Acc. Chem. Res. **1999,** *32,* 155−161

Issues Pertinent to the in Vivo in Situ Spin Trapping of Free Radicals§

SOVITJ POU,† HOWARD J. HALPERN,‡
PEI TSAI,† AND GERALD M. ROSEN*,†

Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, Maryland 21201, and Department of Radiation and Cellular Oncology, The University of Chicago, Chicago, Illinois 60637

Received February 26, 1998

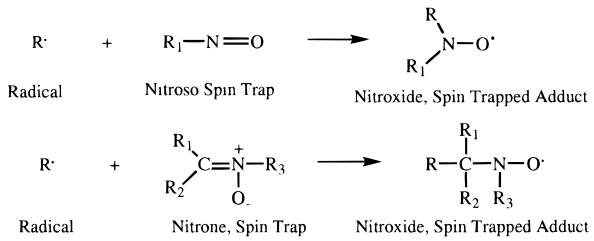
Introduction

In 1954, it was proposed that free radicals were the toxic intermediate associated with oxygen poisoning and ionizing radiation.[1] Surprisingly, this hypothesis and its implication to aerobic organisms seemed remote to the scientists of that time, as more than a decade would elapse before biological sources of superoxide would be linked to the discovery of an enzyme—superoxide dismutase—designed to eliminate this free radical.[2] Now as we approach the 50th anniversary of the Gerschman[1] hypothesis, the importance of free radicals in biology is no longer controversial. These reactive species are common intermediates in cellular metabolism, where they play an essential role in the control of many physiological functions, including the regulation of vascular tone and host immune response.[3]

Identification of free radicals at a cellular level is central to the study of these intermediates. Although there are a wide variety of analytic tools[4] for identifying specific free radicals, spin trapping and low-frequency EPR spectroscopy has emerged as the primary method to characterize free radicals in animals in real time and at their site of evolution. This Account explores this development.

Background

The history of spin trapping traces its origin to a series of publications in the late 1960s when several laboratories reported the addition of free radicals to either nitrosoalkanes or nitrones.[5] The resulting nitroxide—a spin-trapped adduct—was found to exhibit remarkable stability at ambient temperature, far exceeding the lifetime of the parent free radical.[5] Despite the nearly decade delay in adapting this technique to biological systems[6] and the enormous literature devoted thereafter, the birth of spin trapping can be dated from these early investigations.[5,7]



Considering the importance of superoxide dismutase and catalase to life in an aerobic world,[8] it is not surprising that superoxide and hydroxyl radical were the first free radicals of biological derivation to be spin trapped.[9] In subsequent years, an extensive spin trapping literature, devoted to identifying oxygen- and carbon-centered free radicals, has led to new insights into the role of free radicals in biology. Herein, we will not detail these studies as they have been extensively reviewed, but rather we will focus our attention on issues pertaining to the in vivo in situ spin trapping of free radicals in real time using low-frequency EPR spectroscopy.

In Vivo in Situ Spin Trapping of Free Radicals

Low-Frequency EPR Spectroscopy. The in vivo in situ detection of free radicals in real time in tissue samples of dimensions larger than 1 mm requires EPR spectroscopy at lower than conventional X-band (~9.5 GHz) frequencies. Living samples consist primarily of aqueous, conductive material, which absorbs microwave and radio frequency electromagnetic radiation.[10] Under generic conditions, this absorption or loss of electromagnetic energy creates an exponential loss of signal as a function

Sovitj Pou, after receiving his Ph.D. in organic chemistry from the University of Oregon, became a postdoctoral fellow with Gerald Rosen at Duke University. Thereafter, he joined the faculty in the Department of Pharmacology at the University of Maryland School of Pharmacy. He is Assistant Professor in the Medical Biotechnology Institute and a senior scientist at GloboMax. His current research interests center on the synthesis of isotope-labeled spin traps.

Howard J. Halpern received his Ph.D. in high-energy physics from the University of Wisconsin and his M.D. from the University of Miami. He has worked with low-frequency EPR spectroscopy for the past 15 years. His research in this field was initiated at Harvard Medical School and has continued at The University of Chicago, where he is currently Professor of Radiation and Cellular Oncology.

Pei Tsai was educated at the University of Oregon, where she received her Ph.D. in organic chemistry. She is a postdoctoral fellow with Gerald Rosen, developing spin traps for the in vivo in situ detection of free radicals in animal models.

Gerald M. Rosen, after receiving his Ph.D. in organic chemistry from Clarkson University and spending three years of postdoctoral training in pharmacology, joined the Department of Pharmacology, Duke University, where he spent most of his academic career. In 1988, he became Chairman of the Department of Pharmacology at the University of Maryland School of Pharmacy, until 1993, when he returned to full-time research, conducting experiments on the in vivo identification of biologically generated free radicals using spin trapping/EPR spectroscopy. He is currently Isaac E. Emerson Professor of Pharmacology.

* To whom correspondence should be addressed at the Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Medical Biotechnology Center, 725 West Lombard Street, Baltimore, MD 21201. Phone: (410) 706-0514. Fax: (410) 706-8184. E-mail: grosen@umaryland. edu.

† University of Maryland School of Pharmacy.

‡ The University of Chicago.

§ This article is dedicated to the Nobel laureates Robert F. Furchgott, Louis J. Ignarro, and Ferid Murid, who received the 1998 Prize in Physiology or Medicine for their research on the biological role of nitric oxide.

10.1021/ar970251l CCC: $18.00
Published on Web 11/04/1998

of depth of penetration. This loss of energy with depth in the sample makes measurement deep in a living sample virtually impossible for higher frequency radiation. It also reduces the sensitivity of the measurement, even at superficial depths. Animals, which absorb electromagnetic energy, will increase the energy lost to absorption and reduce the variable term *Q*. The *Q* of the EPR resonator is the ratio of stored electromagnetic energy to the energy lost to absorption in a single cycle. Classically, the sensitivity of the magnetic resonance experiment is proportional to *Q*. Thus, the sensitivity of the measurement will diminish with larger samples at higher frequencies.[11]

The distance scale of the exponential energy loss is set by the frequency of the electromagnetic radiation. The scale, referred to as the skin depth, is found to be inversely proportional to the square root of the frequency. This relationship between frequency and skin depth is model dependent, where for many realistic situations, the effective skin depth is a stronger function of frequency.[10,12] Electromagnetic loss will, therefore, diminish as the frequency is lowered. Longer wavelengths will penetrate deeper. At a given depth, there will be less absorption of longer wavelength, lower frequency electromagnetic radiation. At 9.5 GHz, experiments are limited to small volumes of homogeneous solutions, crude enzyme preparations, isolated cell suspensions, and thin tissue slices <1 mm thick.

As the frequency is lowered, paramagnetic species can be identified in larger biological samples with magnetic resonance spectroscopy, in particular EPR spectroscopy. At a frequency of 2−4 GHz (S-band), for instance, detection of free radicals can be obtained at a depth of several millimeters, increasing to approximately 1 cm at 1−2 GHz (L-band). At still lower frequencies such as 250 MHz skin depth absorption in tissues approaches 6−7 cm, making larger animal measurements feasible.[13]

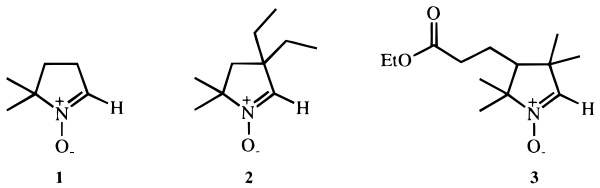
The sensitivity or signal-to-noise ratio (S/N) decreases as the frequency diminishes, the extent of which is controversial.[14] It is commonly held that the sensitivity is proportional to the square of the frequency. Certainly, the signal can be thought of as having this dependence.[15] One factor of frequency in this dependence is derived from the proportionality of the energy in the absorbed quantum to frequency through Planck's relationship, also referred to as the Boltzmann factor. The second frequency factor derives from the coupling to a changing magnetization. With a square rule frequency dependence, low-frequency measurements would, therefore, be very difficult. Bear in mind that a factor of approximately 1600 would be lost in the S/N ratio in changing frequency from 9.5 GHz to 250 MHz. Despite this, it is important to consider sample and resonator noise as a function of frequency in the S/N ratio.[14b,15,16] For animals that fill the resonator, consideration of sample noise under relatively standard operating conditions yields a dependence of the S/N on frequency of less than frequency to the first power.

$$S/N \approx \omega^{0.8}$$

One approach to balance the loss of sensitivity at lower frequencies is to use a loop-gap resonator. In its simplest form the loop-gap resonator is simply an inductive−capacitive (L−C) circuit with the inductive element serving as the sample holder. Loop-gap resonators with their high power density and excellent filling factors are extraordinarily useful at high frequencies.[17] At low frequencies lumped circuit devices such as the loop-gap resonator are required.[18] For homogeneous samples and resonators that are filled nearly entirely by the sample, even weaker dependences of sensitivity on frequency are found. Even with a dependence of sensitivity on frequency this weak, the reduction in frequency still allows sensitive measurements.[19]

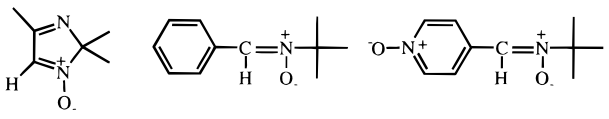
Spin trapping Free Radicals. The ease of spin trapping free radicals in isolated cells cannot readily be adopted for animal models. Such variables as spin trap specificity, sensitivity, and distribution, along with the rate of spin trap adduct formation and stability of the corresponding spin-trapped adduct, take on additional importance when identifying a free radical in animal models in real time. In isolated cell suspensions there are two compartments—intra- and extracellular—into which the spin trap partitions. Through well-designed experiments localization of the corresponding spin-trapped adduct and by inference the site of free radical formation can be deduced.[20] In contrast, such dynamic studies are considerably more complicated in isolated organ preparations and in vivo.[21]

Several classes of spin traps have been developed to react with specific free radicals. For instance, cyclic nitrones such as 5,5-dimethyl-1-pyrroline *N*-oxide (**1**) can spin trap superoxide, hydroxyl, and small carbon-centered free radicals, giving spin-trapped adducts with defined EPR spectra.[9c] Increasing steric hindrance has resulted in nitrone **2** and nitrone **3**, which exhibit specificity toward hydroxyl radical.[22]



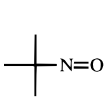
1 **2** **3**

Substituted 2*H*-imidazole *N*-oxides (**4**) have displayed selectivity toward hydroxyl and small carbon-centered free radicals.[23] Although acyclic nitrones, *N*-*tert*-butyl-α-phenylnitrone (**5**) and α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (**6**), can react with superoxide and hydroxyl radical, the half-life of the corresponding nitroxides is, however, under a minute at room temperature.[24] In contrast, the lifetime of spin-trapped adducts derived from the reaction of acyclic nitrones with small carbon-centered free radicals is considerably longer.[25]

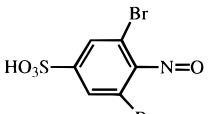


4 **5** **6**

Finally, poor stability of spin-trapped adducts deduced from the spin trapping of oxygen-centered free radicals with nitrosoalkanes, 2-methyl-2-nitrosopropane (**7**) and 3,5-dibromo-4-nitrosobenzenesulfonate (**8**), limits the use of these spin traps to carbon-centered free radicals.[26]



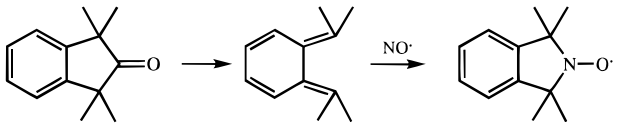
7



8

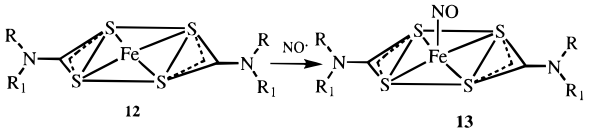
The physiological importance of nitric oxide has spurred the synthesis of unique spin traps, some of which have been used to identify this free radical in animal models in real time. Unlike superoxide and hydroxyl radical, the unexpectedly long lifetime of nitric oxide, an essential prerequisite for a physiological transmitter whose cellular target is often distant from the site of origin, presents unique challenges in the design of spin traps specific for this free radical. In fact, until recently it was questionable whether nitric oxide could be spin trapped, as its fate in aqueous solutions, yet alone in vivo, was uncertain. Even though nitrite is the end product of nitric oxide autoxidation,[27] other intermediates, which may have a direct impact on the spin trapping of this free radical, have only recently been catalogued, including nitrosyloxyl radical ($ONOO^{\bullet}$), nitrosyl radical ($NO_2^{\bullet}$), and dinitrogen trioxide (N_2O_3).[27c]

One of the most innovative schemes for spin trapping nitric oxide draws upon the ease with which "activated" cis-conjugated dienes react with free radicals.[28] Typical of this new class of spin traps is 7,7,8,8-tetramethyl-*o*-quinodimethane (**10**), which is generated upon photolysis of 1,1,3,3-tetramethyl-2-indanone (**9**). Reaction of a solution of **10** with nitric oxide results in the formation of 1,1,3,3-tetramethylisoindolin-2-oxyl (**11**).[28a]



9 **10** **11**

As nitric oxide is known to react with ferrous salts, giving intense EPR spectra in aqueous solutions at ambient temperature, synthetic iron chelates have played an important role in identifying this free radical in biological milieu.[29] One of the better defined complexes is iron(II) diethyldithiocarbamate ($Fe^{2+}(DETC)_2$, **12a**).[29b] Its reaction with nitric oxide is characterized by the low-temperature EPR spectrum at $g_{\perp} = 2.035$ and $g_{||} = 2.02$ with a triplet hyperfine structure at $g_{\perp}$.[29b] At 37 °C, the unresolved hyperfine structure $g_{\perp}$ in the frozen state changes to an isotropic triplet with $g_{av} = 2.03$. Poor water solubility of **12a** has limited its use. Alternative complexes, $Fe^{2+}(MGD)_2$ (**12b**) or $Fe^{2+}(DTCS)_2$ (**12c**), have faired better, as in vivo in situ EPR spectra corresponding to $NO-Fe^{2+}(MGD)_2$ (**13b**) and $NO-Fe^{2+}(DTCS)_2$ (**13c**) have been recorded in real time.[19,30]

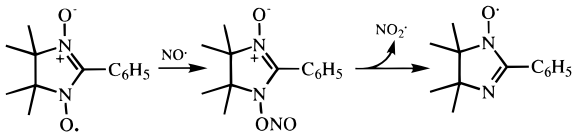


12 **13**

a $R = R_1 = C_2H_5$
b $R = CH_3$, $R_1 = CH_2\text{-}(CHOH)_4\text{-}CH_2OH$
c $R = CH_3$, $R_1 = CH_2CO_2^-$

a $R = R_1 = C_2H_5$
b $R = CH_3$, $R_1 = CH_2\text{-}(CHOH)_4\text{-}CH_2OH$
c $R = CH_3$, $R_1 = CH_2CO_2^-$

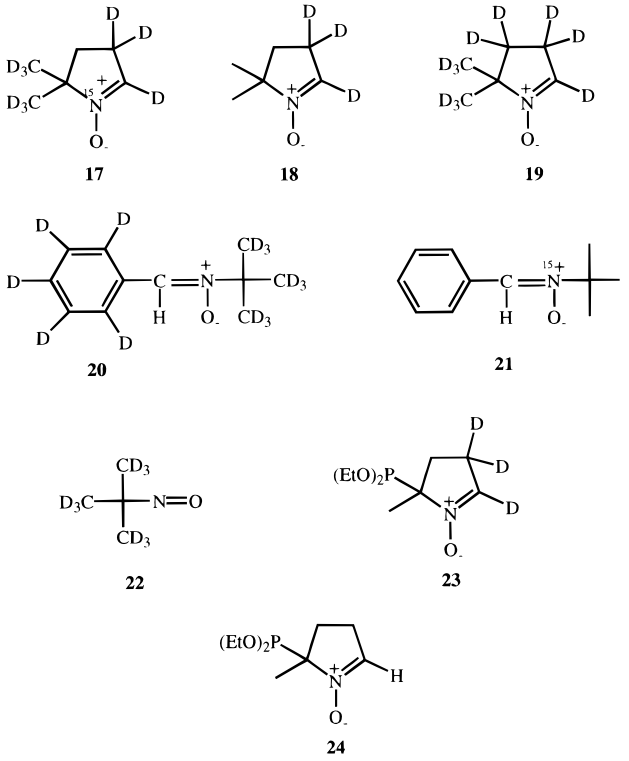
Nitronyl nitroxides such as **14** have been explored as spin traps for nitric oxide.[31] Here, reaction with this free radical results in the loss of $NO_2^{\bullet}$ and the formation of an imino nitroxide **16**, whose EPR spectrum is distinguishable from that of the parent nitroxide **14**.[31]



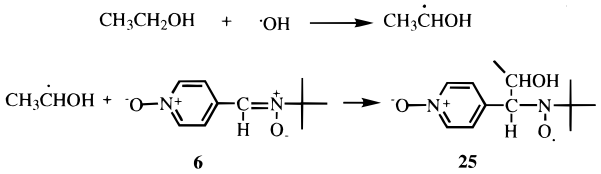
14 **15** **16**

In Vivo Spin Trapping in Real Time. Limitations to Overcome. A loss of sensitivity occurs when the operating frequency decreases from 9.5 GHz to 250 MHz. One approach[32] to compensate for this diminution in signal intensity is to synthesize[22b,33] spin traps with ^2H and ^{15}N in place of ^1H and ^{14}N. Various ring and substitutent aliphatic hydrogens contribute to the unresolved broadening of the EPR spectral lines. Deuterium has a magnetic moment approximately 1/7 that of hydrogen, but higher multiplicity, giving an overall line width reduction by a factor of 4. Further reduction in the hyperfine splitting from 3 to 2 by replacing ^{14}N with ^{15}N gives an additional enhancement of sensitivity by a factor of 1.5. In turn, this has resulted in a proportionately higher sensitivity toward superoxide and hydroxyl radical.[33a,34] A number of isotope-labeled spin traps have been synthesized, including ^{15}N-containing and deuterated 5,5-dimethyl-1-pyrroline *N*-oxide[33a] (**17**), trideuterated 5,5-dimethyl-1-pyrroline *N*-oxide[33a] (**18**), perdeuterated 5,5-dimethyl-1-pyrroline *N*-oxide[33a] (**19**), deuterated *N*-*tert*-butyl-α-phenylnitrone[33b] (**20**), ^{15}N-containing *N*-*tert*-butyl-α-phenylnitrone[33d] (**21**), perdeuterated 2-methyl-2-nitrosopropane[33e] (**22**), and deuterated 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide[33c] (**23**). The significance[34] of this enhanced sensitivity can be seen by low-frequency EPR images from spin-trapped adducts of nitrone **19**, whereas such images were not observed with nitrone **1**.

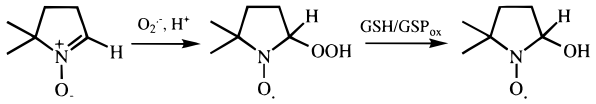
Even with isotope-labeled nitrones, the feasibility of spin trapping superoxide in vivo is problematic. Consider that the rate constant[24a] for spin trapping superoxide by nitrone **1** at physiological pH is no greater than 12 M^{-1} s^{-1}, whereas the disproportionation rate constant[35] for superoxide at this pH is 3×10^5 M^{-1} s^{-1} and its reaction with superoxide dismutase[36] is considerably faster at 2×10^9 M^{-1} s^{-1}. Certainly phosphorylated nitrone **24**, with a 60 M^{-1} s^{-1} rate constant toward superoxide[33c] is a significant improvement over nitrone **1**; however, even under the best of experimental conditions, spin trap **24** will have limited in vivo applications.



From a kinetic standpoint, the spin trapping of hydroxyl radical offers an opportunity to in vivo in situ detect this free radical, as it reacts with both cyclic and acyclic nitrones at near diffusion-controlled rates.[24a] Yet poor stability of the corresponding spin-trapped adducts has greatly restricted this approach to static systems.[24a,37] On the basis of previous reports,[25a,b] a highly promising approach involved an indirect method of hydroxyl radical detection. Here, EtOH was included with nitrone **1**, nitrone **5**, or nitrone **6**. Hydrogen atom abstraction by hydroxyl radical yielded α-hydoxylethyl radical, which was subsequently spin trapped by each of the nitrones. We found nitrone **6** and EtOH was the most sensitive of the spin trapping systems examined with a second-order rate constant of $3.1 \times 10^7\ M^{-1}\ s^{-1}$.[25c] Unlike hydroxyl radical spin-trapped adducts of nitrones **5** and **6**, α-hydroxyethylmethyl α-pyridyl-1-oxide *N-tert*-butylnitroxide (**25**) exhibited remarkable stability, appearing to be unaffected by a myriad of biological reducing agents.[25c]



Even though spin trapping can distinguish among a variety of biologically generated free radicals, cellular metabolism can often disguise the origin of the initial free radical spin trapped. For illustrative purposes, consider the intracellular spin trapping of superoxide.[20b,38] Here, the corresponding nitroxide **26**, a substrate for glutathione peroxidase (GSP_{ox}),[38] is rapidly converted to 2-hydroxy-2,2,dimethy-1-pyrrolidinoxyl (**27**) at such a rate that the EPR spectrum of nitroxide **26** is never recorded with standard EPR spectrometers. In the intervening years, much effort has been put forth to synthesize spin traps with selectivity toward hydroxyl radical, thereby circumventing the masking effects of cellular metabolism.[22a,b] The most successful of these spin traps, nitrone **3**, is resistant to a variety of biological oxidative reactions that would otherwise result in artifactually derived nitroxides,[22b] as is seen with nitrone **1**. Thus, nitrone **3** has the potential to distinguish between intracellularly produced superoxide and hydroxyl radical and may find a niche in the in vivo characerization of hydroxyl radical.



Localization of the spin trap at the site of free radical evolution is one of the most difficult and daunting tasks that awaits attempts to identify the reactive intermediate in isolated organ preparations and in animal models. Consider, for example, the in vivo in situ spin trapping of radiation-induced hydroxyl radical in a mouse in real time.[21b] The experimental design, based on in vitro kinetic and stability studies,[25c] included the introduction of EtOH and nitrone **6** into a mouse extremity tumor, to which was delivered a high, toxic dose of radiation to a substantial bulk of the tumor with minimal effect on the physiology of the animal. On the basis of the observed EPR spectrum[25c] characteristic of nitroxide **25**, it was assumed that nitrone **6** and EtOH were compartmentalized within the same tissue so that radiation-induced α-hydroxyethyl radical could react with acyclic nitrone **6**.[21b]

Even though EtOH can diffuse into many sites within a tissue, nitrone **6**, which has a small 1-octanol/water partition coefficient of ~0.09, does not passively enter cells.[25c] Thus, the detection of hydroxyl radical as nitroxide **25** was solely limited to interstitial and vascular spaces. Poor cellular uptake of nitrone **6** resulted in measurements of spin-trapped adducts that localized away from sites of relevant intracellular production of free radicals. Whatever free radical events that may take place intracelluarly, they would not be discernible with nitrone **6** and EtOH, even though γ-irradiation of water within cells has long been known to produce hydroxyl radical at sensitive loci.[39]

The in vivo activation of intraperitoneal macrophages with high doses of lipopolysaccharide has allowed the in vivo in situ spin trapping of macrophage-derived nitric oxide.[19,30,40] The successful experiment was dependent upon phagocytic release of a high flux of nitric oxide for prolonged periods of time. Diffusion from intracellular sites of formation allowed nitric oxide to be spin trapped in the surrounding milieu by the charged iron chelates **12b** and **12c**.

Significance

In 1895, Röntgen, while investigating the nature of cathode rays, found that this tube was emitting a penetrating form of radiation, which he called the X-ray.[41] Within a month of this publication, physicians in Chicago[42] and Zürich used X-rays to treat cancer patients with this radiation. It was soon recognized that the radiation emitted from Crookes tube and from specific isotopes of various elements had profound effects on water. However, it was not until 1944 that this reaction was found to generate hydroxyl radical.[43]

$$H_2O + h\nu \rightarrow H_2O^{\bullet+} + e^- \ (\rightarrow e_{aq})$$

$$H_2O^{\bullet+} + H_2O \rightarrow HO^{\bullet} + H_3O^+$$

Verification that such reactions take place in vivo would take another 50 years—the 100th anniversary of the discovery of the X-ray—when spin trapping/low-frequency EPR spectroscopy measured the in vivo in situ generation of this free radical in real time.[21b]

In 1980, Furchgott and Zawakzki[44] demonstrated that acetylcholine induced vascular relaxation in precontracted aortic rings. This relaxation was dependent upon the presence of the endothelium. On the basis of their results, it was proposed[3a] that acetylcholine stimulated the release of a factor, known as endothelial-derived relaxation factor (EDRF), which resulted in the observed physiological response. Subsequently, it was demonstrated that the free radical nitric oxide exhibited many of the physiological properties attributed to EDRF.[44] Besides regulating vascular tone, nitric oxide has been shown to be a novel transient biological messenger as this free radical augments cell−cell communications and governs many intracellular events, in addition to its role in host immune defense, particularly effective against a number of intracellular pathogens.[45] Only recently with the development of in vivo stable ferrochelates, such as **12b** and **12c**, has it been possible to in vivo in situ spin trap nitric oxide in real time using low-frequency EPR spectroscopy.[22b,30,40]

When one considers the complexity of the in vivo in situ spin trapping studies described above, the detection of a specific free radical has to be considered a remarkable accomplishment. Yet such successes were achieved with exceedingly high, nonpharmacological doses of γ-irradiation[21b] and cytokine levels[22b,30,40] far in excess of what is considered physiological. One of the great challenges will be the identification and localization of these free radicals at standard clinical doses of radiation and under homeostatic conditions. These goals can be achieved through design of spin traps whose specificity, sensitivity, and lipophilicity will allow detection of specific free radicals at relevant tissue compartments. Likewise, further developments in very low-frequency EPR spectroscopy, such as a pulsed EPR spectrometer[46] operating at 300 MHz, hold great promise for rapid data acquisition, an essential element in improvement of EPR spectroscopic sensitivity.

This research was supported by grants from the National Institutes of Health, CA-69538 and AG-14829.

References

(1) Gerschman, R.; Gilbert, D. L.; Nye, S. W.; Dwyer, P.; Fenn, W. O. Oxygen poisoning and X irradiation: A mechanism in common. *Science* **1954**, *119*, 623−626.

(2) McCord, J. M.; Fridovich, I. Superoxide dismutase − an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **1969**, *244*, 6049−6055.

(3) (a) Furchgott, R. F.; Zawadzki, J. V. The obligatory role of endothelial cells on the relaxation of arterial smooth muscle by acetylcholine. *Nature* **1980**, *288*, 373−376. (b) Babior, B. M.; Kipnes, R. S.; Curnutte, J. T. Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **1973**, *52*, 741−744.

(4) (a) Bors, W.; Saran, M.; Michel, C. Assays of oxygen radicals: Methods and mechanisms. In *Superoxide Dismutase*, Oberley, L. W., Ed.; CRC Press: Boca Raton, FL, 1982; Vol. II, pp 31−62. (b) Archer, A. Measurement of nitric oxide in biological models. *FASEB J.* **1993**, *7*, 349−360.

(5) (a) Mackor, A.; Wajer, Th. A. J. W.; de Boer, Th. J. C−Nitroso compounds Part I. The formation of nitroxides by photolysis of nitroso compounds as studied by electron spin resonance. *Tetrahedron Lett.* **1966**, 2115−2123. (b) Iwamura, M.; Inamoto, N. Novel radical 1,3-addition to nitrones. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 702. (c) Chalfont, G. R.; Perkins, M. J.; Horsfield, A. A probe for homolytic reactions in solution. II. The polymerization of styrene. *J. Am. Chem. Soc.* **1968**, *90*, 7141−7142. (d) Janzen, E. G.; Blackburn, B. J. Detection and identification of short-lived free radicals by an electron spin resonance trapping technique. *J. Am. Chem. Soc.* **1968**, *90*, 5909−5910. (e) Lagerscrantz, C.; Forshult, S. Trapping of free radicals formed by γ-irradiation of organic compounds. *Nature* **1968**, *218*, 1247−1248.

(6) Harbour, J. R.; Bolton, J. R. Superoxide formation in spinach chloroplasts: Electron spin resonance detection by spin trapping. *Biochem. Biophys. Res. Commun.* **1975**, *64*, 803−807.

(7) Janzen, E. G. Spin trapping. *Acc. Chem. Res.* **1971**, *4*, 31−40.

(8) (a) Loewen, P. Isolation of catalase-deficient *Escherichia coli* mutants and genetic mapping of *katE*, a locus that affects catalase activity. *J. Bacteriol.* **1984**, *157*, 622−626. (b) Nakayama, K. Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphysomonas gingivalis*. *J. Bacteriol.* **1994**, *176*, 1939−1943. (c) Longo, V. D.; Gralla, E. B.; Valetine, J. S. Superoxide dismutase activity is essential for stationary phase survival of *Saccharomyces cerevisiae*: Mitochondrial production of toxic oxygen species in vivo. *J. Biol. Chem.* **1996**, *271*, 12275−12280.

(9) (a) Harbour, J. R.; Chow, V.; Bolton, J. R. An electron spin resonance study of the spin adducts of OH and HO_2 radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solutions. *Can. J. Chem.* **1974**, *52*, 3549−3553. (b) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J.; Paxton J. Spin trapping of superoxide. *Mol. Pharmacol.* **1979**, *16*, 676−685. (c) Kalyanaraman, B.; Perez-Reyes, E.; Mason, R. P. Spin-trapping and direct electron spin resonance investigations of the redox metabolism of quinone anticancer drugs. *Biochim. Biophys. Acta* **1980**, *630*, 119−130.

(10) Halpern, H. J.; Bowman, M. K. Low-frequency EPR spectrometers: MHz Range. In *EPR Imaging and In Vivo EPR*; Eaton, G. R., Eaton, S. S., Ohno, K., Eds.; CRC Press: Boca Raton, FL, 1991; pp 45−63.
(11) Feher, G. Sensitivity consideratons in microwave paramagnetic resonance absorption techniques. *Bell Syst. Technol.* **1957**, *36*, 449−484.
(12) (a) Bottomley, P. A.; Reddington, R. W.; Edelstein, W. A.; Schenck, J. F. Estimating radio frequency power deposition in body NMR imaging. *Magn. Reson. Med.* **1985**, *2*, 336−349. (b) Roschmann, P. Radio frequency penetration and absorption in the human body: Limitations to high-field whole-body nuclear magnetic resonance imaging. *Med. Phys.* **1987**, *14*, 922−928.
(13) Halpern, H. J.; Spencer, D. P.; van Polen, J.; Bowman, M. K.; Massoth, R. J.; Nelson, A. C.; Dowey, E. M.; Teicher, B. A. An imaging radio frequency electron spin resonance spectrometer with high resolution and sensitivity for in vivo measurements. *Rev. Sci. Instrum.* **1989**, *60*, 1040−1050.
(14) (a) Hoult, D. I.; R. E. Richards, R. E. The signal-to-noise ratio of nuclear magnetic resonance experiment. *J. Magn. Reson.* **1976**, *24*, 71−85. (b) Hoult, D. I.; Lauterbur, P. C. The sensitivity of the zeugmatographic experiment involving human samples *J. Magn. Reson.* **1979**, *34*, 425−433.
(15) Edelstein, W. A.; Glover, G. H.; Hardy, C. J.; Redington, R. W. The intrinsic signal-to-noise ratio in NMR imaging. *Magn. Reson. Med.* **1986**, *3*, 604−618.
(16) (a) Hoult, D. I.; Chen, C. N.; Sank, V. J. The field dependence of NMR imaging. II. Arguments concerning an optimal filed strength. *Magn. Reson. Med.* **1986**, *3*, 730−746. (b) Foster, T. H. Tissue conductivity modifies the magnetic resonance intrinsic signal-to-noise ratio at high frequencies. *Magn. Reson. Med.* **1992**, *23*, 383−385.
(17) Froncisz, W.; Hyde, J. S. The loop-gap resonator: A new microwave lumped circuit ESR sample structure. *J. Magn. Reson.* **1982**, *47*, 515−521.
(18) Decorps, M.; Fric, C. Low-frequency spectrometer of high sensitivity for the study of ESR. *J. Phys. E* **1972**, *5*, 337−342.
(19) Yoshimura, T.; Yokoyama, H.; Fujii, S.; Takayama, F.; Oikawa, K.; Kamada, H. In vivo EPR detection and imaging of endogenous nitric oxide in lipopolysaccharide-treated mice. *Nat. Biotechnol.* **1996**, *14*, 992−994.
(20) (a) Samuni, A.; Carmichael, A. J.; Russo, A.; Mitchell, J. B.; Reisz, P. On the spin trapping and ESR detection of oxygen-derived free radicals generated inside cells. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7593−7597. (b) Britigan, B. E.; Roeder, T. L.; Shasby, D. M. Insight into the nature and site of oxygen-centered free radical generation by endothelial cell monolayers using a novel spin trapping technique. *Blood* **1992**, *79*, 699−707.
(21) (a) Rosen, G. M.; Halpern, H. J.; Brunsting, L. A.; Spencer, D. P.; Strauss, K. E.; Bowman, M. J.; Wechsler, A. S. Direct measurement of nitroxide pharmacokinetics in isolated hearts situated in a low-frequency electron spin resonance spectrometer: Implications for spin trapping and in vivo oxymetry. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7772−7776. (b) Halpern, H. J.; Yu, C.; Barth, E.; Peric, M.; Rosen, G. M. In situ detection, by spin trapping of hydroxyl radical markers produced from ionizing radiation in the tumor of a living mouse. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 796−800.
(22) (a) Rosen, G. M.; Turner, M. J., III. Synthesis of spin traps specific for hydroxyl radical. *J. Med. Chem.* **1988**, *31*, 428−432. (b) Arya, P.; Stephens, J. C.; Griller, D.; Pou, S.; Ramos, C. L.; Pou, W. S.; Rosen, G. M. Design of modified pyrroline *N*-oxide derivatives as spin traps specific for hydroxyl radical. *J. Org. Chem.* **1992**, *57*, 2297−2301.
(23) Dikalov, S.; Kirilyuk, I.; Grigor'ev, I. Spin trapping of O-, C-, and S-centered radicals and peroxynitrite by 2H-imidazole-1-oxides. *Biochem. Biophys. Res. Commun.* **1996**, *218*, 616−622.
(24) (a) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. Spin trapping. Kinetics of the reaction of superoxide and hydroxyl radicals with nitrones. *J. Am. Chem. Soc.* **1980**, *102*, 4994−4999. (b) Kotake, Y.; Janzen, E. G. Decay and fate of the hydroxyl radical adduct of α-phenyl-N-*tert*-butylnitrone in aqueous media. *J. Am. Chem. Soc.* **1991**, *113*, 9503−9506.
(25) (a) Connor, H. D.; Thurman, R. G.; Galizi, M. D.; Mason, R. P. The formation of a novel free radical metabolite of CCl_4 in the perfused rat liver and *in vivo*. *J. Biol. Chem.* **1986**, *261*, 4542−4548. (b) Britigan, B. E.; Coffman, T. J.; Buettner, G. R. Spin trapping evidence for the lack of significant hydroxyl radical production during the respiration burst of human phagocytes using a spin adduct resistant to superoxide-mediated destruction. *J. Biol. Chem.* **1990**, *265*, 2650−2650. (c) Pou, S.; Ramos, C. L.; Gladwell, T.; Renk, E.; Centra, M.; Young, D., Cohen, M. S.; Rosen, G. M. A kinetic approach to the selection of a sensitive spin trapping system for the detection of hydroxyl radical. *Anal. Biochem.* **1994**, *217*, 76−83.
(26) (a) Wargon, J. A.; Williams F. Electron spin resonance studies of radical trapping in the radiolysis of organic liquids. I. Evidence for the primary formation of the methoxy radical in methanol. *J. Am. Chem. Soc.* **1972**, *94*, 7917−7918. (b) Pou, S.; Keaton, L.; Surichamorn, W.; Frigillana, P.; Rosen, G. M. Can nitric oxide be spin trapped by nitrone and nitroso compounds? *Biochim. Biophys. Acta* **1994**, *1201*, 118−124.
(27) (a) Ignarro, L. J.; Fukuto, J. M.; Griscavage, J. M.; N. E. Rogers, N. E.; Byrns, R. E. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8103−8107. (b) Kharitonov, V. G.; Sundquist, A. R.; Sharma, V. S. Kinetics of nitric oxide autoxidation in aqueous solution. *J. Biol. Chem.* **1994**, *269*, 5881−5883. (c) Goldstein, S.; Czapski, G. Kinetics of nitric oxide autoxidation in aqueous solution in the absence and presence of various reductants. The nature of the oxidizing intermediates. *J. Am. Chem. Soc.* **1995**, *117*, 12078−12084.
(28) (a) Korth, H.-G.; Ingold, K. U.; Sustmann, R.; De Groot, H.; Sies, H. Tetramethyl-ortho-quinodimethane. First member of a family of custom-tailored cheletropic spin traps for nitric oxide. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 891−893. (b) Korth, H. G.; Sustmann, R.; Lommes, P.; Paul, T.; de Groot, H.; Hughes, L.; Ingold, K. U. Nitric oxide cheletropic traps (NOCTs) with improved thermal stability and water solubility. *J. Am. Chem. Soc.* **1994**, *116*, 2767−2777. (c) Paul, T.; Hassan, M. A.; Korth, H.-G.; Sustmann, R.; Avila, D. V. Reaction of phenyl-substituted o-quinodimethanes with nitric oxide. Are benzocyclobutenes suitable precursors for nitric oxide cheletropic traps? *J. Org. Chem.* **1996**, *61*, 6835−6848.

(29) (a) Goodman, B. A.; Raynor, J. B.; Symons, M. C. R. Electron spin resonance of bis(N,N-diethylthiocarbamato)nitrosyliron. *J. Chem. Soc.* **1969**, 2572−2575. (b) Mordvintcev, P.; Mülsch, A.; Busse, R.; Vanin, A. On-line detection of nitric oxide formation in liquid aqueous phase by electron paramagnetic resonance spectroscopy. *Anal. Biochem.* **1991**, *199*, 142−146.

(30) Komarov, A.; Mattson, D.; Jones, M. M.; Singh, P. K.; Lai, C.-S. *In vivo* spin trapping of nitric oxide in mice. *Biochem. Biophys. Res. Commun.* **1993**, *195*, 1191−1198.

(31) (a) Ullman, E. F.; Osiecki, J. H.; Boocock, G. B.; Darcy, R. Studies of stable free radicals. X. Nitronyl nitroxide monoradicals and biradicals as possible molecule spin labels. *J. Am. Chem. Soc.* **1972**, *94*, 7049−7059. (b) Akaike, T.; Maeda, H. Quantitation of nitric oxide using 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). *Methods Enzymol.* **1996**, *268*, 211−221.

(32) Beth, A. H.; Venkataramu, S. D.; Balasubramanian, K.; Dalton, L. R.; Robinson, B. H.; Pearson, D. E.; Park, C. R.; Park, J. H. ^{15}N- and ^{2}H-substituted maleimide spin labels: Improved sensitivity and resolution for biological EPR studies. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 967−971.

(33) (a) Pou, S.; Rosen, G. M.; Wu, Y.; Keana, J. F. W. Synthesis of deuterium- and ^{15}N-containing pyrroline 1-oxides: A spin trapping study. *J. Org. Chem.* **1990**, *55*, 4438−4443. (b) Janzen, E. G.; Oehler, U. M.; Haire, D. L.; Kotake, Y. ENDOR spectra of aminoxyls. Conformational study of alklyl and aryl spin adducts of deuterated α-phenyl-N-*tert*-butylnitrone (PBN) based on proton and β-^{13}C hyperfine splittings. *J. Am. Chem. Soc.* **1986**, *108*, 6858−6863. (c) Frejaville, C.; Karoui, H.; Tuccio, B.; Le Moigne, F.; Culcasi, M.; Pietri, S.; Lauricella, R.; Tordo, P. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO): A new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals. *J. Med. Chem.* **1995**, *38*, 258−265. (d) Zhang, Y.-K. First synthesis of C-phenyl *N*-*tert*-butyl[^{15}N]nitrone (PBN-^{15}N) for the EPR spin trapping methodology. *Z. Naturforsch.* **1996**, *51b*, 139−143. (e) Holman, R. J.; Perkins, M. J. A probe for homolytic reactions in solution. Part. V. Perdeuterionitrosobutane. An improved spin trap. *J. Chem. Soc. C* **1971**, 2324−2326.

(34) Halpern, H. J.; Pou, S.; Peric, M.; Yu, C.; Bath, E.; Rosen, G. M. Detection and imaging of oxygen-centered free radicals with low-frequency electron paramagnetic resonance and signal-enhancing deuterium- containing spin traps. *J. Am. Chem. Soc.* **1993**, *115*, 218−223.

(35) Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; Ross, A. B. Reactivity of HO_2/O_2^- radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041−1100.

(36) Bull, C.; Fee, J. A. Steady-state kinetic studies of superoxide dismutases: Properties of the iron containing protein from *Escherichia coli*. *J. Am. Chem. Soc.* **1985**, *107*, 3295−3304.

(37) Janzen, E. G.; Kotake, Y.; Hinton, R. D. Stabilities of hydroxyl radical spin adducts of PBN-type spin traps. *Free Radical Biol. Med.* **1992**, *12*, 169−173.

(38) Rosen, G. M.; Freeman, B. A. Detection of superoxide generated by endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 7269−7273.

(39) Roots, R.; Okada, S. Protection of DNA molecules of cultured mammalian cells from radiation-induced single strand scissions by various alcohols and SH compounds. *Int. J. Radiat. Biol.* **1992**, *21*, 329−342.

(40) Fujii, H.; Koscielniak, J.; Berliner, L. J. Determination and characterization of nitric oxide generation in mice by in vivo L-band EPR spectroscopy. *Magn. Reson. Med.* **1997**, *38*, 565−568.

(41) Röntgen, W.C. Über eine neue Art von Strahlen. *Würzberger Phys.-Med. Ges.* **1895**, *9*, 132−141.

(42) Grubbé, E. H. Priority in the therapeutic use of X-rays. *Radiology* **1933**, *21*, 156−162.

(43) Weiss, J. Radiochemistry of aqueous solutions. *Nature* **1944**, *153*, 748−750.

(44) (a) Furchgott, R. F. Studies on relaxation of rabbit aorta by sodium nitrite: The basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In *Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium*; Vanhoutte, P. M., Ed.; Raven Press: New York, 1988; pp 401−414. (b) Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265−9269. (c) Palmer, R. M. J.; Ferrige, A. G.; Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **1987**, *327*, 524−526.

(45) (a) Huang, P. L.; Dawson, T. M.; Bredt, D. S.; Snyder, S. H.; Fishman, M. C. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* **1993**, *75*, 1273−1286. (b) Huang, P. L.; Huang, Z.; Mashimo, H.; Bloch, K. D.; Moskowitz, M. A.; Bevan, J. A.; Fishman, M. C. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* **1995**, *377*, 239−242. (c) Huang, Z.; Huang, P. L.; Panahian, N.; Dalkara, T.; Fishman, M. C.; Moskowitz, M. A. Effects of cerebral ischemia in mice deficient in neuron nitric oxide synthase. *Science* **1994**, *265*, 1883−1885. (d) Lander, H. M. An essential role for free radicals and derived species in signal transduction. *FASEB J.* **1997**, *11*, 118−124.

(46) Murugesan, R.; Cook, J. A.; Devasahayam, N.; Afeworki, M.; Subramanian, S.; Tschudin, R.; Larsen, J. H. A.; Mitchell, J. B.; Russo, A.; Krishna, M. C. In vivo imaging of a stable paramagnetic probe by pulsed-radio frequency electron paramagnetic resonance spectroscopy. *Magn. Res. Med.* **1997**, *38*, 409−414.

AR970251L