroscopy is the most commonly used method for detection (65, 89) of paramagnetic species. This involves absorption of microwave energy by paramagnetic species in the presence of an external magnetic field resulting in the transition of spin states. This transition is described by the fundamental Eq. 1:

$$\Delta E = h\nu = g\beta H \qquad (1)$$

where ΔE corresponds to the energy of absorbed microwave energy, h is Planck's constant, ν is the frequency of applied electromagnetic radiation, g is a factor equal to 2.0023 for a free electron, and H is the applied field strength. Conventional X-band ~9.5 GHz frequency is often employed for *in vitro* or *ex vivo* applications. At this frequency the penetration of the sample by the applied radiofrequency radiation is limited, and only small, millimeter-in-diameter, samples can be studied. For *in vivo* applications, however, of larger samples lower frequencies can be used. Studies of isolated organs or live mice or rats have been performed at L-band (1–2 GHz) or lower frequencies. This low-frequency EPR approach enables penetration of much larger samples (100), living animals, and even human subjects (14).

Spin trapping is a technique in which transient radicals, *e.g.,* $^{\bullet}OH$, $O_2^{\bullet-}$, ROO·, or relatively stable radicals such as NO that are undetectable under normal conditions react with spin traps such as nitrone and nitroso compounds, or metal complexes such as in the case of NO, *e.g.,* Fe^{2+}-*N*-methyl-D-



SCHEME 1. Spin trapping mechanisms.

glucamine dithiocarbamate (MGD) or Fe^{2+}-*N*-(dithiocarboxy)sarcosine (DTCS), to form a radical adduct that is detectable by EPR. A covalent bond is formed from the reaction of nitrones and nitroso with reactive radicals, while a coordination bond is formed between NO and Fe^{2+} complexes (see Scheme 1).

Characteristic EPR spectral profiles can be observed for different spin adducts, and therefore transient radicals that are otherwise undetectable under normal conditions can now be observed. Figure 3 shows typical spectra of some of these spin adducts. The ability of these spectra to discern the type of radicals being trapped can provide a wealth of information in terms of mechanism, kinetics, and dose dependence of radical production in biological systems. However, this straight-

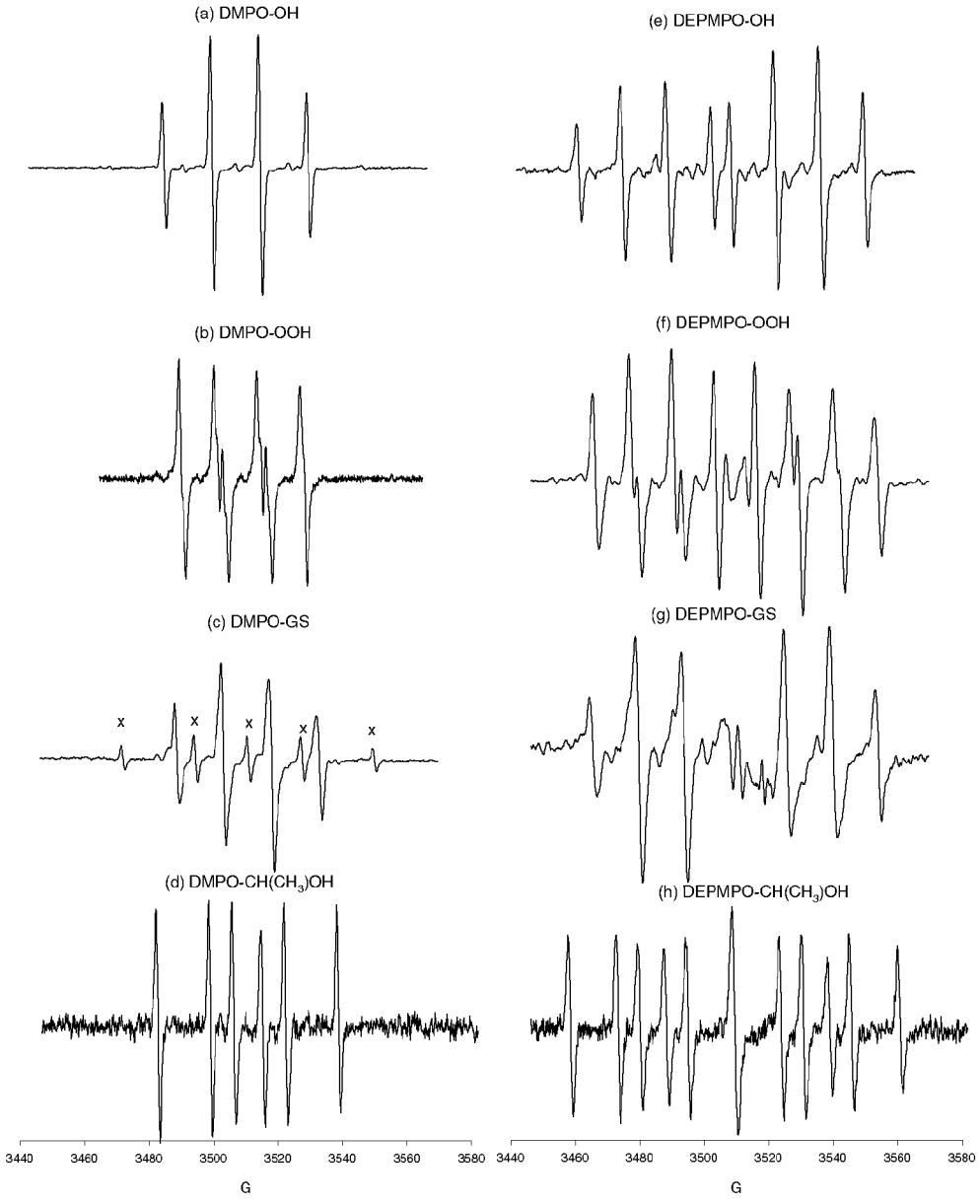


FIG. 3. Typical spin-adduct EPR of ·OH (a and e), $O_2^{\cdot-}$ (b and f), ·SG (c and g) (peaks marked with "x" are dimers), and CH_3·CHOH (d and h) adducts of DMPO and DEPMPO in phosphate buffer at pH 7.4 from various radical generating systems.

forward identification of radicals being trapped requires some caution as will be discussed in the subsequent section.

DETECTION OF OXYGEN RADICALS

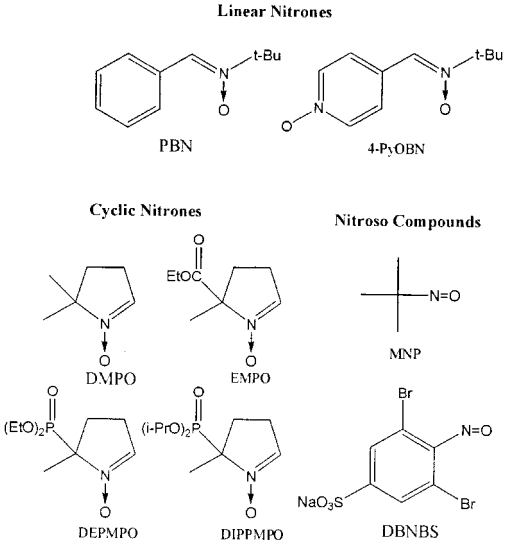
Classes of spin traps

Several excellent reviews have appeared over the past 2 decades on EPR spin trapping methods of oxygen radical detection (31, 32, 64, 65). Nitrones have been the molecule of choice due to their specificity and ability to quantify transient OFRs. Two classes of spin traps are commonly used: the linear nitrones, PBN and α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (4-PyOBN); and the pyrroline-based cyclic nitrones, DMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), and 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (EMPO). Nitroso compounds are also another class of spin traps, but are limited by their instability, their tendency to form dimers in solution and subsequent disproportionation to give EPR-active species, and short-half-lived radical adducts, as well as their high toxicity, particularly in the case of nitrosobenzenes (Scheme 2).

Isotopically labeled spin traps such as those of ^{2}H and ^{15}N-labeled DMPO (58) can exhibit a significant increase in detection sensitivity due to reduction in the number of hyperfine splittings. Replacement of ^{1}H with ^{2}H eliminates the doublet proton splittings, while substitution of ^{14}N with ^{15}N results in doublet rather than triplet splitting. It has also been reported for the spin trap EMPO that the stability of the $O_2^{\bullet-}$ adduct is improved with ^{15}N-substitution and deuteration (98).

Spin trap toxicity

Cellular toxicity of nitrones such as DMPO and PBN is low, hence making them suitable for application in biological systems. Cytotoxicity studies on bovine aortic endothelial cells have been performed for some of these nitrones and have shown DMPO to have the least toxicity with a 50% inhibitory concentration of approximately 140 m*M* compared with PBN and 4-PyOBN with 50% inhibitory concentrations of 9 m*M* and 5 m*M,* respectively (29) (see Table 2). Evidence from gross pathology and histopathological examinations showed that the spin traps DMPO (232 mg/100 g by weight) and PBN (100 mg/100 g by weight) injected intraperitoneally gave no cellular damage, while 4-PyOBN was found to be lethal at 100 and 200 mg/100 g by weight (71).



SCHEME 2. Various types of nitrones and nitroso spin traps.

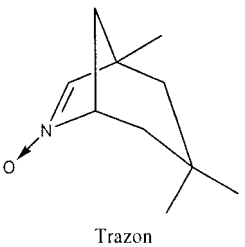
Detection in lipid phase

One of the challenges in spin trapping is the ability to detect radical formation in the lipid phase that would allow trapping of lipid-derived radicals from lipid peroxidation processes. Limitations due to the relatively hydrophilic character of some of the commonly used nitrones can be overcome by derivatization of DMPO (56, 81), DEPMPO (76, 95), or EMPO (77) with bulky or long alkyl chains enabling them to have improved lipophilicity. The partition coefficient K_p, as a measure of spin trap lipophilicity, is defined as the ratio of the spin trap concentration (based on its UV absorbance) in 1-octanol to that in water. Table 1 shows the partition coefficients of the commonly used cyclic and linear nitrones, with PBN being the most lipophilic, giving it an obvious disadvantage of having poor solubility in water. The 1-octyloxy derivative of EMPO has a high K_p value of 42 (77), and the stability of alkoxyl adducts is increased compared with other analogues with shorter alkyl chain lengths.

High stability of the superoxide adduct of EMPO was observed compared with its derivatives with higher alkyl chain length. Results show that the 1-octyloxy EMPO derivative is more suitable for detection of lipid alkoxy radicals (77), while DEPMPO is not suitable for trapping applications in the lipid phase because of its strong hydrophilic character (76). The bicyclic nitrone Trazon (70) (Scheme 3) gave characteristic EPR spectra for various alkoxyl and lipid-derived radicals (78), and spin trapping in a liposomal environment has been carried out using lipid-soluble spin traps such as 2-methyl-nitrosopropane (MNP) (17, 72).

Competitive trapping

Endogenous cellular superoxide radical scavengers such as superoxide dismutase (SOD), ferricytochrome *c,* epinephrine, L-ascorbic acid, hydroxylamine, or pyrogallol can com-



SCHEME 3. Structure of Trazon.

TABLE 1. RADICAL GENERATING SYSTEMS IN AQUEOUS MEDIA

Radical	*Generating system*	*Reference*
·OH	Fe^{2+}/H_2O_2; $H_2O_2/h\nu$	18
$O_2^{\bullet-}$, ·OOH	Hypoxanthine/xanthine oxidase; riboflavin/light	18, 67, 86
$\cdot CH_3$	Fe^{2+}/H_2O_2/dimethyl sulfoxide	55
$CO_2^{\bullet-}$	$Fe^{2+}/H_2O_2/NaHCO_3$	55
$CH_3\cdot CHCOH$	Fe^{2+}/H_2O_2/ethanol	59
RO·	ROH/Fe^{3+}; $ROH/Pb(OAc)_4$	12, 95
GS·, RS·	GSSG/UV; RSSR/$h\nu$	95
$SO_3^{\bullet-}$	SO_3^{2-}/Cr^{4+}; $Fe^{2+}/H_2O_2/SO_3^{2-}$	45, 55
H·	$(n\text{-}Bu)_3SnH/h\nu$	95
·Ph, X-Ph·	$Ph(O)COSnCy_3/h\nu$	95

pete with the nitrone spin traps. However, knowledge of the second order rate constants k_s of these scavengers with $O_2^{\bullet-}$ can help greatly in setting up experimental design, *e.g.,* type or concentration of trap to be employed. Mitsuta *et al.* (52) reported important kinetic data involving reaction of $O_2^{\bullet-}$ with SODs and SOD-mimic molecules. Data show that SODs have k_s values on the order of $1.6–3.0 \times 10^9\ M^{-1}\ s^{-1}$ at pH 7.8, while reactivity of other SOD-mimics with $O_2^{\bullet-}$ are relatively lower compared with the SODs, with k_s values ranging only from 0.86 to $43 \times 10^5\ M^{-1}\ s^{-1}$, and for hydroxylamine hydrochloride, which has almost the same rate of reaction as DMPO, with k_s of about $1.6 \times 10^2\ M^{-1}\ s^{-1}$. For *in vitro* studies, 10–50 m*M* concentrations of the spin trap are competitive enough and usually give reasonable peak intensity.

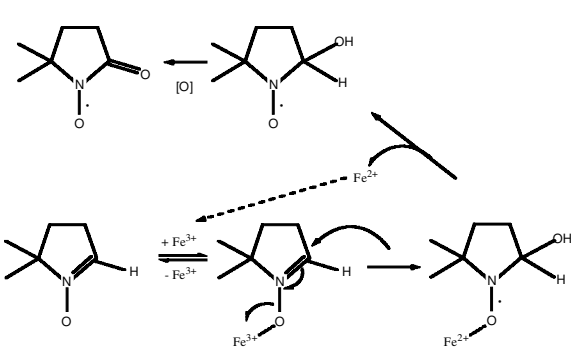
Artifacts in spin trapping

Artifacts are commonly encountered in spin trapping experiments. The cyclic nitrone DMPO, for example, is known to undergo the Forrester-Hepburn reaction (63), in which a radical adduct is produced from the Fe^{3+}-catalyzed nucleophilic addition of water to nitrone and subsequent oxidation to the EPR-active nitroxide according to Scheme 4 (15). While catalase does not affect this adduct formation, the presence of metal chelators exerts inhibition. Formation of the DMPO-OH adduct due to the nucleophilic addition of H_2O in the presence of Fe^{3+} or Cu^{2+} was confirmed using ^{17}O-enriched water (28). Since Fe^{3+} impurities are only present at micromolar levels under typical experimental conditions, the use of chelators such as diethylenetriaminepentaacetic acid in 100 μ*M* concentrations can avoid formation of this artifactual DMPO-OH.

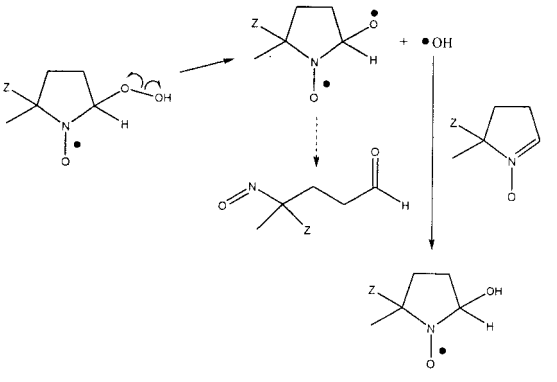
Accurate assignments of spin adducts can be confirmed by independently generating the radical adducts of interest, and computer simulation of the observed EPR spectra (1, 16). Several established procedures have been reported in the literature on how to generate radicals independently and are summarized in Table 1.

Use of scavengers such as SOD and catalase inhibits $O_2^{\bullet-}$ and ·OH adduct formation, respectively. Secondary radical formation from ethanol, methanol, or dimethyl sulfoxide can be added to confirm the generation of ·OH. Formation of C-centered adducts by addition of these secondary radicals, or inhibition of ·OH adduct formation by catalase, can rule out the possibility that the ·OH adduct formation resulted from the decomposition of the $O_2^{\bullet-}$ adduct (6) *via* a mechanism (3, 65) shown in Scheme 5, or by some nucleophilic addition reaction.

The formation of EPR-detectable nitroxides during spin trapping experiments may not necessarily result from the direct addition of radical to the spin trap. For example, polyunsaturated fatty acid (PUFA) peroxyl radicals give rise to PUFA-derived alkoxyl radical adducts rather than primary PUFA peroxyl radical adducts, and this initially resulted in



SCHEME 4. Forrester-Hepburn reaction for the formation of hydroxyl adduct via a non-spin trapping mechanism.



SCHEME 5. Hypothetical mechanism for the unimolecular decomposition of the $O_2^{\cdot-}$/HOO· adduct of DMPO.

some confusion regarding the spectral assignments of these adducts in the literature (12). Because of the instability of the primary peroxyl radical, conversion to its corresponding alkoxyl radical adduct occurs. In the case of DEPMPO, the EPR signals of the •OH-DEPMPO adduct can also lead to erroneous interpretation, especially in the presence of high-valency metal ions such as Fe^{3+}, Cu^{2+}, or Mn^{4+}. These metal ions can oxidize DEPMPO to form the radical cation species $DEPMPO^{\bullet+}$, hence making this species susceptible to hydration to form the •OH adduct. In addition, this can lead to nucleophilic substitution by anions to give, for example, the DEPMPO-SO_3^- radical adduct. Intramolecular rearrangements have been reported in which the ethyl moiety from the phosphoryl group can migrate to the nitronyl carbon to yield a C-centered radical adduct (9).

Kinetic considerations

The apparent rate constant k_{app} values for the second order reaction of various nitrones with $O_2^{\bullet-}$ and •OH radicals as well as the half-lives of their respective spin adducts are shown in Table 2. It should be noted, however, that rate constants can vary from one radical generating system to another because of the effect of several factors such as the presence of metal ions, ionic strength, pH, or endogenous radical scavengers. The pH dependence of the rate of $O_2^{\bullet-}$ trapping by DMPO shows that the rate of trapping is relatively faster in acidic media (18) as determined using the xanthine–xanthine oxidase superoxide generating system. This is due to the formation of hydroperoxyl radical (•OOH), which is far more oxidizing than the unprotonated form, *i.e.,* E^o_{red} = 1.06 V for •OOH versus E^o_{red} = 0.94 V for $O_2^{\bullet-}$ (5). Furthermore, reaction of DMPO with •OOH predominates below pH 7.7, while reaction between DMPO with $O_2^{\bullet-}$ occurs above this pH (18).

Other applications

Spin trapping using kinetic analysis has been employed to deduce the stoichiometry of O_2 reduction by NADPH to generate $O_2^{\bullet-}$. It was shown in the NADPH–cytochrome P-450 reductase–paraquat system that the stoichiometry is as follows:

$$NADPH + 2\ O_2 \rightarrow NADP^+ + H^+ + 2\ O_2^{\bullet-}$$

as obtained from the rate extrapolated to infinite [DMPO] (96). The rate of $O_2^{\bullet-}$ production from 1-methyl-4-phenyl-2,3-dihydropyridinium, a bioactivated intermediate of the parkinsonian-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, was found to be about $k_1 = 3.97 \times 10^{-6}$ s^{-1} from kinetic spin trapping methods using DMPO as a spin trap (97). Spin trapping with DMPO has enabled identification of $O_2^{\bullet-}$ generation from endothelial NOS (94), neuronal NOS (60), and inducible NOS (93) and characterization of the mechanisms of this process. Recently, DMPO spin trapping has been used to quantitate $O_2^{\bullet-}$ generation from the vascular NADPH oxidase (74). Simultaneous real-time measurements of free radicals and cardiac contractile function have been performed using *in vivo* spin trapping in isolated beating hearts. *In vivo* EPR techniques have enabled determination of the kinetics of free radical metabolism in normally perfused and globally ischemic hearts at 1–2 GHz EPR frequency (100).

NO DETECTION

NO is generated by the enzyme NOS from the substrates L-Arg, O_2, and NADPH in the presence of calcium ions, calmodulin, and tetrahydrobiopterin. NO can also be generated from other molecules such as nitroglycerine, nitrites, *S*-nitrosothiols, diazeniumdiolates, and transition metal nitrosyl complexes. NO production in various *in vivo* and *in vitro* systems has been extensively studied by EPR spin trapping methods. NO has been detected in intact mitochondria, mitochondrial homogenates, and submitochondrial particles with great success (24). A wide variety of literature describes the use of EPR spin trapping methods to detect NO in many ap-

TABLE 2. APPARENT SECOND ORDER RATE CONSTANTS (K_{APP}) FOR THE TRAPPING OF SUPEROXIDE OR HYDROXYL RADICALS, PARTITION COEFFICIENTS (K_p), AND TOXICITY OF COMMONLY USED NITRONE SPIN TRAPS, AND HALF-LIVES ($T_{1/2}$) OF THEIR CORRESPONDING SPIN ADDUCTS

	*$O_2^{\bullet-}$ trapping**		*·OH trapping†*			*Toxicity*	
Spin traps	k_{app} (M^{-1} s^{-1})	$t_{1/2}$ (min)	k_{app} ($/10^{-9}$ M^{-1} s^{-1})	$t_{1/2}$ (min)	*Partition coefficient* (K_p)‡	*Cells [IC_{50} (mM)]*	*Animals (mg/100 g)§*
DMPO	50[86]–60[18]	1[86]	1.9[87]–3.4[18]	55[87]	0.1[34]	138[29]	<200[71]
EMPO	—	8.6[77, 98]	5.0[87]	127[87]	0.15[77]	—	—
DEPMPO	60[86]–90[19]	14[86]	4.8[87]–7.8[19]	132[87]	0.06[1]	<25[45]	—
DIPPMPO	—	~23[7]	4.6[87]	158[87]	2.1[7]	—	—
PBN	N/A	N/A	6.1–8.5[65]	0.8[33]	15[34]	9.4[29]	<100[71]
4-PyOBN	N/A	N/A	4.0[65]	0.2[33]	0.15[34]	5.4[29]	<100[71]

References are given as superscripts. A dash indicates data not reported. N/A, not applicable.

*Using a riboflavin-light radical generating system in phosphate buffer, pH 7.0.

†Radical sources: Janzen *et al.* (33) and Villamena *et al.* (87) with UV photolysis of H_2O_2; Rosen *et al.* (65) with ionizing radiation-N_2O; Finkelstein *et al.* (18) and Frejaville *et al.* (19) with Fe^{2+}-H_2O_2.

‡In 1-octanol/phosphate buffer system, pH 7.4.

§On Sprague-Dawley rats as lethal doses.

plications, including tumors (46) and models of sepsis such as lipopolysaccharide-induced injury (44), as well as from environmental pollution and cigarette smoke (73). NO formation in whole mice has been measured using L-band EPR spin trapping (20, 40). In rats induced with lipopolysaccharide, spin-trapped NO was observed in the liver, kidney, urinary bladder, and head regions (21).

NO is a relatively inert gas and is highly diffusible in cells and cell membranes because of its moderate solubility in water (approximately 2.0 m*M* atm^{-1}) at ambient temperature, or 1.4 m*M* atm^{-1} at 37°C (11) and with higher solubility in non-polar media. Several NO traps such as the complexes of iron with dithiocarbamate ligands (Scheme 6) have been developed that allow NO detection in both polar and non-polar environments. Vanin and co-workers (53, 83, 85) first demonstrated the use of Fe(diethyldithiocarbamate)$_2$ [Fe(DETC)$_2$] for NO detection in biological systems. *In vivo* EPR experiments on mice showed that NO trapping by hydrophobic Fe^{2+}-DETC is more efficient than by hydrophilic Fe^{2+}-MGD due to higher stability of this complex in animal tissues (49). In addition to NO trapping forming mononitrosyl iron complexes, dinitrosyl iron complexes can be formed typically from iron sulfur proteins. Dinitrosyl iron complexes have been observed in liver and kidney and show high affinity and membrane permeability in these organs (82).

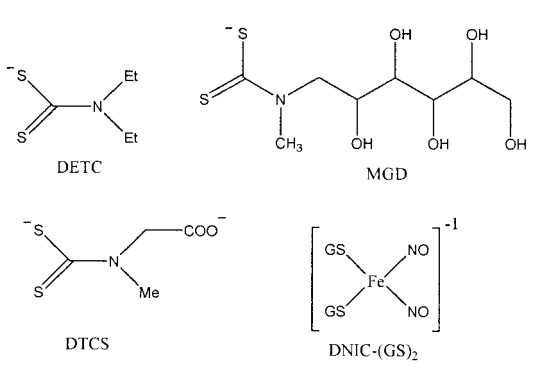
The redox state of Fe-dithiocarbamates, either of MGD or DETC, plays a critical role in the accurate quantification of NO production. The Fe^{2+} complex can be readily oxidized under aerobic conditions to form Fe^{3+}-dithiocarbamate, which can form NO-Fe^{3+} complexes as observed by using various spectroscopic and electrochemical methods (84). Conversion of the EPR-silent NO-Fe^{3+}-MGD complex to an EPR-detectable NO-Fe^{2+}-MGD can be accomplished with 50% yield by NO itself and by reductants such as ascorbate, hydroquinone, or cysteine with conversion efficiency of up to 99.9%. To limit these redox reactions with oxygen, it has been suggested that under aerobic conditions, the oxidized ferric form of these complexes should be used for cellular or tissue measurements. Moreover, dithiocarbamates are efficient Cu(II) chelators and can inhibit copper enzymes such as SOD (10).

NO can also be generated non-enzymatically (105) in tissues by either direct disproportionation, or reduction of nitrite (NO_2^-) under acidic and highly states, which can occur in ischemic conditions. Nitrite anions can also form NO at physiological pH, but this only occurs at a very low rate (80) since its rate of NO formation is a function of H^+ and nitrite concentrations. As a caution, it has also been reported that under some conditions Fe^{2+}-MGD can significantly catalyze the reduction of nitrite to NO (78).

Because of the facile reaction of O_2 with NO to form more reactive nitrogen oxide species such as NO_2, N_2O_3, and N_2O_4, preparations of NO stock solutions and of their Fe^{2+} complexes should be made in deoxygenated water. Introduction of these spin trap complexes either *in vitro* or *in vivo* usually involve premixing of the $FeSO_4$ with excess dithiocarbamate co-ligand. Water-insoluble Fe(DETC)$_2$ can be introduced as a suspension with serum albumin (79) and has been reported to measure NO in porcine aorta with high sensitivity of 10 pmol/ml. Detection of NO in blood vessels as well as human umbilical endothelial cells has been successfully demonstrated using colloidal Fe^{2+}-DETC prepared by mixing DETC and Fe^{2+} in concentrated Krebs-HEPES solution (38, 39).

Hemoglobin has also been employed as a spin trap and has relatively high specificity and affinity ($K_a = 3 \times 10^{10}\ M^{-1}$) for NO and slow NO dissociation rate ($K_D = 10^3$–$10^5\ s^{-1}$) (42, 43). The highly oxygen-sensitive glucose chars, which are solid-state paramagnetic probes, have also been employed for NO detection with sensitivity of up to 10–100 n*M* levels in anaerobic solutions but are not sensitive enough to measure physiological NO concentrations <10 n*M* (104).

Several non-conventional traps or probes, such as dendrimeric-containing nitronyl nitroxides (66), diazoketones (88), cheletropic *o*-quinodimethane derivatives (2), nitronyl-nitroxides (35, 91), diradicals (48), and nitromethane (62), have also been employed to detect NO, but are limited by their availability, specificity, instability in biological milieu, and short EPR signal half-lives (90).



SCHEME 6. Various types of dithiocarbamates used as ligands for NO trapping.

MISCELLANEOUS SPIN TRAPPING DETECTION METHODS

Both ^{31}P- and ^{19}F-nuclear magnetic resonance (NMR) spectroscopy have been employed in the detection of various radicals through analysis of the decomposition products of their respective adducts with DEPMPO and a fluorinated DMPO analogue (36, 37). In the case of ^{31}P-NMR, O-centered radical, *e.g.,* •OH and $O_2^{\bullet -}$, adducts of DEPMPO gave characteristic peaks at 27.05 ppm that disappeared over time to only a peak signal corresponding to DEPMPO. Potapenko *et al.* (57) had proposed a non-radical mechanism for the formation of $SO_3^{\bullet -}$-DEPMPO from the initial nucleophilic addition of the SO_3^{2-} anion to DEPMPO to form the corresponding hydroxylamine as a mixture of diastereomers as characterized by ^{31}P-, ^{13}C-, and ^1H-NMR spectroscopy, and its subsequent oxidation leads to the formation of its corresponding nitroxides. Using electrochemical (47) and liquid chromatography with electrochemical detection (54) techniques, spin adducts and their decomposition products have been analyzed. Lipid derived C-centered pentadienyl radicals have been detected and identified *in vitro* and *in vivo* by liquid chromatography/EPR and liquid chromatography/mass spectrometry (61) using

4-PyOBN as a spin trap. Proton magnetic resonance imaging detection of NO production based on $(MGD)_2$-Fe^{2+}-NO complex formation and the effect of these complexes on proton relaxation have been reported in rats (22).

Cyclic hydroxylamines are also employed as OFR probes although not spin traps *per se,* since the nitroxide formation results from the oxidation of the hydroxylamine by OFRs to give an EPR signal, and there is no trapping/bonding of the radical. The specificity of these cyclic hydroxylamines is still not clear at the moment since the effect of other potential oxidizing agents in the biological milieu has not yet been reported. A negatively charged cyclic hydroxylamine, 1-hydro-4-phosphonooxy-2,2,6,6-tetramethylpiperidine, has been used to detect ROS for diagnosis of extracellular inflammation-induced oxidative stress in cultured macrophages, blood, and *in vivo* applications (13).

CONCLUSION

EPR spin trapping is a powerful technique for measuring free radicals in biomedical applications. It enables sensitive and specific detection of free radicals and characterization of the mechanisms of their formation in a broad range of applications. In spite of the limitations associated with spin trapping techniques such as the need to improve efficiency of radical trapping, selectivity and availability of spin traps, the limited stability of spin adducts, and possible formation of artifactual spin adducts, the use of this technique for detection of free radicals has become an indispensable tool for the detection of OFRs and NO in biological systems. There is an ongoing need for improved spin traps and related development efforts. Further progress in spin trap development together with advances in EPR technology, including low-frequency EPR instrumentation, for *in vivo* applications should enable this technique to be extended to address many critical questions regarding the role of free radicals in cellular function and disease in animal models and in humans.

ABBREVIATIONS

L-Arg, L-arginine; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DETC, diethyldithiocarbamate; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTCS, *N*-(dithiocarboxy)sarcosine; EMPO, 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; MGD, *N*-methyl-D-glucamine dithiocarbamate; NMR, nuclear magnetic resonance; NO, nitric oxide; NOS, nitric oxide synthase; OFR, oxygen free radical; PBN, *N-tert*-butyl-α-phenyl nitrone; PUFA, polyunsaturated fatty acid; 4-PyOBN, α-(4-pyridyl-1-oxide)-*N-tert*-butyl nitrone; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; UV, ultraviolet.

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