

# Issues Pertinent to the *in Vivo* in Situ Spin Trapping of Free Radicals<sup>§</sup>

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## Introduction

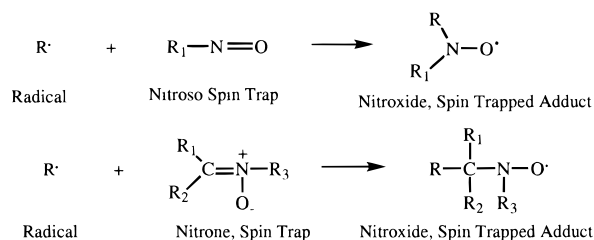
In 1954, it was proposed that free radicals were the toxic intermediate associated with oxygen poisoning and ionizing radiation.<sup>1</sup> Surprisingly, this hypothesis and its implication to aerobic organisms seemed remote to the scientists of that time, as more than a decade would elapse before biological sources of superoxide would be linked to the discovery of an enzyme—superoxide dismutase—designed to eliminate this free radical.<sup>2</sup> Now as we approach the 50th anniversary of the Gerschman<sup>1</sup> 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.<sup>3</sup>

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<sup>4</sup> 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.<sup>5</sup> 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.<sup>5</sup> Despite the nearly decade delay in adapting this technique to biological systems<sup>6</sup> and the enormous literature devoted thereafter, the birth of spin trapping can be dated from these early investigations.<sup>5,7</sup>



Considering the importance of superoxide dismutase and catalase to life in an aerobic world,<sup>8</sup> it is not surprising that superoxide and hydroxyl radical were the first free radicals of biological derivation to be spin trapped.<sup>9</sup> 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.<sup>10</sup> Under generic conditions, this absorption or loss of electromagnetic energy creates an exponential loss of signal as a function

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<sup>§</sup> 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.

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.<sup>11</sup>

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.<sup>10,12</sup> 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.<sup>13</sup>

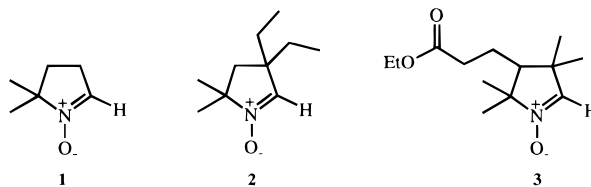
The sensitivity or signal-to-noise ratio (S/N) decreases as the frequency diminishes, the extent of which is controversial.<sup>14</sup> It is commonly held that the sensitivity is proportional to the square of the frequency. Certainly, the signal can be thought of as having this dependence.<sup>15</sup> 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.<sup>14b,15,16</sup> 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.

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

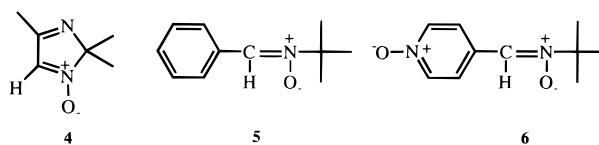
One approach to balance the loss of sensitivity at lower frequencies is to use a loop-gap resonator. In its simplest form the loop-gap resonator is simply an inductive–capacitive (L–C) circuit with the inductive element serving as the sample holder. Loop-gap resonators with their high power density and excellent filling factors are extraordinarily useful at high frequencies.<sup>17</sup> At low frequencies lumped circuit devices such as the loop-gap resonator are required.<sup>18</sup> 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.<sup>19</sup>

**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.<sup>20</sup> In contrast, such dynamic studies are considerably more complicated in isolated organ preparations and in vivo.<sup>21</sup>

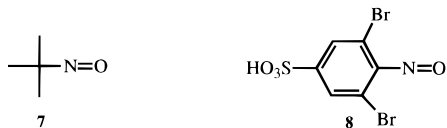
Several classes of spin traps have been developed to react with specific free radicals. For instance, cyclic nitrones such as 5,5-dimethyl-1-pyrroline *N*-oxide (**1**) can spin trap superoxide, hydroxyl, and small carbon-centered free radicals, giving spin-trapped adducts with defined EPR spectra.<sup>9c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



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

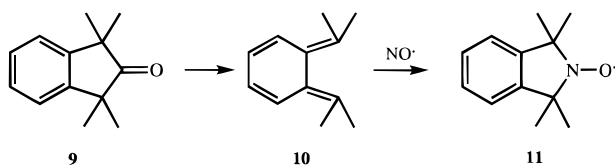


Finally, poor stability of spin-trapped adducts deduced from the spin trapping of oxygen-centered free radicals with nitrosoalkanes, 2-methyl-2-nitrosopropane (**7**) and 3,5-dibromo-4-nitrosobenzenesulfonate (**8**), limits the use of these spin traps to carbon-centered free radicals.<sup>26</sup>

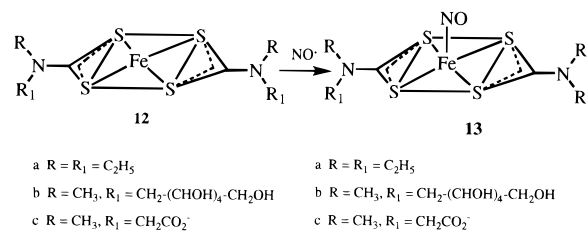


The physiological importance of nitric oxide has spurred the synthesis of unique spin traps, some of which have been used to identify this free radical in animal models in real time. Unlike superoxide and hydroxyl radical, the unexpectedly long lifetime of nitric oxide, an essential prerequisite for 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,<sup>27</sup> 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<sup>•</sup>), nitrosyl radical (NO<sup>•</sup>), and dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>).<sup>27c</sup>

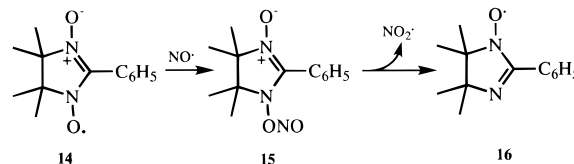
One of the most innovative schemes for spin trapping nitric oxide draws upon the ease with which “activated” cis-conjugated dienes react with free radicals.<sup>28</sup> Typical of this new class of spin traps is 7,7,8,8-tetramethyl-*o*-quinodimethane (**10**), which is generated upon photolysis of 1,1,3,3-tetramethyl-2-indanone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



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.<sup>29</sup> One of the better defined complexes is iron(II) diethyldithiocarbamate (Fe<sup>2+</sup>(DETC)<sub>2</sub>, **12a**).<sup>29b</sup> Its reaction with nitric oxide is characterized by the low-temperature EPR spectrum at  $g_{\perp} = 2.035$  and  $g_{\parallel} = 2.02$  with a triplet hyperfine structure at  $g_{\perp}$ .<sup>29b</sup> 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<sup>2+</sup>(MGD)<sub>2</sub> (**12b**) or Fe<sup>2+</sup>(DTCS)<sub>2</sub> (**12c**), have fared better, as in vivo in situ EPR spectra corresponding to NO-Fe<sup>2+</sup>(MGD)<sub>2</sub> (**13b**) and NO-Fe<sup>2+</sup>(DTCS)<sub>2</sub> (**13c**) have been recorded in real time.<sup>19,30</sup>



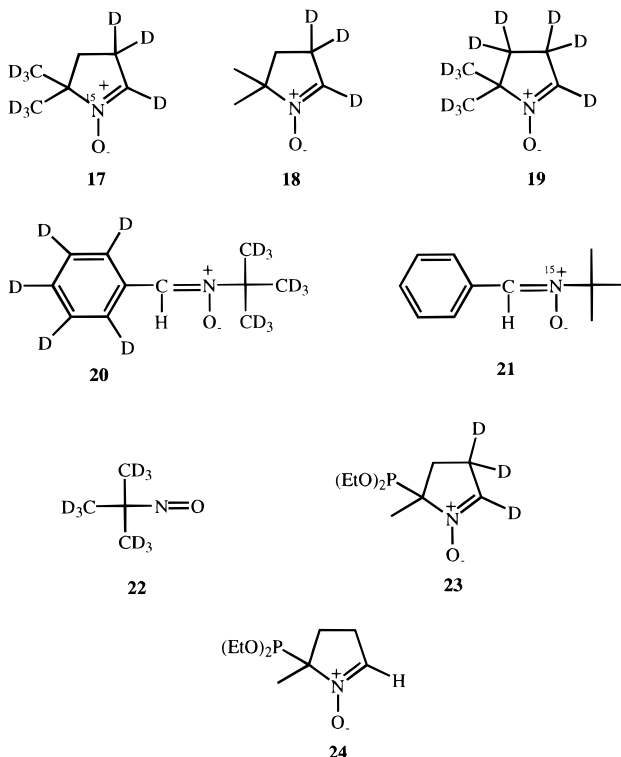
Nitronyl nitroxides such as **14** have been explored as spin traps for nitric oxide.<sup>31</sup> Here, reaction with this free radical results in the loss of NO<sup>•</sup> and the formation of an imino nitroxide **16**, whose EPR spectrum is distinguishable from that of the parent nitroxide **14**.<sup>31</sup>



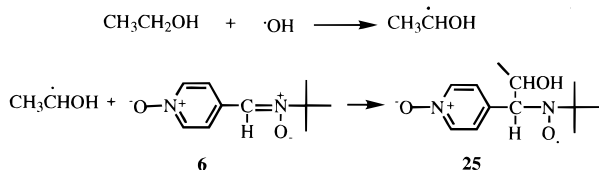
**In Vivo Spin Trapping in Real Time. Limitations to Overcome.** A loss of sensitivity occurs when the operating frequency decreases from 9.5 GHz to 250 MHz. One approach<sup>32</sup> to compensate for this diminution in signal intensity is to synthesize<sup>22b,33</sup> spin traps with <sup>2</sup>H and <sup>15</sup>N in place of <sup>1</sup>H and <sup>14</sup>N. Various ring and substituent 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 <sup>14</sup>N with <sup>15</sup>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.<sup>33a,34</sup> A number of isotope-labeled spin traps have been synthesized, including <sup>15</sup>N-containing and deuterated 5,5-dimethyl-1-pyrroline *N*-oxide<sup>33a</sup> (**17**), trideuterated 5,5-dimethyl-1-pyrroline *N*-oxide<sup>33a</sup> (**18**), perdeuterated 5,5-dimethyl-1-pyrroline *N*-oxide<sup>33a</sup> (**19**), deuterated *N-tert*-butyl- $\alpha$ -phenylnitronyl<sup>33b</sup> (**20**), <sup>15</sup>N-containing *N-tert*-butyl- $\alpha$ -phenylnitronyl<sup>33d</sup> (**21**), perdeuterated 2-methyl-2-nitrosopropane<sup>33e</sup> (**22**), and deuterated 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide<sup>33c</sup> (**23**). The significance<sup>34</sup> of this enhanced sensitivity can be seen by low-frequency EPR images from spin-trapped adducts of nitronyl **19**, whereas such images were not observed with nitronyl **1**.

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



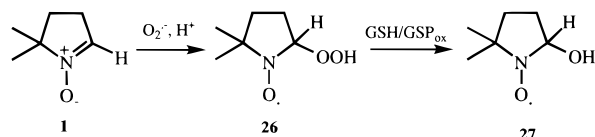


From a kinetic standpoint, the spin trapping of hydroxyl radical offers an opportunity to in vivo in situ detect this free radical, as it reacts with both cyclic and acyclic nitrones at near diffusion-controlled rates.<sup>24a</sup> Yet poor stability of the corresponding spin-trapped adducts has greatly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



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.<sup>20b,38</sup> Here, the corresponding nitroxide **26**, a substrate for glutathione peroxidase (GSP<sub>ox</sub>),<sup>38</sup> is rapidly converted to 2-hydroxy-2,2-dimethyl-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.<sup>22a,b</sup> The most successful of these spin traps, nitron **3**, is resistant to a variety of biological oxidative reactions that would otherwise result in artifactually derived nitroxides,<sup>22b</sup> as is seen with nitron **1**. Thus, nitron **3** has the potential to distinguish between intracellularly produced superoxide and hydroxyl radical and may find a niche in the in vivo characterization of hydroxyl radical.



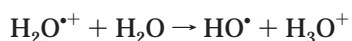
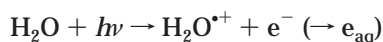
Localization of the spin trap at the site of free radical evolution is one of the most difficult and daunting tasks that awaits attempts to identify the reactive intermediate in isolated organ preparations and in animal models. Consider, for example, the in vivo in situ spin trapping of radiation-induced hydroxyl radical in a mouse in real time.<sup>21b</sup> The experimental design, based on in vitro kinetic and stability studies,<sup>25c</sup> included the introduction of EtOH and nitron **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<sup>25c</sup> characteristic of nitroxide **25**, it was assumed that nitron **6** and EtOH were compartmentalized within the same tissue so that radiation-induced  $\alpha$ -hydroxyethyl radical could react with acyclic nitron **6**.<sup>21b</sup>

Even though EtOH can diffuse into many sites within a tissue, nitron **6**, which has a small 1-octanol/water partition coefficient of  $\sim 0.09$ , does not passively enter cells.<sup>25c</sup> Thus, the detection of hydroxyl radical as nitroxide **25** was solely limited to interstitial and vascular spaces. Poor cellular uptake of nitron **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 intracellularly, they would not be discernible with nitron **6** and EtOH, even though  $\gamma$ -irradiation of water within cells has long been known to produce hydroxyl radical at sensitive loci.<sup>39</sup>

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.<sup>19,30,40</sup> 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.<sup>41</sup> Within a month of this publication, physicians in Chicago<sup>42</sup> 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.<sup>43</sup>



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.<sup>21b</sup>

In 1980, Furchgott and Zawadzki<sup>44</sup> 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<sup>3a</sup> 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.<sup>44</sup> 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.<sup>45</sup> Only recently with the development of in vivo stable ferrocenyls, 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.<sup>22b,30,40</sup>

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  $\gamma$ -irradiation<sup>21b</sup> and cytokine levels<sup>22b,30,40</sup> 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<sup>46</sup> operating at 300 MHz, hold great promise for rapid data acquisition, an essential element in improvement of EPR spectroscopic sensitivity.

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