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Original Contribution

DETAILED METHODS FOR THE QUANTIFICATION OF NITRIC OXIDE IN AQUEOUS SOLUTIONS USING EITHER AN OXYGEN MONITOR OR EPR

SUJATHA VENKATARAMAN, SEAN M. MARTIN, FREYA Q. SCHAFER, and GARRY R. BUETTNER
Free Radical Research Institute, The University of Iowa, Iowa City, IA, USA

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Abstract—The interest in nitric oxide has grown with the discovery that it has many biological functions. This has heightened the need for methods to quantify nitric oxide. Here we report two separate methods for the quantification of aqueous stock solutions of nitric oxide. The first is a new method based on the reaction of nitric oxide with oxygen in liquid phase ($4^{\bullet}NO + O_2 + 2H_2O \rightarrow 4HNO_2$); an oxygen monitor is used to measure the consumption of oxygen by nitric oxide. This method offers the advantages of being both simple and direct. The presence of nitrite or nitrate, frequent contaminants in nitric oxide stock solutions, does not interfere with the quantification of nitric oxide. Measuring the disappearance of dissolved oxygen, a reactant, in the presence of known amounts of nitric oxide has provided verification of the 4:1 stoichiometry of the reaction. The second method uses electron paramagnetic resonance spectroscopy (EPR) and the nitric oxide trap $\{Fe^{2+}\text{-}(MGD)_2\}$, (MGD = N-methyl-D-glucamine dithiocarbamate). The nitrosyl complex is stable and easily quantitated as a room temperature aqueous solution. These two methods are validated with Sievers 280 Nitric Oxide Analyzer and cross-checked with standards using UV-Vis spectroscopy. The practical lower limits for measuring the concentration of nitric oxide using the oxygen monitor approach and EPR are approximately 3 μM and 500 nM, respectively. Both methods provide straightforward approaches for the standardization of nitric oxide in solution. © 2000 Elsevier Science Inc.

Keywords—Nitric oxide, Nitrite, N-methyl-D-glucamine dithiocarbamate (MGD), EPR, Oxygen monitor, Free radicals

INTRODUCTION

Nitric oxide ($^{\bullet}NO$) has been shown to have a role in many biochemical processes [1,2]. To investigate the biochemistry and biology of $^{\bullet}NO$, researchers often employ saturated solutions of $^{\bullet}NO$, but the standardization of these solutions can be problematic. A common method employed to determine the concentration of $^{\bullet}NO$ in near-saturated solutions is to estimate the concentration of $^{\bullet}NO$ using physical-chemical data [3]. These estimations can be inaccurate due to insufficient removal of oxygen during solution preparation, inadequate bubbling with $^{\bullet}NO$ gas, changes in temperature, or changes in pressure. Another frequently used method is to measure nitrite formation that results from the aerobic oxidation of $^{\bullet}NO$ [4]. This approach can also lead to erroneous results because trace amounts of oxygen present during the initial preparation of the $^{\bullet}NO$ solution will lead to nitrite formation and therefore an overestimation of the actual concentration of $^{\bullet}NO$. Other approaches use instrumentation that is not available in many laboratories, such as a nitric oxide analyzer (NOA) [5]. Thus, the advent of a new technique that would eliminate the hindrances and uncertainties associated with the quantification of $^{\bullet}NO$ in aqueous solution would be of benefit to researchers using such solutions.

The objective of this work is to provide a convenient and accurate method to quantify $^{\bullet}NO$ in aqueous solutions. To accomplish this we have used two methods. The first uses the widely available oxygen monitor (Clark electrode) to determine the concentration of $^{\bullet}NO$ in aqueous samples. The measurement relies on the accepted stoichiometry of the reaction of nitric oxide with dioxygen, reaction 1 [6,7].

$$4^{\bullet}NO + O_2 + 2H_2O \rightarrow 4HNO_2 \qquad (1)$$

The concentration of $^{\bullet}NO$ determined from the consumption of oxygen, using the oxygen monitor, was

Address correspondence to: Garry R. Buettner, Ph.D., Free Radical Research Institute, EMRB 68, The University of Iowa, Iowa City, IA 52242-1101, USA; Tel: (319) 335-6749; Fax: (319) 335-8049; E-Mail: garry-buettner@uiowa.edu.

verified with a NOA. A second method, EPR with $\{Fe^{2+}\text{-}(MGD)_2\}$ as a trap for •NO [8], was also investigated as a means for standardizing aqueous •NO solutions. This technique was verified with both the NOA and the spin probe 3-carboxy proxyl. Both the oxygen monitor and EPR techniques are well suited for the determination of the concentration of •NO in aqueous solutions.

MATERIALS AND METHODS

Ferrous sulfate and sodium nitrite were purchased from Fischer Scientific (Pittsburgh, PA, USA). Catalase (bovine liver) was obtained from Sigma (St. Louis, MO, USA). Pure nitric oxide gas was purchased from Matheson (Cleveland, OH, USA) and argon was purchased from Air Products (Allentown, PA, USA). The spin label, 3-carboxy proxyl (3-CP) was purchased from Aldrich (Milwaukee, WI, USA).

Preparation of •NO stock solution

Nitric oxide gas was either obtained from a nitric oxide gas tank or prepared from acidified sodium nitrite solutions [9]. To prepare gaseous nitric oxide, 50 ml of deoxygenated 2 M H_2SO_4 was introduced into a 250 ml gas sampling bottle containing 100 ml of deoxygenated 4 M $NaNO_2$ solution. During this addition •NO gas is produced. The rate of addition was carefully controlled to keep a modest pressure inside the system to avoid stoppers from popping open.

Since the •NO gas from either source can be contaminated with other oxides of nitrogen, it was purified by passing it through 100 ml of 4 M NaOH solution and then through 100 ml DDI water that was previously purged with argon for at least 30 min [10]. The NaOH solution and DDI water were in 250 ml gas washing bottles. Finally, the purified •NO gas was bubbled through approximately 300 ml of DDI water in a 500 ml gas sampling bottle for at least 20 min. This procedure produced stock solutions of 1.6 to 1.8 mM •NO, as measured by the NOA. The gas sampling bottle (Alltech Co., cat. No. 6944, Deerfield, IL, USA) has two stopcocks and a side arm with a septum through which the solution was accessed. The •NO stored in this way was stable for at least one week at room temperature. The concentration of nitrite, present as a contaminant, varied between 50 to 100 μM initially, but increased up to 300 μM if stored for 2 to 3 weeks, as determined by the NOA.

Aliquots of the •NO stock solutions were delivered to the various assay systems using argon-flushed gastight microliter syringes (Hamilton Co., Reno, NV, USA). Prior to aspiration of the •NO sample, an equal volume of argon was injected into the bottle containing •NO stock solution to minimize contamination with oxygen.

Nitric oxide analyzer

A Sievers 280 NOA was used to standardize the •NO stock solution and to measure nitrites. The •NO solution was injected into the empty purge vessel of the analyzer and measured directly. To measure nitrite, the purge vessel contained a reducing agent (1% potassium iodide in glacial acetic acid) to convert nitrite to nitric oxide. The •NO produced is swept into the NOA where it reacts with ozone, forming electronically excited nitrogen dioxide; the associated emission is proportional to the amount of •NO present in the sample. The amount of •NO present was determined by integrating the emission signal over time and was calibrated using known amounts of nitrite as a source of •NO. The •NO solution standardized by the NOA served as a standard for the validation of the oxygen monitor and EPR techniques.

Oxygen monitor

The YSI 5300 Biological Oxygen Monitor (Yellow Springs Instruments, Inc., Yellow Springs, OH, USA) uses a Clark-type polarographic oxygen probe immersed in a magnetically stirred sample chamber to measure oxygen concentration in aqueous solutions. The electrode has a 90% response time of < 60 s. To determine the concentration of •NO in a stock solution, 2.80 to 3.00 ml of DDI water were introduced into the sample chamber and stirred for approximately 3 min to allow for temperature equilibration. Next, the probe was placed in the sample chamber and the recorder output was followed for 1–2 min at 100% air saturation to ensure system stability. Once steady-state conditions were achieved, 50–200 μl of •NO stock solution were injected and changes in the oxygen concentration recorded. This change in the oxygen concentration is a result of a dual effect. While the injection of aliquots of anaerobic •NO solution causes the oxygen level to decrease due to reaction 1, the oxygen level will also decrease due to dilution of the aerobic solution. To account for the latter effect, 50–200 μl of argon-bubbled DDI water were added to the sample chamber containing air-saturated DDI water. The corresponding changes in the oxygen concentration due to this addition were measured and used as a correction factor to calculate the exact concentration of •NO in the anaerobic stock solution.

The quality of the oxygen probe measurements was assured by consecutive injections of the same volume of the $^{\bullet}NO$ stock solutions into the sample chamber, allowing 3–4 min between additions. If drastic, inappropriate changes in oxygen concentration were observed, the membrane was changed. The initial concentration of oxygen in air-saturated DDI water was taken as 250 μM under our conditions of room temperature (25°C), atmospheric pressure, and ionic strength. This value was obtained by interpolation from the plots of $[O_2]$ vs. temperature at various ionic strengths and then corrected for atmospheric pressure [11]. The oxygen monitor was calibrated with the amount of oxygen liberated when 500 U/ml of catalase were added to the sample chamber containing 50 μM hydrogen peroxide, at the same temperature and pressure as the $^{\bullet}NO$ sample measurements. The catalase/H_2O_2 reaction will produce one equivalent of O_2 for every two equivalents of H_2O_2 added. The hydrogen peroxide stock solution in DDI water was standardized spectrophotometrically using $\varepsilon_{240} = 43.6$ M^{-1} cm^{-1} [12].

Preparation of {NO-Fe^{2+}-$(MGD)_2$} complex and EPR

N-Methyl-D-glucamine dithiocarbamate (MGD) sodium salt was synthesized by the method of Shinobu et al. [13]. Stock solutions of {Fe^{2+}-$(MGD)_2$} were prepared by dissolving MGD sodium salt and ferrous sulfate in DDI water, molar ratio 5:1, respectively. All solutions were prepared with argon-purged DDI water. Because MGD is in excess, the concentration of the {Fe^{2+}-$(MGD)_2$} complex is always expressed with respect to [Fe(II)]. In general, the concentration of {Fe^{2+}-$(MGD)_2$} stock solutions prepared for the experiments ranged from 2–10 mM depending on the experimental conditions.

Although $^{\bullet}NO$ by itself is a paramagnetic compound, it is EPR silent at room temperature in aqueous solutions. However, when $^{\bullet}NO$ is trapped with {Fe^{2+}-$(MGD)_2$}, the resulting complex {NO-Fe^{2+}-$(MGD)_2$} is EPR-detectable [8]. The {NO-Fe^{2+}-$(MGD)_2$} samples were 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, various volumes of $^{\bullet}NO$ stock solutions were added using gastight syringes, resulting in the formation of the {NO-Fe^{2+}-$(MGD)_2$} complex. The pH of this solution varied between 7.5 and 8.0. To generate standards for quantitative measurement of $^{\bullet}NO$, various dilutions of known concentrations of $^{\bullet}NO$ stock solutions were used to prepare {NO-Fe^{2+}-$(MGD)_2$} samples. The concentrations of the $^{\bullet}NO$ stock solutions were standardized using the NOA. The concentration of {Fe^{2+}-$(MGD)_2$} was always in 3- to 5-fold excess of the $^{\bullet}NO$ added to ensure that all the $^{\bullet}NO$ was trapped. The spin label 3-CP was used as an additional standard to verify the concentrations of {NO-Fe^{2+}-$(MGD)_2$} standards, correcting for the difference in their g-values [14]. The 3-CP solution was standardized spectrophotometrically using $\varepsilon_{234} = 2370 \pm 50$ M^{-1} cm^{-1}.

Once formed, the {NO-Fe^{2+}-$(MGD)_2$} samples were quickly transferred to the degassed flat cell to avoid oxidation of the complex, and EPR spectra were recorded within 5–10 min. This {NO-Fe^{2+}-$(MGD)_2$} complex gives a 3-line EPR spectrum. Control experiments were done by adding various volumes of $NaNO_2$ to the {Fe^{2+}-$(MGD)_2$} solution, and EPR spectra were recorded. Pilot experiments were also done to see if {Fe^{2+}-$(MGD)_2$} could be used as the limiting reagent when studying the {NO-Fe^{2+}-$(MGD)_2$} complex. It was found that when the $^{\bullet}NO$ is added in great excess to {Fe^{2+}-$(MGD)_2$}, the resulting complex is EPR-silent under our instrument settings. We hypothesize that this may be due to the formation of diamagnetic dinitrosyl complexes similar to those reported by Vanin et al. [15]. In our experiments nitric oxide was always the limiting reagent.

EPR spectra were obtained using a Bruker X-band 300-EMX spectrometer (Karlsruhe, Germany) at room temperature. The typical instrument settings were: 9.75 GHz microwave frequency; 100 kHz modulation frequency; 10 mW, a nonsaturating microwave power [16], 1 G modulation amplitude; 3418 G center field for {NO-Fe^{2+}-$(MGD)_2$} and 3475 G for 3-CP; 100 G/84 s scan rate; and 82 ms time constant.

RESULTS

Oxygen monitor

To determine the concentration of a stock solution of $^{\bullet}NO$, 100 μl of the solution was introduced into 2.90 ml of DDI water in the oxygen monitor chamber. The percent total change in oxygen concentration was recorded as Y_{A+B}. As a control for the effect of anaerobic dilution (see Methods), 100 μl of nitrogen- or argon-bubbled DDI water were added to the chamber instead of the $^{\bullet}NO$ solutions, and the corresponding change in the oxygen concentration was recorded as Y_B. The concentration of $^{\bullet}NO$ in the original solution was then calculated by the following formula:

$$[^{\bullet}NO] = ((Y_{A+B} - Y_B)/100) \times [O_2]_{air} \times DF \times 4 \quad (2)$$

Where:

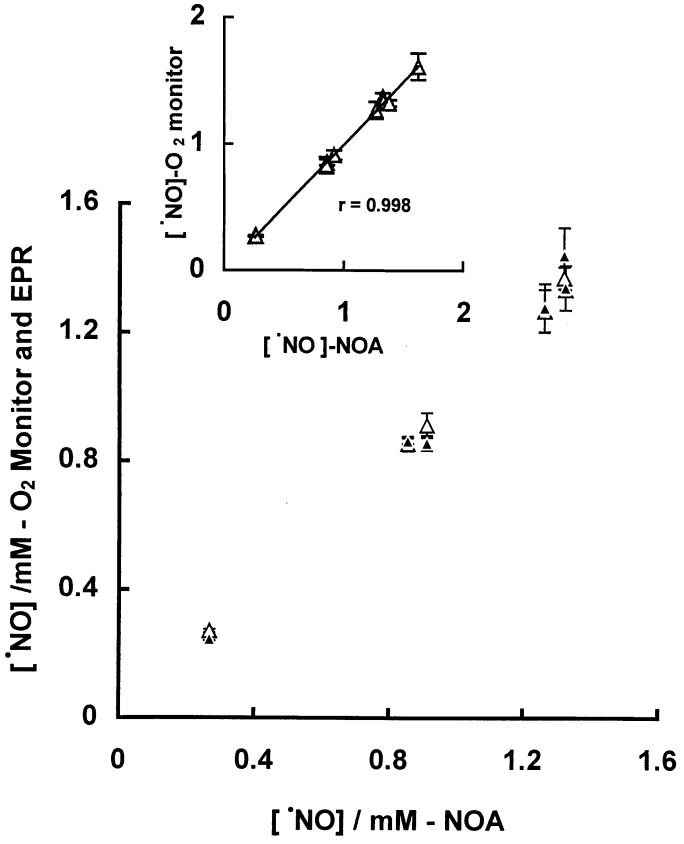

Fig. 1. Standardization of oxygen monitor technique with NOA and EPR. The data represent the concentrations of various stock solutions of •NO determined by the NOA (abscissa) and the oxygen monitor (Δ) and EPR (▲) (ordinate). The inset shows the best-fit line for the oxygen monitor results alone. The units on all axes are in mM. Identical volumes of various •NO stock solutions were injected into the NOA. The oxygen monitor analyses were performed by adding 100 μl of various •NO stock solutions to 2.90 ml of air-saturated DDI. EPR analyses were performed by adding 100 μl of •NO stock solution to a solution of $\{Fe^{2+}\text{-}(MGD)_2\}$ under argon. Each point represents the mean of three independent measurements and the bars the standard error. The size of the error bars in some data points is smaller than that of the symbols used.

Y_{A+B} = percent change in oxygen concentration upon addition of •NO stock solution;

Y_B = percent change in oxygen concentration due to anaerobic dilution;

$(Y_{A+B} - Y_B)$ = percent change in oxygen concentration due to the reaction of •NO with oxygen alone;

$[O_2]_{air}$ = the oxygen concentration of an air-saturated solution under the physical conditions of the experiment (for this work it was taken to be 250 μM);

DF = dilution factor of •NO (for example, in our experiments it is (2900 μl + 100 μl)/100 μl);

and "4" accounts for the stoichiometry of the reaction (reaction 1).

The concentration of the •NO stock solutions determined by the oxygen monitor method correlates ideally with that obtained from the NOA, Fig. 1 (see also inset). The slope of the best-fit line being 0.997 indicates that the oxygen monitor is a reliable method for the quantification of •NO stock solutions. Neither nitrite nor nitrate interfere with this quantitation, as control experiments in which 100 μl of various concentrations of $NaNO_2$ or $NaNO_3$ solutions were added to achieve a final concentration of 500–1000 μM showed no electrode response. Our results suggest that the oxygen monitor can be used to standardize •NO stock solutions with concentrations in the range of approximately 10 μM–1.9 mM at room temperature. The higher concentration of 1.9 mM is the upper limit of the solubility of •NO in aqueous solution at room temperature.

The focus of this work is the quantification of nitric oxide in aqueous solution. The slope in Fig. 1 (0.997) coupled with the stoichiometric factor used in equation 2,

is the first verification of the 4•NO:1O_2 stoichiometry of reaction 1 determined by measuring the consumption of oxygen, a reactant.

EPR quantitation of •NO with {Fe^{2+}-$(MGD)_2$}

To determine the concentration of •NO using EPR, 100 μl of various concentrations of •NO stock solutions were introduced to a solution of {Fe^{2+}-$(MGD)_2$} complex in water. A three-line EPR spectrum was observed corresponding to the {NO-Fe^{2+}-$(MGD)_2$} complex at g_{iso} = 2.04, a^N = 12.8 G. The concentration of this nitrosyl complex was obtained by double integration of the three lines, calibrated against standards of {NO-Fe^{2+}-$(MGD)_2$}, and cross-checked with 3-CP. The concentration of •NO solutions determined by EPR measurements correlates well (slope = 1.08) with those determined from the NOA, and are plotted in Fig. 1 along with the results from the oxygen monitor.

It has been shown that nitrite in the presence of {Fe^{2+}-$(MGD)_2$} could be an additional source of •NO, which in turn would form the {NO-Fe^{2+}-$(MGD)_2$} complex [17,18,19]. Small amounts of nitrite, invariably present in •NO stock solutions, could lead to an overestimation of nitric oxide. It is therefore important to determine whether or not the presence of small amounts of nitrite will interfere with the standardization of •NO solutions using {Fe^{2+}-$(MGD)_2$} as a •NO trap. To check whether the presence of nitrite as a contaminant interferes with the estimation of •NO, $NaNO_2$ was tested for its reactivity with the {Fe^{2+}-$(MGD)_2$} complex in the time frame of our experimental conditions. The {Fe^{2+}-$(MGD)_2$}/$NaNO_2$ solutions produced no detectable EPR signals with our instrument settings until [$NaNO_2$] $>$ 500 μM. At 1 mM $NaNO_2$, an EPR spectrum corresponding to the {NO-Fe^{2+}-$(MGD)_2$} complex was detected within the first 3 min after addition of $NaNO_2$. The signal intensity increased approximately 5-fold over 30 min. The signal intensity after 30 min corresponded to 110 μM of {NO-Fe^{2+}-$(MGD)_2$}. This observation is consistent with the conversion of nitrite to •NO in the presence of {Fe^{2+}-$(MGD)_2$} [18]. In another control experiment, the •NO stock solution was purged with argon for 30 min to ensure the solution was free of •NO and contained only nitrite as a contaminant. When this solution was added to the {Fe^{2+}-$(MGD)_2$} solution no EPR signals were observed, although the NOA revealed the presence of nitrite (150–250 μM). Because the formation of nitric oxide from nitrite depends on the concentration of {Fe^{2+}-$(MGD)_2$}, we added different concentrations of {Fe^{2+}-$(MGD)_2$} (0.3 mM to 0.6 mM) to the above solution. No detectable {NO-Fe^{2+}-$(MGD)_2$} signal was observed under our experimental conditions. This demonstrates that small amounts of nitrite, present as contaminants in the •NO stock solutions, do not interfere with the quantification of •NO by EPR. When the modulation amplitude and the time constant of the EPR spectrometer were increased, then the 3-line {NO-Fe^{2+}-$(MGD)_2$} signal could be seen from solutions containing nitrite as low as 100 μM. Thus, the potential interference from nitrite must be considered. With appropriate protocols, {Fe^{2+}-$(MGD)_2$} can be used to quantitate •NO stock solutions in the range of 500 nM (by peak height measurements) to 1.9 mM.

DISCUSSION

How does the oxygen monitor method compare to other methods for quantification of •NO in stock solution? The oxygen monitor method is an economical and accurate approach to quantify •NO in only a few minutes. In comparison to EPR, the oxygen monitor is the method of choice when contaminating nitrite levels are high ($\geq$ 1 mM), because there is no interference from nitrite. However, under our experimental conditions, i.e., the sample solution with pH = 7.5–8.0, at room temperature and when the nitrite is less than approximately 500 μM, there appears to be no interference with the {NO-Fe^{2+}-$(MGD)_2$} measurement by EPR. This is made clear in Fig. 1 where the concentrations of •NO from both methods are compared with that from the NOA. At low nitrite levels, both methods are excellent. Recall that both methods were cross-checked using UV-Vis spectroscopy, i.e., the oxygen monitor was calibrated using concentrations of hydrogen peroxide that were determined spectrophotometrically and EPR measurements were referenced to 3-CP, which was again standardized by absorbance measurements. The oxygen monitor can be used to estimate the concentrations of aqueous •NO solutions having concentrations of $\approx$ 10 μM to 1.9 mM.

Although there have been numerous studies using EPR with {Fe^{2+}-$(MGD)_2$} for the detection of •NO in biological systems, we have extended this work by developing a protocol to quantify aqueous •NO stock solutions. Nitric oxide must be the limiting reagent for success in the {Fe^{2+}-$(MGD)_2$}/•NO reaction; {Fe^{2+}-$(MGD)_2$} should be at least 3- to 5-fold excess to ensure that all the •NO is trapped. Nitrite can interfere with the {NO-Fe^{2+}-$(MGD)_2$} measurement, but if it is present at less than approximately 500 μM, pH = 7.5–8.0, using our EPR parameters we found no significant interference. Both the oxygen monitor [20] and EPR [8] have been used in the study of the biochemistry of nitric oxide. Here we use these methods for the quantification of •NO. In conclusion, the concentration of •NO determined using the oxygen monitor and EPR correlated ideally with the results obtained from the NOA. The oxygen monitor

provides a simple and fast approach to the standardization of aqueous solutions of nitric oxide.

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REFERENCES

[1] Stamler, J. S.; Singel, D. J.; Loscalzo, J. Biochemistry of nitric oxide and its redox-activated forms. *Science* **258:**1898–1902; 1992.
[2] Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric oxide: physiology, pathology and pharmacology. *Pharmacol. Rev.* **43:**109–142; 1991.
[3] Lange, N. A. *Handbook of chemistry* (4th eds.). Sandusky, OH: Handbook Publishers, Inc.; 1941.
[4] Archer, S. L.; Shultz, J. P.; Warren, J. B.; Hampl, V.; DeMaster, G. E. Preparation of standards and measurement of nitric oxide, nitroxyl and related oxidation products. *Methods* **7:**21–34; 1995.
[5] Fontijin, A.; Sabadell, A. J.; Ronco, R. J. Homogenous chemiluminescent measurement of nitric oxide with ozone. *Anal. Chem.* **42:**575–579; 1970.
[6] Pogrebnaya, V. L.; Usov, A. P.; Baranov, A. V.; Nesterenko, A. I.; Bez'yzzychnyi, P. I. Oxidation of nitric oxide by oxygen in the liquid phase. *J. Appl. Chem. USSR.* **48:**1004–1007; 1995.
[7] Ford, P. C.; Wink, D. A.; Stanbury, D. M. Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett.* **326:**1–3; 1993.
[8] Komarov, A.; Mattson, D.; Jones, M. M. *In vivo* spin trapping of nitric oxide in mice. *Biochem. Biophys. Res. Commun.* **195:**1191–1198; 1993.
[9] Schenk, P. W. Nitrogen. In: Brauer, G., ed. *Handbook of preparative inorganic chemistry* (2nd ed.). New York: Academic Press; 1963:457–517.
[10] Kelm, M.; Schrader, J. Control of coronary vascular tone by nitric oxide. *Circ. Res.* **66:**1561–1575; 1990.
[11] Koppenol, W. H.; Butler, J. Energetics of interconversion reactions of oxyradicals. *Adv. Free Radic. Biol. Med.* **1:**91–121; 1985.
[12] Hildebrandt, A. G.; Roots, I. Reduced NADPH-dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reaction in liver microsomes. *Arch. Biochem. Biophys.* **171:**385–397; 1975.
[13] Shinobu, L. A.; Jones, S. G.; Jones, M. M. Sodium N-methyl-D-glucamine dithiocarbamate and cadmium intoxication. *Acta. Pharmacol. Toxicol.* (Copehn.) **54:**189–194; 1984.
[14] Eaton, S. S.; Eaton, G. R. Signal area measurement in EPR. *Bull. Magn. Reson.* **1:**130–138; 1980.
[15] Vanin, A. F.; Malenkova, I. V.; Serezhenkov, V. A. Iron catalyzes both decomposition and synthesis of S-nitrosothiols: optical and electron paramagnetic resonance studies. *Nitric Oxide* **1:**191–203; 1997.
[16] Buettner, G. R.; Kiminyo, K. P. Optimal EPR detection of weak nitroxide spin adduct and ascorbyl free radical signals. *J. Biochem. Biophys. Methods* **24:**147–151; 1992.
[17] Samouilov, A.; Kuppusamy, P.; Zweier, J. L. Evaluation of the magnitude and rate of nitric oxide production from nitrite in biological systems. *Arch. Biochem. Biophys.* **357:**1–7; 1998.
[18] Tsuchiya, K.; Yoshizumi, M.; Houchi, H.; Mason, R. P. Nitric oxide forming reactions between the iron-N-methyl-D-glucamine dithiocarbamate complex and nitrite. *J. Biol. Chem.* **275:**1551–1556; 2000.
[19] Hiramoto, K.; Tomiyama, S.; Kikugawa, K. Appearance of electron spin resonance signals in the interaction of dithiocarbamate-Fe(II) with nitrogen dioxide and nitrite. *Free Radic. Res.* **27:**505–509; 1997.
[20] Kelley, E. E.; Wagner, B. A.; Buettner, G. R.; Burns, C. P. Nitric oxide inhibits iron-induced lipid peroxidation in HL-60 cells. *Arch. Biochem. Biophys.* **370:**97–104; 1999.

ABBREVIATIONS

3-CP—3-carboxy proxyl
DDI—De-ionized distilled water
EPR—Electron Paramagnetic Resonance
a^N—Hyperfine splitting constant of nitrogen
MGD—N-methyl-D-glucamine dithiocarbamate
NOA—Nitric Oxide Analyzer