[1] Electron Paramagnetic Resonance for Quantitation of Nitric Oxide in Aqueous Solutions

By SUJATHA VENKATARAMAN, SEAN M. MARTIN, and GARRY R. BUETTNER

Introduction

Nitric oxide ($^{\bullet}NO$) has many complex and diverse biological functions.[1-3] For example, it functions as an endothelium-derived relaxing factor (EDRF), a vascular antioxidant, and as a messenger molecule in the cardiovascular and immune systems. Nitric oxide also plays important roles in the biochemical aspects of a plethora of disorders. A molecule of such significance needs reliable methods for its detection and quantitation so that detailed mechanistic studies are possible.

Because $^{\bullet}NO$ is a reactive free radical and oxidizes rapidly to nitrite and nitrate, the quantitation of $^{\bullet}NO$ in an aqueous solution is challenging. There are a number of methods for the detection of nitric oxide,[4-8] but due to lack of specificity and sensitivity, only a few are able to provide actual absolute quantitation of $^{\bullet}NO$.[9-11] The choice of the method depends on the objective of the analysis: whether it is qualitative or quantitative.

Of the methods proposed so far, electron paramagnetic resonance (EPR) appears to be very useful and reliable. EPR has been used extensively to unravel the role of $^{\bullet}NO$ in biological systems.[12,13] Despite $^{\bullet}NO$ being a paramagnetic compound, it cannot be detected directly using EPR. However, diamagnetic compounds that form stable paramagnetic adducts with $^{\bullet}NO$ can be used to detect $^{\bullet}NO$ by EPR. The high affinity of $^{\bullet}NO$ for certain classes of Fe^{2+} complexes plays an

[1] S. Moncada and A. Higgs, *N. Engl. J. Med.* **329,** 2002 (1993).
[2] S. Moncada, R. M. J. Palmer, and E. A. Higgs, *Pharmacol. Rev.* **43,** 109 (1991).
[3] Q. Feng and T. Hedner, *Clin. Physiol.* **10,** 407 (1990).
[4] T. Malinski and L. Czuchajowski, *in* "Methods in Nitric Oxide Research" (M. Feelisch and J. S. Stamler, eds.), p. 319. Wiley, New York, 1996.
[5] J. F. Leikert, T. R. Rathel, C. Muller, A. M. Vollmar, and V. M. Dirsch, *FEBS Lett.* **506,** 131 (2001).
[6] D. M. Hall and G. R. Buettner, *Methods Enzymol.* **268,** 188 (1996).
[7] A. F. Vanin, *Methods Enzymol.* **301,** 269 (1999).
[8] B. Kalyanaraman, *Methods Enzymol.* **268,** 168 (1996).
[9] R. W. Nims, J. C. Cook, M. C. Krishna, D. Christodoulou, C. M. B. Poore, A. M. Miles, M. B. Grisham, and D. A. Wink, *Methods Enzymol.* **268,** 93 (1996).
[10] S. Venkataraman, S. M. Martin, F. Q. Schafer, and G. R. Buettner, *Free Radic. Biol. Med.* **29,** 580 (2000).
[11] K. Tsuchiya, M. Takasugi, K. Minakuchi, and K. Fukuzawa, *Free Radic. Biol. Med.* **21,** 733 (1996).
[12] Y. Henry, C. Ducrocq, J.-C. Drapier, D. Servent, C. Pellat, and A. Guissani, *Eur. Biophys. J.* **20,** 1 (1991).
[13] R. F. Lin, T.-S. Lin, R. G. Tilton, and A. H. Cross, *J. Exp. Med.* **178,** 643 (1993).

0076-6879/02 $35.00

important role in biological processes. The most frequently used traps for ·NO also take advantage of this property. For example, ·NO reacts with Fe^{2+} hemoglobin and other ferrous hemes, as well as Fe^{2+}(dithiocarbamate)$_2$ complexes forming stable paramagnetic species. These complexes have been widely used in the study of the chemical and biological aspects of ·NO. The use of iron complexes to detect ·NO in animal studies was first reported by Vanin *et al.*[14]

A new series of paramagnetic compounds, nitronyl nitroxides (NNR), has been used to detect ·NO. Although the chemistry of these compounds with ·NO is quite different from that of iron complexes, these compounds have good specificity and can be used to quantitate ·NO in aqueous solutions using EPR. Extensive studies have been reported on the use of nitronyl nitroxides as a probe for vasodilation and as a ·NO antagonist in perfused organs.[15,16] Nitronyl nitroxides have also been used in other areas of biology to study the antimicrobial action of ·NO.[17]

This article presents methods using Fe^{2+}(MGD)$_2$ (MGD, *N*-methyl-D-glucamine dithiocarbamate) and nitronyl nitroxides to quantitate ·NO in aqueous solution by EPR. Our goal is to provide methods that will direct the chemistry involved to obtain reliable and accurate results.

·NO Chemistry and Detection Strategy

It is always important to understand the chemistry behind methods of quantitation. Nitric oxide has a relatively short half-life,[18] it reacts spontaneously with dioxygen in the gas phase with a stoichiometry of 2 : 1 [Eq. (1)] and 4 : 1 in water [Eq. (2)].[10,19]

$$2\,{\cdot}NO + O_2 \rightarrow 2\,{\cdot}NO_2 \qquad \text{(gas phase)} \qquad (1)$$

$$4\,{\cdot}NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \qquad \text{(aqueous phase)} \qquad (2)$$

These reactions are the basis for several methods of detection and quantitation of ·NO, including the estimation of [·NO] using an oxygen monitor,[10] spectrophotometric methods such as UV-Vis,[9] or fluorescence[20] analysis of nitrite using various substrates.

[14] A. F. Vanin, P. I. Mordvintcev, and A. L. Kleschyov, *Stud. Biophys.* **102,** 135 (1984).

[15] A. Konorev, M. M. Tarpey, J. Joseph, J. E. Baker, and B. Kalyanaraman, *Free Radic. Biol. Med.* **18,** 169 (1995).

[16] E. A. Konorev, M. M. Tarpey, J. Joseph, J. E. Baker, and B. Kalyanaraman, *J. Pharmacol. Exp. Ther.* **274,** 200 (1995).

[17] K. Yoshida, T. Akaiki, T. Doi, K. Sato, S. Uiri, M. Suga, M. Ando, and H. Maeda, *Infect. Immunol.* **61,** 3552 (1993).

[18] L. J. Ignarro, *Annu. Rev. Pharmacol. Toxicol.* **30,** 535 (1990).

[19] P. C. Ford, D. A. Wink, and D. M. Stanbury, *FEBS Lett.* **326,** 1 (1993).

[20] A. M. Miles, D. A. Wink, J. C. Cook, and M. B. Grisham, *Methods Enzymol.* **268,** 105 (1996).

Nitrite is also measured based on the Griess reaction.[21] In this diazotization method, nitrite is reacted with Griess reagent to form a colored azo product that is measured spectrophotometrically.[22,23]

A chemiluminescent method of detection of gas-phase $^{\bullet}NO$ uses its reaction with ozone. In the gas phase, O_3 reacts with $^{\bullet}NO$ according to Eqs. (3) and (4):

$$^{\bullet}NO + O_3 \rightarrow {}^{\bullet}NO_2^* + O_2 \quad (3)$$

$$^{\bullet}NO_2^* \rightarrow {}^{\bullet}NO_2 + h\nu \quad (4)$$

where $^{\bullet}NO_2^*$ denotes the $^{\bullet}NO_2$ molecule in the excited state and $h\nu$ represents an emitted photon. Chemiluminescence resulting from these reactions provides a basis for the Sievers NOA detection system for $^{\bullet}NO$.[24] This method is ideal for anaerobic $^{\bullet}NO$ solutions and is highly sensitive, but can be time-consuming and expensive for occasional quantitative analysis.

Nitric oxide reacts spontaneously with heme proteins. This forms the basis of the spectrophotometric method that measures the amount of metHb formed from the reaction of $^{\bullet}NO$ with oxyhemoglobin.[25,26] Also, $^{\bullet}NO$ reacts with deoxyhemoglobin and forms a stable nitrosylated adduct, which is measured using EPR.[27,28]

Nitric oxide is also measured by other techniques, such as electrochemical detection[4] and other spectroscopic methods based on its physical and chemical properties.

EPR spectroscopy continues to play a significant role in the evolution of our understanding of $^{\bullet}NO$ biology. EPR faithfully reproduces the spectral features of the indicator molecule, revealing the identity of the $^{\bullet}NO$ complexes formed, a result not achieved by other methods. Because $^{\bullet}NO$ is a paramagnetic compound, it should be detectable by EPR. However, the quantum mechanical properties of diatomic free radicals (fast spin-orbit relaxation resulting in very, very broad lines) render these radicals essentially EPR silent at room temperature. Therefore, a spin trap is needed that has high affinity for $^{\bullet}NO$ and also forms a stable paramagnetic complex. Classic spin traps such as 5,5-dimethyl-1-pyrroline N-oxide (DMPO) do not trap $^{\bullet}NO$, as $^{\bullet}NO$ has little tendency to form the covalent bond with non-radical molecules required by the typical spin-trapping reaction. However, $^{\bullet}NO$ rapidly forms relatively stable ligand bonds, forming nitrosylates, with certain

[21] J. P. Griess, *Phil. Trans. R. Soc. (Lond.)* **154,** 667 (1864).
[22] S. Archer, *FASEB. J.* **7,** 349 (1993).
[23] J. M. Hevel and M. A. Marletta, *Methods Enzymol.* **233,** 250 (1994).
[24] A. Fontijn, A. J. Sabadell, and R. J. Ronco, *Anal. Chem.* **42,** 575 (1970).
[25] M. E. Murphy and E. Noack, *Methods Enzymol.* **233,** 240 (1994).
[26] M. Feelisch, D. Kubitzek, and J. Werringloer, *in* "Methods in Nitric Oxide Research" (M. Feelisch and J. S. Stamler, eds.), p. 455. Wiley, New York, 1996.
[27] A. Wennmalm, B. Lanne, and A.-S. Petersson, *Anal. Biochem.* **187,** 359 (1990).
[28] Q. Wang, J. Jacobs, J. DeLeo, H. Kruszyna, R. Smith, and D. Wilcox, *Life Sci.* **49,** PL55 (1991).

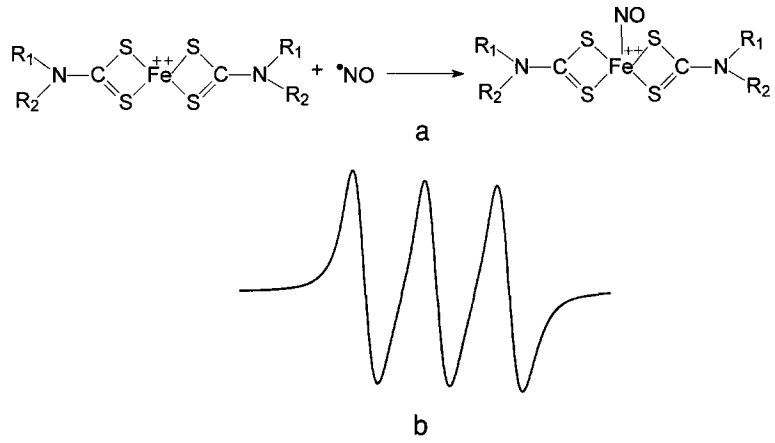

FIG. 1. (a) The trapping reaction of $^{\bullet}NO$ by $Fe^{2+}(MGD)_2$ [$R_1 = CH_3$ and $R_2 = CH_2(CHOH)_4CH_2OH$] or $Fe^{2+}(DETC)_2$, [$R_1 = R_2 = C_2H_5$]. (b) Representative room temperature EPR spectrum for NO-$Fe^{2+}(MGD)_2$ ($a^N = 12.9$ G; $g_{iso} = 2.04$) or for NO-$Fe^{2+}(DETC)_2$ ($a^N = 12.7$ G; $g_{iso} = 2.04$).

iron complexes. Stable iron(II) complexes of $^{\bullet}NO$ yield characteristic EPR spectra. Without $^{\bullet}NO$ as a ligand, these ferrous complexes are usually EPR inactive. Example complexes are the ferrous dithiocarbamate products NO-$Fe^{2+}(DETC)_2$ and NO-$Fe^{2+}(MGD)_2$, which give EPR spectra that can be used to identify and quantitate $^{\bullet}NO$.

Nitronyl nitroxides are also used to quantitate $^{\bullet}NO$ with EPR. These are organic compounds that have both nitrone, $\gtrless N^{+}—O^{-}$ (a functional group corresponding to a spin trap), and nitroxide, $>N—O^{\bullet}$ (a functional group corresponding to a spin label or spin adduct) moieties. Both the NNR and the product formed after its reaction with $^{\bullet}NO$ are stable nitroxides that have distinguishable EPR spectral features.

This article presents the chemistry behind $^{\bullet}NO$ and its reactions with ferrous dithiocarbamate complexes, $Fe^{2+}(DETC)_2$ and $Fe^{2+}(MGD)_2$ (Fig. 1), and nitronyl nitroxides (Fig. 2) used to detect $^{\bullet}NO$. Representative EPR spectra and their parameters are presented. Also, important rate constants related to $^{\bullet}NO$ quantitation are summarized in Table I.

Materials

The nitric oxide donor, DEANO [2-(*N,N*-diethylamino)diazenolate-2-oxide, sodium salt], and PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) are from Alexis Corporation (San Diego, CA). The spin label 3-CP (3-carboxy proxyl) is from Aldrich (Milwaukee, WI). Sodium nitrite, ferrous sulfate, glacial acetic acid, and potassium iodide are from Fisher Scientific (Pittsburgh, PA). The chelating resin is obtained from Sigma Chemical Co. (St. Louis, MO). All

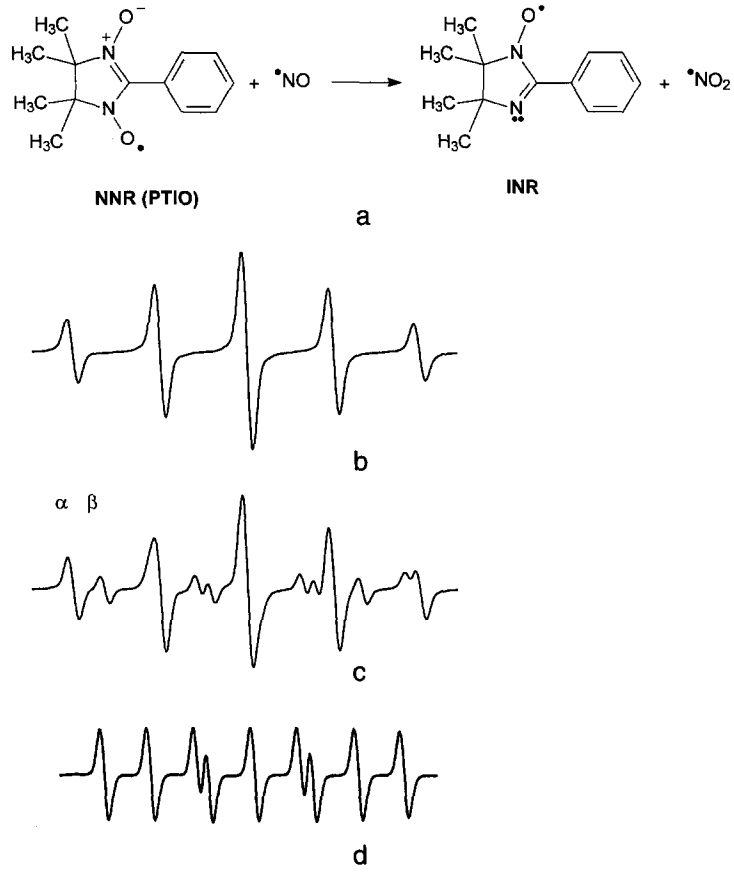

FIG. 2. (a) Reaction of $^{\bullet}NO$ with nitronyl nitroxide (NNR), (b) the ESR spectrum of NNR alone ($a^N = 8.1$ G), (c) mixture of NNR and imino nitroxide (INR) with α and β indicating the low field lines of NNR and INR, respectively, and (d) INR alone ($a_1^N = 4.4$ G and $a_2^N = 9.9$ G).

chemicals and reagents used are of analytical grade. High-purity gaseous nitric oxide is obtained from AGA Specialty Gas (Maumee, OH). Argon and nitrogen are from Air Products (Allentown, PA).

Methods

Phosphate-buffered saline (PBS) (pH 7.4) is prepared and stirred very gently overnight in the presence of the chelating resin to remove adventitious catalytic metals. The ascorbate test is used to verify their removal.[29]

[29] G. R. Buettner, *J. Biochem. Biophys. Methods* **16,** 27 (1988).

TABLE I
REACTIONS AND RATE CONSTANTS OF 'NO IN AQUEOUS SOLUTION[a]

Reaction	Rate constant	Ref.[b]
$^{\cdot}NO + {}^{\cdot}NO_2 \rightarrow N_2O_3$	$k = 1.1 \times 10^9\ M^{-1}\ s^{-1}$	(a)
$^{\cdot}NO_2 + {}^{\cdot}NO_2 \rightarrow N_2O_4$	$k = 4.5 \times 10^8\ M^{-1}\ s^{-1}$	(a)
$N_2O_4 \rightarrow {}^{\cdot}NO_2 + {}^{\cdot}NO_2$	$k = 6.9 \times 10^3\ s^{-1}$	(b)
$N_2O_3 \rightarrow {}^{\cdot}NO + {}^{\cdot}NO_2$	$k = 8.0 \times 10^4\ s^{-1}$	(b)
$N_2O_3 + H_2O \rightarrow 2HNO_2$	$k = 5.3 \times 10^2\ s^{-1}$	(b)
$N_2O_4 + H_2O \rightarrow HNO_2 + H^+ + NO_3^-$	$k = 1.0 \times 10^3\ s^{-1}$	(c)
$4{}^{\cdot}NO + O_2 + 2H_2O \rightarrow 4HNO_2$	$k = 1.58 \times 10^6\ M^{-2}\ s^{-1}$	(d)
$Fe^{2+}(MGD)_2 + O_2 \rightarrow Fe^{3+}(MGD)_2 + O_2^{\cdot -}$	$k = 5 \times 10^5\ M^{-1}\ s^{-1}$	(e)
$Fe^{2+}(MGD)_2 + {}^{\cdot}NO \rightarrow NO\text{-}Fe^{2+}(MGD)_2$	$k = 1.21 \times 10^6\ M^{-1}\ s^{-1}$	(f)
$Fe^{2+}(DETC)_2 + {}^{\cdot}NO \rightarrow NO\text{-}Fe^{2+}(DETC)_2$	$k \approx 10^8\ M^{-1}\ s^{-1}$	(g)
$Fe^{2+}(DTCS)_2 + {}^{\cdot}NO \rightarrow NO\text{-}Fe^{2+}(DTCS)_2$	$k = 1.71 \times 10^6\ M^{-1}\ s^{-1}$	(f)
$NNR + {}^{\cdot}NO \rightarrow INR + {}^{\cdot}NO_2$	$k \approx 10^4\ M^{-1}\ s^{-1}$	(h)
$^{\cdot}NO + O_2^{\cdot -} \rightarrow O{=}NOO^-$	$k \approx 6.7 \times 10^9\ M^{-1}\ s^{-1}$	(i)

[a] MGD, *N*-Methyl-D-glucamine dithiocarbamate; DETC, diethyl dithiocarbamate; DTCS, *N*-(dithiocarboxy)sarcosine; NNR, nitronyl nitroxide; INR, imino nitroxide.

[b] Key to references: (a) M. P. Doyle and J. W. Hoeksta, *J. Inorg. Biochem* **14,** 351 (1981). (b) M. Grätzel, J. Henglein, J. Lillie, and G. Beck, *Ber. Bunseges. Phys. Chem.* **73,** 646 (1969). (c) P. C. Ford, D. A. Wink, and D. M. Standbury, *FEBS Lett.* **326,** 1 (1993). (d) D. A. Wink, J. F. Darbyshire, R. W. Nims, J. E. Saavedra, and P. C. Ford, *Chem. Res. Toxicol.* **6,** 23 (1993). (e) K. Tshuchiya, J. J. Jiang, M. Yoshizumi, T. Tamaki, H. Houchi, K. Minakuchi, K. Fukuzawa, and R. P. Mason, *Free Radic. Biol. Med.* **27,** 367 (1999). (f) S. Pou, P. Tsai, S. Porusuphatana, H. J. Halpern, G. V. R. Chandramouli, E. D. Barth, and G. M. Rosen, *Biochim. Biophys. Acta* **1427,** 216 (1999). (g) A. F. Vanin, *Methods Enzymol.* **301,** 269 (1999). (h) T. Akaike, M. Yoshida, Y. Miyamato, K. Sato, M. Kohno, K. Sasamoto, K. Miyazaki, S. Ueda, and H. Maeda, *Biochemistry* **32,** 827 (1993); Y. Y. Woldman, V. V. Khramtsov, I. A. Grigor'ev, I. A. Kiriljuk, and D. I. Utepbergenov, *Biochem. Biophys. Res. Commun.* **202,** 195 (1994). (i) R. E. Huie and S. Padmaja, *Free Radic. Res. Commun.* **18,** 195 (1993).

Nitric Oxide Stock Solutions

Nitric oxide gas is either obtained from a nitric oxide gas tank or prepared from an acidified sodium nitrite solution.[30,10] Because the nitric oxide from either source can be contaminated with other oxides of nitrogen, it is purified by passing it through NaOH (4 *M*) and then deionized (DI) water. The purified 'NO gas is then bubbled through a gas sampling bottle containing degassed DI water and stored. DEANO is used as a source of 'NO for some experiments. DEANO has a half-life of about 2 min in PBS, pH 7.4, at 37°, ideally, releasing two molecules of 'NO

[30] G. Brauer (ed.), "Handbook of Preparative Inorganic Chemistry," 2nd Ed., p. 486. Academic Press, New York, 1963.

and one molecule of diethylamine[31] (under our conditions 1.5 molecules of $^{\bullet}NO$ per one molecule of diethylamine).

Aliquots of the $^{\bullet}NO$ stock solutions are delivered to the various assay systems using argon-flushed, gas-tight microliter syringes (Hamilton Co., Reno, NV). Prior to filling the syringes with the $^{\bullet}NO$ stock solution, an equal volume of argon is injected into the bottle containing $^{\bullet}NO$ solution to minimize contamination with oxygen. It should be kept in mind that the container to which the $^{\bullet}NO$ stock solution is added should have a small surface-to-volume ratio so that the amount of $^{\bullet}NO$ escaping to the headspace is minimized.

Preparation of NO-Fe^{2+}(Dithiocarbamate)$_2$ Complexes

Diethyldithiocarbamate (DETC) is water soluble, but when complexed with Fe^{2+}, the $Fe^{2+}(DETC)_2$ formed is insoluble. To overcome the solubility problem of $Fe^{2+}(DETC)_2$, Mordvintcev *et al.*[32] incubated the complex with yeast membranes, thereby making a usable suspension. For the same reason, Tsuchiya *et al.*[33] used porcine serum albumin (PSA) to carry $Fe^{2+}(DETC)_2$ into aqueous solutions. Once in solution, oxygen should be minimized to prevent any side reactions with $^{\bullet}NO$. $^{\bullet}NO$ can then be added to these solutions. It is worth mentioning that if the physical arrangement of the EPR cavity allows the flat cell to be placed horizontally, potential sedimentation problems can be avoided.[34]

MGD, a derivative of DETC, is synthesized as described previously.[35] All solutions are prepared with argon-purged DI water. Stock solutions of $Fe^{2+}(MGD)_2$ are prepared by dissolving MGD sodium salt and ferrous sulfate in DI water, with a molar ratio of 5 : 1, respectively. The NO-$Fe^{2+}(MGD)_2$ samples are prepared by first pipetting different amounts of $Fe^{2+}(MGD)_2$ stock solution (50–400 μl) into a 10-ml test tube kept under argon. To this solution, the desired volume of $^{\bullet}NO$ stock solution (to achieve a specific final [$^{\bullet}NO$]) is added using gas-tight syringes, resulting in the formation of NO-$Fe^{2+}(MGD)_2$. The concentration of $Fe^{2+}(MGD)_2$ is always in three- to fivefold excess of the [$^{\bullet}NO$] to ensure that all the $^{\bullet}NO$ is trapped. The NO-$Fe^{2+}(MGD)_2$ samples, once formed, are transferred quickly to an argon-filled flat cell to avoid oxidation of the complex, and spectra are recorded within 5–10 min.

3-Carboxy proxyl is used as an additional standard to verify the concentrations of NO-$Fe^{2+}(MGD)_2$ standards,[10] after correcting for the difference in their g values using the factor g(3-CP)/g (NO-$Fe^{2+}(MGD)_2$) = 2.006/2.04.[36,37]

[31] L. K. Keefer, R. W. Nims, K. M. Davies, and D. A. Wink, *Methods Enzymol.* **268,** 281 (1996).
[32] P. Mordvintcev, A. Mulsch, R. Busse, and A. Vanin, *Anal. Biochem.* **199,** 142 (1991).
[33] K. Tsuchiya, M. Takasugi, K. Minakuchi, and K. Fukuzawa, *Free Radic. Biol. Med.* **21,** 733 (1996).
[34] Y. Kotake, L. A. Reinke, T. Tanigawa, and H. Koshida, *Free Radic. Biol. Med.* **17,** 215 (1995).
[35] L. A. Shinobu, S. G. Jones, and M. M. Jones, *Acta Pharmacol. Toxicol.* **54,** 189 (1984).
[36] J. A. Weil, J. R. Bolton, and J. E. Wertz, "Electron Paramagnetic Resonance: Elementary Theory, and Practical Applications," p. 498. Wiley-Interscience, New York, 1994.
[37] S. S. Eaton and G. R. Eaton, *Bull. Magn. Reson.* **1,** 130 (1980).

Preparation of INR Compound

In experiments in which PTIO is used to quantitate •NO, DEANO is used as the source of •NO. The concentration of PTIO used is always at least three times of that needed to capture all the •NO. A known concentration of PTIO is aliquoted into a 2-ml gas-tight, screw-capped vial containing PBS and is deoxygenated with N_2. A known amount of deoxygenated DEANO solution is injected into the vial to release •NO. The sample is then incubated for 20 min at 37°. This solution is transferred to the flat cell and EPR spectra are recorded. Spectra are recorded over time to ensure the completion of the reaction and to verify the stability of the product.

Measurements

Optical Aspects

All UV-Vis spectrometric measurements are done at room temperature using an HP 8453 diode-array spectrophotometer (Hewlett-Packard, Wilmington, DE.). The PTIO solutions are standardized using $\varepsilon_{560} = 1020 \pm 50\, M^{-1}\text{cm}^{-1}$ in water. The optical parameters of other compounds used in this study are 3-CP ($\varepsilon_{234} = 2370\, M^{-1}\text{cm}^{-1}$)[10] in water and DEANO ($\varepsilon_{250} = 6500\, M^{-1}\text{cm}^{-1}$)[31] in dilute alkali.

Aqueous nitric oxide solutions are standardized using a Sievers 280 nitric oxide analyzer (NOA) as described.[10]

EPR Spectral Aspects

The first derivative EPR spectra are obtained using a Bruker EMX spectrometer (X-band) at room temperature. Typical instrument settings are 9.75 GHz microwave frequency; 100 kHz modulation frequency; 10 mW, a nonsaturating microwave power,[38] 1 G modulation amplitude; 3418 G center field for NO-$Fe^{2+}(MGD)_2$, 3475 G for 3-CP; 100 G/84 sec scan rate; and 82 ms time constant. Settings for PTIO-•NO experiments are 0.5 G modulation amplitude; 3480 G center field; 50 G/168 sec scan rate; and a time constant of 164 ms.

Computer Aspects

The computer simulation of EPR spectra is done using the program PEST Winsim and LMB-EPR.[39,40] Deconvolution and double integration of peaks are performed using WINEPR (Bruker) software.

[38] G. R. Buettner and K. P. Kiminyo, *J. Biochem. Biophys. Methods* **24,** 147 (1992).
[39] epr.niehs.nih.gov
[40] D. R. Duling, *J. Magn Reson. B* **104,** 105 (1994).

Results

Comparison of Various Available Methods

Quantitation of 'NO with $Fe^{2+}(DETC)_2$. Mordvitcev *et al.*[32] were among the first to use iron dithiocarbamate complexes to quantitate 'NO by EPR. They used DETC with ferrous iron to trap 'NO. A detailed method to synthesize $Fe^{2+}(DETC)_2$ complex with 'NO is given by Vanin *et al.*[41] The complex NO-$Fe^{2+}(DETC)_2$ gives a three-line EPR spectrum at room temperature ($g_{iso} = 2.04$; $a^N = 12.7$ G) (Fig. 1).

The actual quantitation of 'NO, as NO-$Fe^{2+}(DETC)_2$, is accomplished using $Fe^{2+}(NO)(S_2O_3)_2$ as an EPR standard.[32] Although $Fe^{2+}(DETC)_2$ has had many successes as a tool to study nitric oxide chemistry and biology, its solubility in aqueous solution poses problems. This issue has limited the use of DETC and spurred the development and use of $Fe^{2+}(MGD)_2$ as a tool.

Quantiation of 'NO with $Fe^{2+}(MGD)_2$. MGD is a derivative of DETC introduced by Komarov *et al.*[42] for the detection of 'NO to overcome the solubility problems with $Fe^{2+}(DETC)_2$. It is readily soluble in water and forms a water-soluble NO-$Fe^{2+}(MGD)_2$ complex. It has an EPR spectrum very similar to that of NO-$Fe^{2+}(DETC)_2$, a three-line spectrum with $g_{iso} = 2.04$, $a^N = 12.9$ G in aqueous solution (Fig. 1).

Quantitation of 'NO using $Fe^{2+}(MGD)_2$ is done at room temperature under anaerobic conditions. The concentration of 'NO is determined by adding various amounts of 'NO stock solutions to solutions of $Fe^{2+}(MGD)_2$ in DI water and their EPR spectra are recorded. The concentration can be determined using peak intensity or by double integration. The concentration of this nitrosyl complex, NO-$Fe^{2+}(MGD)$, is obtained by double integration of the three lines and is calibrated against 3-CP standards. The concentration of 'NO solutions determined by EPR measurements correlates well (slope = 1.06) with those determined from the NOA (Fig. 3). The double integrated areas of EPR spectra for various concentrations of 'NO vary linearly with ['NO] (Fig. 4). Thus, standard curves such as this can be used to extrapolate the concentration of the unknown 'NO solutions as NO-$Fe^{2+}(MGD)_2$.

It has been shown that nitrite in the presence of $Fe^{2+}(MGD)_2$ could be a source of 'NO, which in turn would form NO-$Fe^{2+}(MGD)_2$.[43–45] Therefore, it is important to determine whether the presence of nitrite, as a contaminant, interferes with the estimation of 'NO. The $Fe^{2+}(MGD)_2 - NaNO_2$ solutions produce no detectable EPR signals with our instrument settings and experimental protocols until

[41] A. F. Vanin, P. I. Mordvintcev, and A. L. Kleshcev, *Stud. Biophys.* **102,** 135 (1984).
[42] A. Komarov, D. Mattson, and M. M. Jones, *Biochem. Biophys. Res. Commun.* **195,** 1191 (1993).
[43] A. Samouilov, P. Kuppusamy, and J. L. Zweier, *Arch. Biochem. Biophys.* **357,** 1 (1998).
[44] K. Tsuchiya, J. Jiang, M. Yoshizumi, T. Tamaki, H. Houchi, K. Minakuchi, K. Fukuzawa, and R. P. Mason, *Free Radic. Biol. Med.* **27,** 347 (1999).
[45] K. Hiramoto, S. Tomiyama, and K. Kikugawa, *Free Radic. Res.* **27,** 505 (1997).

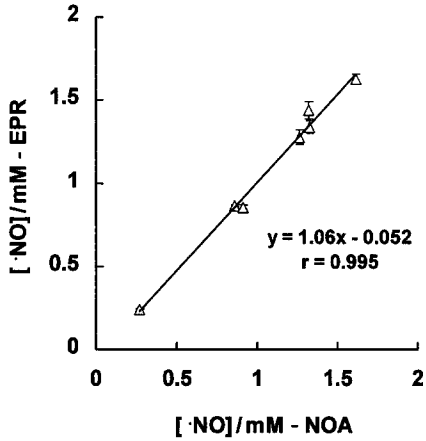

FIG. 3. Standardization of the EPR $Fe^{2+}(MGD)_2$ spin-trapping technique for the quantitation of ·NO with a NOA [from S. Venkataraman, S. M. Martin, F. Q. Schafer, and G. R. Buettner, *Free Radic. Biol. Med.* **29,** 580 (2000)]. Each data point represents the mean of three independent measurements. Error bars represent standard error.

$[NaNO_2] > 500\ \mu M$. EPR spectra corresponding to the NO-$Fe^{2+}(MGD)_2$ complex are detected only when the concentration of $NaNO_2$ is 1 mM or greater in the time frame of these measurements. However, with time the signal intensity increases. The signal intensity after 30 min corresponds to 110 μM of NO-$Fe^{2+}(MGD)_2$. This observation is consistent with the conversion of nitrite to ·NO in the presence

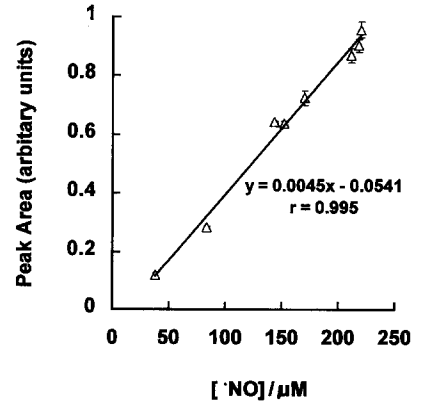

FIG. 4. Double integrated EPR area measurements of NO-$Fe^{2+}(MGD)_2$ spectra. [·NO] varied from 38 to 220 μM as determined by chemiluminescence measurement from a nitric oxide analyzer. The $Fe^{2+}(MGD)_2$ concentration was three times that of the respective ·NO concentration. Each data point represents $n = 3$. Error bars represent standard error.

of $Fe^{2+}(MGD)_2$.[46] In another control experiment, the $^{\bullet}NO$ stock solution is purged with argon for 30 min to ensure that the solution is free of $^{\bullet}NO$ and contains only nitrite, present as a contaminant. When this solution is added to the $Fe^{2+}(MGD)_2$ solution, no EPR signals are observed, even though the NOA reveals the presence of nitrite to be 150–250 μM. However, when the modulation amplitude and the time constant of the EPR spectrometer are increased, 1 to 3 G and 82 to 327 ms, respectively, the three-line NO-$Fe^{2+}(MGD)_2$ signal can be seen from solutions containing nitrite as low as 100 μM. Thus, the potential interference from nitrite must be considered. Use of reducing agents that will convert NO_2^- back to $^{\bullet}NO$ result in overestimating the amount of free $^{\bullet}NO$ in aqueous solution. Care should be taken to use these traps in anaerobic environments to avoid oxidation of Fe^{2+} to Fe^{3+}, resulting in loss of trap as well as subsequent production of superoxide (Table I). However, with appropriate protocols, $Fe^{2+}(MGD)_2$ can be used to quantitate $^{\bullet}NO$ stock solutions in the range of 500 nM to 1.9 mM (by peak height measurements or double integration).

Nitronyl Nitroxides (NNR) as Nitric Oxide Probe. Although EPR quantitation of $^{\bullet}NO$ by various Fe^{2+} complexes can be sensitive and reliable, their use is limited by the possible overestimation of $^{\bullet}NO$ in the presence of nitrite. An alternate approach is to use nitronyl nitroxides to quantitate $^{\bullet}NO$. Nitronyl nitroxides were first synthesized by Ulmann and co-workers[47] and are now available commercially. Since their discovery, this class of stable radicals has attracted considerable interest as probes for $^{\bullet}NO$. An example structure is provided in Fig. 2. These compounds are paramagnetic and give a characteristic five-line EPR spectrum. Nitronyl nitroxides react and form an unstable adduct with $^{\bullet}NO$ that rearranges to give an imino nitroxide (INR) and $^{\bullet}NO_2$.[48] Both nitronyl nitroxides and imino nitroxides are stable and have distinctly different EPR spectra, which forms the basis for their quantitation of $^{\bullet}NO$. The advantages of using these compounds are that they have a high efficiency of trapping $^{\bullet}NO$, most are crystalline and water soluble, and they do not react with nitrite or $^{\bullet}NO_2$.

The important aspect of using nitronyl nitroxide to quantitate $^{\bullet}NO$ is to find the exact stoichiometry of the reaction between NNR and $^{\bullet}NO$. Akaike and Maeda[49] first reported that the reaction between NNR and $^{\bullet}NO$ is in the ratio of 1 : 1. Because one of the products, $^{\bullet}NO_2$, has the propensity to react with $^{\bullet}NO$ spontaneously (Table I), the ratio of $^{\bullet}NO$ reacting with NNR was actually found to be close to 2 : 1[50]; 1 : 1 is observed only at low concentrations of $^{\bullet}NO$,[51] where the amount of

[46] K. Tsuchiya, M. Yoshizumi, H. Houchi, and R. P. Mason, *J. Biol. Chem.* **275,** 1551 (2000).
[47] E. F. Ullman, J. H. Osiecki, D. G. B. Boocock, and R. Darcy, *J. Am. Chem. Soc.* **94,** 7049 (1972).
[48] J. Joseph, B. Kalyanaraman, and J. S. Hyde, *Biochim. Biophys. Res. Commun.* **192,** 926 (1993).
[49] T. Akaike and H. Maeda, *Methods Enzymol.* **268,** 211 (1996).
[50] N. Hogg, R. J. Singh, J. Joseph, F. Neese, and B. Kalyanaraman, *Free Radic. Res.* **22,** 47 (1995).
[51] Y. Y. Woldman, V. V. Khramtsov, I. A. Grigor'ev, I. A. Kiriljuk, and D. I. Utepbergenov, *Biochem. Biophys. Res. Commun.* **202,** 195 (1994).

$^{\bullet}NO$ reacting with $^{\bullet}NO_2$ is negligible. These seemingly disparate observations on the stoichiometry of the reaction between $^{\bullet}NO$ and NNR leave the quantitation of $^{\bullet}NO$ in question. We examined this stoichiometry so that $^{\bullet}NO$ could be quantitated with confidence.

We found that as the concentration of $^{\bullet}NO$ increases, the ratio between $^{\bullet}NO$ and NNR gradually changes from 1 : 1 to 2 : 1. We present here a step-by-step method to determine $^{\bullet}NO$ concentrations using NNR.

The representative nitronyl nitroxide (NNR) used for this study is PTIO. The concentration of PTIO is determined spectrophotometrically ($\varepsilon_{560} = 1020 \pm 50\ M^{-}cm^{-1}$). Different amounts of DEANO at pH 7.4 are used as the source of $^{\bullet}NO$. The concentration of PTIO used is at least three times that of the "total" concentration of $^{\bullet}NO$. The EPR sample is prepared as described in the methods section.

Because the unpaired electron of PTIO interacts with two equivalent nitrogens, the EPR spectrum of PTIO consists of five lines with intensities 1 : 2 : 3 : 2 : 1.[52,53] The EPR spectrum of the product (INR) consists of nine lines with intensities 1 : 1 : 1 : 1 : 1 : 1 : 1 : 1 : 1, which is distinctly different from that of PTIO.

When the concentration of PTIO is in excess of $^{\bullet}NO$, then the resulting EPR spectrum is due to the spectrum of the unreacted NNR and the spectrum of the product, INR (Fig. 2). The low field line of both species (NNR and INR) is taken as the representative peak for the individual compounds as they have the least overlap. The low field lines, marked α (NNR) and β (INR) in Fig. 2(c), correspond to one-ninth of the sum of total intensities of all peaks for each species. In general, an easy way to estimate the concentrations is by measuring the EPR peak heights. However, in this case, NNR and INR have different line widths and line shapes so direct comparison of peak height measurements will not yield correct information on relative concentrations. Double integration of the individual peaks would usually allow determination of the concentration of these species. However, the two peaks overlap; this overlap in conjunction with their different line shapes provides a challenge. For exact quantitation the peaks have to be deconvoluted.

For this purpose, EPR spectra recorded at different concentrations of $^{\bullet}NO$ are simulated. The simulation of spectra gives the concentration of the two species by area calculation. The spectral parameters used to simulate the two species are for PTIO ($a_1^N = a_2^N = 8.2$ G) and INR ($a_1^N = 4.4$ G and $a_2^N = 9.9$ G). The line shape and line width and g variation are optimized, and the spectra are simulated with a correlation coefficient of 0.999. The ratio of the two species, [$^{\bullet}NO$]/[NNR], changes as a result of the addition of different amounts of $^{\bullet}NO$, showing varying stoichiometry (Fig. 5) obtained from simulation of spectra and double integration of low field lines. The stoichiometry of the reaction of $^{\bullet}NO$ with NNR is close to

[52] T. Akaike, M. Yoshida, Y. Miyamato, K. Sato, M. Kohno, K. Sasamoto, K. Miyazaki, S. Ueda, and H. Maeda, *Biochemistry* **32,** 827 (1993).

[53] J. S. Nadeau and D. G. B. Boocock, *Anal. Chem.* **49,** 1672 (1977).

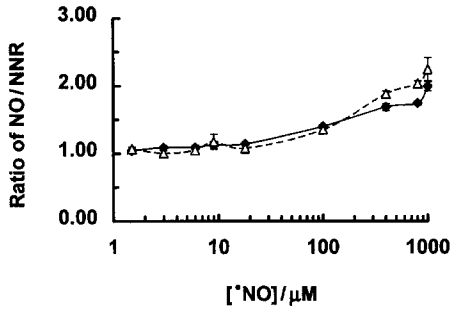

FIG. 5. The stoichiometry of •NO to NNR as determined by (Δ) simulation of experimental EPR spectra and (◆) double integration of deconvoluted low-field EPR lines of imino and nitronyl nitroxides. EPR spectra were obtained by adding different concentrations of DEANO to an anaerobic PBS (pH 7.4) solution containing nitronyl nitroxide [PTIO] and incubating for 20 min at 37°. The [PTIO] was always 3× [•NO] based on maximal release from DEANO. EPR spectra were simulated with a correlation coefficient of 0.999. Values represent mean of $n = 3$ experiments. Error bars represent standard error.

1 : 1 at low concentrations, [•NO] < 20 μM and [PTIO] = 60 μM, but thereafter the ratio increases gradually with the increase in [•NO] and approaches 2 : 1 at high •NO levels.

It is clear that one can use the ratio of 1 : 1 at low [•NO], ≤20 μM, and 2 : 1 at high [•NO], ≥100 μM, but the range in between is a problem. To quantitate •NO in a systematic manner and to avoid errors due to the apparent change in stoichiometry, the following method is recommended for quantitating standard solutions of •NO using PTIO. Because the concentration of •NO in saturated aqueous solution cannot exceed ≈2 mM (room temperature and 1 atm), we first assume an •NO concentration of approximately 2 mM. Second, into a PTIO solution of 60 μM (or greater), introduce a volume of •NO solution to achieve a dilution of about 100-fold. Even if the concentration of unknown is only 100 μM, this dilution will result in 1 μM •NO; EPR is sensitive enough to measure this amount of INR easily. These conditions should result in a reaction ratio close to 1 : 1 between •NO and NNR. Then by double integrating the deconvoluted low field line of PTIO and comparing the area with the standard area obtained from known concentrations of PTIO, the amount of •NO reacted with PTIO can be estimated. Another way of calculating the [•NO] is by double integrating the area under the low field line of INR and standardizing it with the area of known concentrations of a stable standard nitroxide radical such as 4-phenyl-2,2,5,5-tetramethylimidazoline-1-oxyl,[49] which is available commercially. With this dilution, if no INR is detected with EPR, the [•NO] is too low and the amount added will need to be increased. However, this first dilution step will lead to a point where a decision can be made on how to achieve a more favorable dilution. The important aspect is to direct the chemistry so that the stoichiometry of the reaction of •NO with NNR is 1 : 1.

To check whether $^{\bullet}NO_2$ competes with NNR compounds, in a separate experiment we introduced gaseous $^{\bullet}NO_2$ to an NNR solution and the EPR spectrum was recorded. As reported earlier,[48] no significant reaction of $^{\bullet}NO_2$ with PTIO was found. There also appears to be no interference from the presence of nitrite for the quantitation of $^{\bullet}NO$ in aqueous solution using PTIO.

Taken together, the use of $Fe^{2+}(MGD)_2$ or PTIO (NNR) combined with EPR can be used for the quantitation of $^{\bullet}NO$. The choice of method for quantitation will depend on the nature of the system being studied.

Other Potential Traps for $^{\bullet}NO$. Another method developed to quantitate $^{\bullet}NO$ is based on the use of cheletropic spin traps. A compound such as *o*-quinodimethane, otherwise called nitric oxide cheletropic trap (NOCT), reacts with $^{\bullet}NO$ and forms stable nitroxide radicals. These traps are formed by the photolysis of a parent ketone of the type 1,1,3,3-tetramethylind-2-one.[54] These cheletropic traps are water soluble, thermally stable at physiological temperature, and can be used to monitor $^{\bullet}NO$ with EPR techniques. The spectrum of the $^{\bullet}NO$ adduct consists of three lines with $a^N = 13–15$ G and a g value of 2.005, similar to other nitroxides.[8] These cheletropic traps react with both $^{\bullet}NO$ and $^{\bullet}NO_2$, but the EPR spectrum of both have different a^N values that make it easy to differentiate between the two. The intermediate, which traps the $^{\bullet}NO$ radical, is short-lived and needs to be produced *in situ* by photolysis for each experiment. These compounds are not susceptible to reactions with O_2, $O_2^{\bullet-}$, NO_2^-, or NO_3^-.[55] Although NOCT can be an ideal trap for the quantitation of $^{\bullet}NO$, this method can be time-consuming and the exact amount of intermediate formed is difficult to quantitate.

Another potential probe for the detection of nitric oxide in aqueous solutions is the *aci* form of nitromethane, which reacts with $^{\bullet}NO$ in alkaline solutions (pH > 12) and gives a stable adduct. The EPR signal of the resulting spin adduct gives characteristic hyperfine splitting, which facilitates the detection of $^{\bullet}NO$.[56,57]

Use of classic spin traps, nitrone and nitroso compounds, for the detection of $^{\bullet}NO$ as reported earlier[58,59] are not suitable for quantitation of $^{\bullet}NO$ simply because they do not form stable adducts.[60]

[54] H.-G. Korth, R. Sustman, P. Lommes, T. Paul, A. Ernst, H. de Groot, L. Hughes, and K. U. Ingold, *J. Am. Chem. Soc.* **116,** 2767 (1994).

[55] H.-G. Korth and H. Weber, *in* "Methods in Nitric Oxide Research" (M. Feelisch and J. S. Stamler, eds.), p. 383. Wiley, New York, 1996.

[56] K. J. Reszka, C. F. Chignell, and P. Bilski, *J. Am. Chem. Soc.* **116,** 4119 (1994).

[57] K. J. Reszka, P. Bilski, and C. F. Chignell, *J. Am. Chem. Soc.* **118,** 8719 (1996).

[58] C. M. Arroyo and M. Kohno, *Free Radic. Res. Commun.* **14,** 145 (1991).

[59] L. Pronai, K. Ichimori, H. Nozaki, H. Nakazawa, H. Okino, A. J. Carmichael, and C. M. Arroyo, *Eur. J. Biochem.* **202,** 923 (1991).

[60] S. Pou, L. Keaton, W. Surichamorn, P. Frigillana, and G. M. Rosen, *Biochim. Biophys. Acta* **1201,** 118 (1994).

Discussion

The EPR methods presented here to quantitate or detect $^{\bullet}NO$ are good and can be made ideal if they are tailored to avoid shortcomings. The issues to address to achieve accurate quantitation of nitric oxide are highlighted here.

1. Designing the protocol to ensure that all $^{\bullet}NO$ reacts with the trap (or probe) under study: This is done by keeping the $^{\bullet}NO$ trap (or probe) in excess ($\geq 3\times$) of $^{\bullet}NO$ to increase the probability of $^{\bullet}NO$ reacting with the trap. Also, the headspace volume of the air-tight container should be minimized to reduce the partitioning of $^{\bullet}NO$ from the aqueous phase. Nitric oxide quantitation is ideally accomplished using anaerobic solutions as $^{\bullet}NO$ reacts spontaneously with oxygen. When $Fe^{2+}(MGD)_2$ is used as a trap, the conversion of Fe^{2+} to Fe^{3+} will be minimized under anaerobic conditions. As seen in Table I, $Fe^{2+}(MGD)_2$ autoxidizes to produce a molecule of superoxide ($O_2^{\bullet -}$). This $O_2^{\bullet -}$ will interfere with $^{\bullet}NO$ measurement due to very fast reactions to produce peroxynitrite. Reducing agents such as $Na_2S_2O_4$ can be used to convert Fe^{3+} back to Fe^{2+}.

2. Ensuring stability of the product. To ensure product stability ($^{\bullet}NO$ + trap), EPR spectra should be collected over a period of time. This will also provide assurance that the reaction of $^{\bullet}NO$ is complete.

3. Avoiding chemistry that may alter the apparent $^{\bullet}NO$ concentration. $Fe^{2+}(MGD)_2$ will reduce nitrite to $^{\bullet}NO$. Unreacted $Fe^{2+}(MGD)_2$ will react with this $^{\bullet}NO$, resulting in overestimation of the true concentration of nitric oxide. However, by setting up the EPR experiment appropriately, potential interference from nitrite can be avoided. Nitrite does not interfere with the nitronyl nitroxide assay. However, $^{\bullet}NO_2$, one of the products of $^{\bullet}NO$ reacting with NNR, can compete for $^{\bullet}NO$. If experiments are carried out at low concentrations of $^{\bullet}NO$ and excess NNR is used, this problem is avoided and the 1 : 1 stoichiometry holds. Also, when using the $^{\bullet}NO$ solution, the slow addition of $^{\bullet}NO$ and vigorous stirring are preferred to minimize locally high concentrations of $^{\bullet}NO$.

4. Producing a standard curve from EPR measurements. In cases where $Fe^{2+}(MGD)_2$ is used, 3-CP or EPR active iron complexes that have comparable EPR spectral/line parameters can be used as a standard to calculate the concentrations of $^{\bullet}NO$. Similarly, when using nitronyl nitroxides to quantitate $^{\bullet}NO$, stable nitroxide radicals that can be accurately quantified spectrophotometrically are used for EPR standards.

Cautionary Notes

Room temperature for EPR studies of $Fe^{2+}(DETC)_2$ is preferred over low temperature because additional signals from $Cu^{2+}(DETC)_2$, from the yeast, could complicate the spectrum at low temperature. Considering the complexity due to

the precipitation of NO-$Fe^{2+}(DETC)_2$ from aqueous solutions, other spin traps may be preferred.

Because $Fe^{2+}(MGD)_2$ is toxic above a certain concentration,[61] care needs to be taken in use of this spin trap for *in vivo* and *in vitro* cell studies. Also, care should be taken to avoid or understand possible interference from nitrite.

Use of NNR as a spin trap for $^{\bullet}NO$ in biology is limited by possible interference from $O_2^{\bullet-}$ in the assay.[62] Also, NNR and INR compounds are susceptible to various reducing agents, such as thiols and ascorbate.

Acknowledgment

This work was supported by NIH Grants CA66801, CA81090, and CA84462.

[61] J. L. Zweier, P. Wang, and P. Kuppusamy, *J. Biol. Chem.* **270,** 304 (1995).
[62] R. F. Haselof, S. Zollner, I. A. Kirilyuk, I. A. Grigor'ev, R. Reszka, R. Bernhardt, K. Mertsch, B. Roloff, and I. E. Blasig, *Free. Radic. Res.* **26,** 7 (1997).

[2] *In Vitro* Detection of Nitric Oxide and Nitroxyl by Electron Paramagnetic Resonance

By ANDREI M. KOMAROV, ANDREAS REIF, and HARALD H. H. W. SCHMIDT

Introduction

Nitric oxide (NO) and nitroxyl (NO^-) are closely related reactive nitrogen species, but their chemistry is distinctly different. NO detection in the aqueous phase with the use of electron paramagnetic resonance (EPR) spectroscopy requires NO trapping by iron complexes forming paramagnetic mononitrosyl- or dinitrosyl-iron derivatives.[1] Likewise, nitroxyl reacts with metals to give nitrosyl complexes. Trapping of nitroxyl with ferrihemoproteins and the dithiocarbamate–iron complex can therefore be used for EPR detection of NO^-.[2,3] This article describes the methodology and several caveats of EPR detection of both NO and NO^-, with a special emphasis on their reaction with iron and dithiocarbamate–iron complexes.

[1] A. M. Komarov, *Methods Enzymol.* **359,** [6], 2002 (this volume).
[2] D. A. Bazylinski and T. C. Hollocher, *J. Am. Chem. Soc.* **107,** 1982 (1985).
[3] A. M. Komarov, D. A. Wink, M. Feelisch, and H. H. H. W. Schmidt, *Free Radic. Biol. Med.* **28,** 739 (2000).

0076-6879/02 $35.00