# The reduction potential of nitric oxide (NO) and its importance to NO biochemistry

**Michael D. Bartberger[†‡], Wei Liu[§], Eleonora Ford[¶], Katrina M. Miranda[‖], Christopher Switzer[¶], Jon M. Fukuto[¶**], Patrick J. Farmer[§], David A. Wink[‖], and Kendall N. Houk[†**]**

Departments of [†]Chemistry and Biochemistry and [¶]Molecular and Medical Pharmacology, Center for the Health Sciences, University of California, Los Angeles, CA 90095; [§]Department of Chemistry, University of California, Irvine, CA 92697; and [‖]Tumor Biology Section, Radiation Biology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Edited by Louis J. Ignarro, University of California School of Medicine, Los Angeles, CA, and approved June 17, 2002 (received for review February 19, 2002)

**A potential of about −0.8 (±0.2) V (at 1 M versus normal hydrogen electrode) for the reduction of nitric oxide (NO) to its one-electron reduced species, nitroxyl anion ($^3NO^-$) has been determined by a combination of quantum mechanical calculations, cyclic voltammetry measurements, and chemical reduction experiments. This value is in accord with some, but not the most commonly accepted, previous electrochemical measurements involving NO. Reduction of NO to $^1NO^-$ is highly unfavorable, with a predicted reduction potential of about −1.7 (±0.2) V at 1 M versus normal hydrogen electrode. These results represent a substantial revision of the derived and widely cited values of +0.39 V and −0.35 V for the NO/$^3NO^-$ and NO/$^1NO^-$ couples, respectively, and provide support for previous measurements obtained by electrochemical and photoelectrochemical means. With such highly negative reduction potentials, NO is inert to reduction compared with physiological events that reduce molecular oxygen to superoxide. From these reduction potentials, the pKa of $^3NO^-$ has been reevaluated as 11.6 (±3.4). Thus, nitroxyl exists almost exclusively in its protonated form, HNO, under physiological conditions. The singlet state of nitroxyl anion, $^1NO^-$, is physiologically inaccessible. The significance of these potentials to physiological and pathophysiological processes involving NO and $O_2$ under reductive conditions is discussed.**

Nitric oxide (NO) is an endogenously generated species with a diverse array of biological functions (1). NO is one of the primary regulators of vascular tone, is involved in signal transduction in both the peripheral and central nervous system, and is an integral part of the immune response system associated with macrophage and neutrophil activation. More recently, NO has been proposed to be involved in the regulation of mitochondrial function (2, 3). Problems in NO homeostasis have been implicated in the development of a variety of diseases and disorders such as hypertension and atherosclerosis (4), diabetes (5), and many neurodegenerative diseases (6). NO is also thought to be a cytoprotective agent, capable of inhibiting radical-induced damage and oxidative stress (7). To understand the actions of NO as a physiological messenger and a cytotoxic or cytoprotective effector molecule, it is essential to understand its basic chemical interactions with biological systems and its metabolic fate.

NO and its reduced derivative $NO^-$ (and/or its conjugate acid, HNO) have very different chemical properties and display distinct and often opposite effects in cells. For example, HNO/$NO^-$ has been found to be toxic under conditions where NO is cytoprotective (8). HNO/$NO^-$ reacts with $O_2$ to generate potent oxidizing species, capable of damaging DNA and causing cellular thiol depletion, whereas NO does neither under similar conditions (9–11). HNO has been found to be a thiophilic electrophile (12), readily capable of modifying cellular thiol functions (13, 14), whereas NO reacts only indirectly with thiols. HNO/$NO^-$ has been suggested to act as a potent, redox-sensitive intropic agent; in contrast, NO induces negligible inotropic response (15).

Chemically, NO is similar to molecular oxygen ($O_2$) in that it is a gas at room temperature and pressure, is a radical species with one unpaired electron ($O_2$ possesses two), and binds to a variety of metal centers in proteins. NO has been used extensively as an $O_2$ mimic to examine oxygen binding in metalloproteins, because of its ability to associate with $O_2$-binding centers and form stable metal-nitrosyl complexes (16).

Nitroxyl anion ($NO^-$) is isoelectronic with dioxygen; it possesses a triplet ground state ($^3NO^-$) and singlet excited state ($^1NO^-$) with a singlet-triplet gap of about 17−21 kcal/mol (12). The reduction potential for the $O_2/O_2^-$ couple is −0.16 V [1 M versus normal hydrogen electrode (NHE)] and −0.33 V (1 atm versus NHE) (17, 18). Numerous experimental values of the reduction potential of NO exist in the literature, scattered over a range of about +0.4 to −1 V at 1 M versus NHE (19–25). This finding has led to confusion with respect to the biological accessibility of the one-electron reduced form of NO, namely $NO^-$. Values at the highly positive end of the potential range have been determined under conditions where formation of adsorbed species is likely (see below) (19–22), whereas chroniopotentiometric and controlled-potential coulometric studies by Ehman and Sawyer (23) and photoelectrochemical measurements by Benderskii *et al*. (24) on free NO have yielded substantially more negative reduction potential values.

Stanbury (25) has furnished a compendium of reduction potentials for a number of organic and inorganic species. Values for NO were derived based on a literature pKa value for HNO of 4.7, an estimate of the H—NO bond dissociation energy, approximation of the aqueous solvation energy of HNO, and the assumption that the relevant acid-base equilibrium involves the singlet state of both HNO and $NO^-$. The reduction potentials derived in this manner for the NO/$^3NO^-$ and NO/$^1NO^-$ couples are +0.39 V and −0.35 V (1 M versus NHE, respectively) and have been widely cited in the biological literature of NO. Compared with the reduction of $O_2$ to $O_2^-$, the reduction of NO to $^3NO^-$ would be expected to be highly favorable. It has therefore been difficult to explain why NO is not significantly reduced under physiological conditions in a manner similar to that seen with $O_2$.

The primary metabolic fate of $O_2$ in aerobic organisms is reduction to water, through intermediates such as superoxide ($O_2^-$) and peroxide ($O_2^{2-}/H_2O_2$). It is through this mechanism that aerobic organisms derive energy from $O_2$. Typically, $O_2$ reduction occurs via metal-$O_2$ complexes, for example, the cytochrome *c* oxidase heme-$O_2$ complex in mitochondria. Interestingly, despite the general similarities between NO and $O_2$, the primary metabolic fate of NO appears to be oxidative. That is,

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: NHE, normal hydrogen electrode; MSHA, methanesulfohydroxamic acid.

[‡]Present address: Department of Molecular Structure and Design, Amgen, Inc., M/S 29-M-B, One Amgen Center Drive, Thousand Oaks, CA 91320.

**To whom reprint requests may be addressed. E-mail: jfukuto@mednet.ucla.edu or houk@chem.ucla.edu.

 www.pnas.org/cgi/doi/10.1073/pnas.162095599

conversion of NO to oxidized congeners such as nitrogen dioxide ($NO_2$), nitrite ($NO_2^-$) and nitrate ($NO_3^-$) comprise the predominant pathways for degradation of physiologically produced NO. From the widely cited literature values for the reduction potentials of NO and $O_2$, NO would be expected to be reduced much more easily than $O_2$.

Theoretical calculations (12) have predicted that the pKa of HNO was considerably higher than an experimental value of 4.7 obtained by Grätzel *et al*. (26) by pulse radiolysis of aqueous NO. A revised pKa of 7.2 (±1.0) was proposed, suggesting a significant concentration of protonated nitroxyl (HNO) at physiological pH. Subsequent calculations by Dixon and coworkers at the Pacific Northwest National Laboratory, Richland, WA (D. A. Dixon, personal communication) have suggested an even higher pKa. Additionally, based on analogy with $O_2$ and experiments demonstrating the triplet ground-state nature of $NO^-$, it was suggested that the relevant aqueous acid-base equilibrium of HNO should instead involve the triplet form of its conjugate base, $^3NO^-$ (12). Fundamental changes to the acid-base equilibria of HNO and the spin states involved will result in a significant change in the resulting derived reduction potentials for NO. We have now obtained theoretical, chemical, and electrochemical measurements of the reduction potentials of NO and show that they are considerably different from the commonly accepted data. These additional values are reconciled with existing and relevant experimental and biological data.

## Materials and Methods

**Theoretical Calculations.** Reaction energetics were computed by using the complete basis set (CBS-QB3) method of Petersson and coworkers (27). To account for the effects of aqueous solvation, single-point self-consistent reaction field calculations were performed on CBS-QB3 optimized geometries with the polarizable continuum model of Tomasi and coworkers (28), using 100 tesserae per sphere and Pauling (Mertz-Kollman) atomic radii scaled by a factor of 1.2. Solvation energies were computed by using the B3LYP/6–311+G* density. All calculations were performed with the GAUSSIAN 98 program system (29). Solution-phase reduction potentials for $NO_X$ and $CO_2$ were then determined from the corresponding electron affinities and solvation energies, with the standard-state reduction potential for $O_2/O_2^-$ (17) used as a reference value.

**Electrochemistry.** Cyclic voltammetric analyses were conducted in dry, degassed acetonitrile by using a glassy carbon working electrode, platinum mesh counter electrode, Ag/AgCl wire reference electrode, and a scan rate of 0.1 V/sec in the presence of 0.1 M tetrabutylammonium hexafluorophosphate. Ferrocene ($1 \times 10^{-4}$ M) was used as a standard ($FeCp_2/FeCp_2^+$, $E_{1/2}$ = +0.400 V versus NHE). Linear sweep voltammetric analyses were performed by using a glassy carbon working electrode, platinum mesh counter electrode, Ag/AgCl reference electrode, and a scan rate of 3 mV/sec in 0.1 M NaCl in 0.05 M buffer.

The intermediacy of the coupled byproduct of NO reduction, hyponitrite ($N_2O_2^{2-}$), in the observed chemistry was discounted by direct measurement of its electrochemical oxidation under the same conditions. Cyclic voltammograms (−1.0 to + 1.2 V) were measured in 1 M NaOH aqueous solution, with $5 \times 10^{-3}$ M $Na_2N_2O_2$ and 0.05 M NaCl, using a pyrolytic graphite working electrode, a Pt counter electrode, and a standard calomel electrode reference electrode separated from the analyte solution by a fine glass frit. Sodium hyponitrite was synthesized as described (30). Purity was >92% as measured by its absorbance at 248 nm. The apparent $E_{1/2}$ ($N_2O_2^{2-}$) was found to be at +0.4 V versus NHE. Scan rate versus current plots show that this oxidation is diffusion controlled and possesses a reduction wave at about −0.2 V versus NHE, corresponding to a surface-adsorbed species (data not shown).

**Chemical Reduction Experiments.** Angeli's salt (sodium trioxodinitrate, $Na_2N_2O_3$) was synthesized as described (9). Methanesulfohydroxamic acid (MSHA) was a generous gift from H. Nagasawa from the University of Minnesota, Minneapolis. Reduction potentials of the bridged 2,2′-bipyridinium compounds, 7,8-dihydrodipyrido[1,2-*a*:2′,1′-*c*][1,4]diazepinediium ($V1^{2+}$) and 7,8-dihydro-2,12-dimethyldipyrido[1,2-*a*:2′,1′-*c*][1,4]diazepiinedium ($V2^{2+}$) were determined electrochemically versus Ag/AgCl in 0.1 M NaOH/0.1 M NaCl and calibrated to 1,1′-dimethyl-4,4′-bipyridinium (methyl viologen, $MV^{2+}$, $E_{1/2}$ = −0.44 V versus NHE). The reduction potentials of $V1^{2+}$ and $V2^{2+}$ were found to be −0.547 V and −0.690 V, respectively.

Aliquots of anaerobic solutions of MSHA ($10^{-4}$ M in pH 7 phosphate buffer solution) were mixed with anaerobic alkaline solutions of each viologen acceptor, each of which was present in a 10-fold excess ($10^{-3}$ M in 0.1 M NaOH). The final pH of the reaction solution was approximately 12–13. Formation of the corresponding radical cations $MV^{\cdot+}$, $V1^{\cdot+}$, and $V2^{\cdot+}$ obtained upon one-electron reduction were monitored at 603, 503, and 516 nm, respectively. Such radical cations were also observed upon reduction with Zn(Hg). Experiments performed similarly with Angeli's salt at reaction pH of 7 showed no reaction of $MV^{2+}$, as indicated by the absence of the radical cation $MV^{\cdot+}$ at 603 nm.

## Results

**Quantum Mechanical Calculations.** Table 1 lists the calculated gas-phase electron affinities and aqueous reduction potentials for the series of nitrogen oxides $NO_X$ ($x$ = 1−3), $O_2$, and $CO_2$, along with available experimental values. It is seen that the calculated gas-phase electron affinities of $O_2$, NO, and $NO_3$ are found to be accurate to within the experimental error of the literature values. Our calculated solvation free energies for $NO_2^-$, $NO_3^-$, and $O_2^-$ are within 5 kcal/mol of the measured or derived experimental quantities (35, 36), and thus the calculated aqueous reduction potentials for $NO_2$, $NO_3$, and $CO_2$ are found to be accurate to within about ±0.2 V of available experimental data (Table 1). From the experimental standard state (1 M) reduction potential of $O_2$ of −0.16 V versus NHE (17, 18), our calculations predict a potential value for reduction of NO to $^3NO^-$ of −0.76 (±0.2) V versus NHE, 0.6 V more negative than the analogous reduction of $O_2$.

CASMP2 calculations predict a singlet-triplet gap for $NO^-$ of 20.9 kcal/mol (0.906 V) (12). From this value, a highly negative potential of −1.7 (±0.2) V is obtained for the reduction of NO to the excited-state $^1NO–$ anion. Accordingly, this species is physiologically inaccessible because of its very negative reduction potential and high pKa values.

**Electrochemical Measurements.** Numerous electrochemical investigations have shown that irreversible reduction of NO occurs at about +0.3 V versus NHE on platinum or other noble metal electrodes (19). This potential varies significantly with electrode material (20–22), because of multielectron reductions of absorbed NO. Such potentials cannot be ascribed to the one-electron reduction of free NO to $NO^-$. On electrode materials such as mercury or carbon, or in free solution where adsorbates are not formed, the reduction of NO occurs at much lower potentials.[††]

The reversible, single-electron reduction of $O_2$ in acetonitrile occurs at −0.65 V versus NHE. Under identical conditions (Fig. 1), NO is reduced irreversibly at a potential that is 0.4 V more

[††]An apt analogy is the two-electron reduction of $H^+$ to $H_2$ on a platinum surface. This process carries a potential of 0 V versus NHE, but is mediated by stable Pt-H species on the surface of the electrode. Conversely, the single-electron reduction of free $H^+$, producing highly reactive hydrogen atoms, occurs at <−1 V versus NHE at physiological pH.

**Table 1. Experimental and calculated electron affinity and reduction potential values for $O_2$, nitrogen oxides $NO_x$, ($x$ = 1–3) and $CO_2$**

| Species | Experimental electron affinities (gas phase), eV* | Calculated electron affinities (gas phase), eV | Experimental reduction potential (1 M, $H_2O$), V | Calculated reduction potential (1 M, $H_2O$), V |
|---|---|---|---|---|
| $O_2/O_2^-$ | 0.451 (± 0.007) | 0.450 | −0.16† | −0.16† |
| $NO/^3NO^-$ | 0.026 (± 0.005) | 0.0254 | +0.39‡ | −0.76 |
| | | | +0.3§ | |
| | | | −0.7¶ | |
| | | | −0.81‖ | |
| $NO/^1NO^-$ | | | −0.35‡ | −1.67 |
| $NO_2/NO_2^-$ | 2.273 (± 0.005) | 2.26 | +1.04** | +1.19 |
| $NO_3/NO_3^-$ | 3.91 (± 0.22) | 3.92 | +2.5‡ | +2.66 |
| $CO_2/CO_2^-$ | −0.600 | −0.463 | −1.8†† | −1.68 |

*Ref. 31.
†Reduction potential of $O_2$ (−0.16 V at 1 M versus NHE) used as calibration standard, ref. 17.
‡Ref. 25.
§Ref. 19.
¶Ref. 23.
‖Ref. 24.
**Ref. 32.
††Refs. 33 and 34. A similar value of −1.9 V has been determined.

negative than that for $O_2$. Even at scan rates of 0.1 V/sec, no indication of reversibility was seen for NO reduction, indicating that the reduced form has a lifetime of less than milliseconds in saturated NO solution. This irreversibility is likely caused by the series of rapid catenation reactions of $NO^-$ with NO, forming $N_2O_2^-$ and $N_3O_3^-$, previously observed by pulse radiolysis studies (26, 37).

A comparison of $O_2$ and NO reduction in aqueous solution is provided in Fig. 2. The reduction of NO is found to be at a significantly lower potential than $O_2$ at pH 7. The measured half-wave potential is essentially unchanged over a pH range of 4 to 7, but varies between pH 10 and pH 13. This variance in the measured potential is suggestive of a pKa value for $NO^-$ of greater than 9. A decrease in pH facilitates the protonation of $NO^-$ formed upon reduction of NO, leading to somewhat more positive potential values. Using either the approximate half-wave potentials, or those corresponding to peak values at maximum current, the reduction of NO occurs at a potential about 0.4 V more negative that that of $O_2$, in good agreement with theoretical prediction (Table 1).

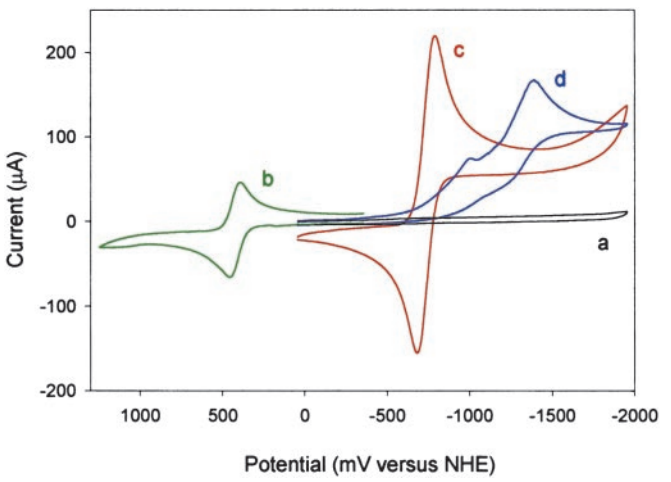


**Fig. 1.** Cyclic voltammograms for reduction of NO and $O_2$. (*a*) Background. (*b*) $10^{-4}$ M ferrocene ($FeCp_2/FeCp_2^+$, $E_{1/2}$ = +0.400 V). (*c*) Saturated $O_2$, about $3 \times 10^{-3}$ M ($O_2/O_2^-$, $E_{1/2}$ = −0.65 V). (*d*) Saturated NO, about $3 \times 10^{-3}$ M ($E_{irrev}$ = −1.02 and −1.41 V).

These irreversible voltammograms (Fig. 1) demonstrate that the reduction of NO occurs at significantly lower potentials than that of $O_2$, but cannot quantitatively identify the potential for reduction of NO nor allow for mechanistic interpretation. Previous voltammetric or polarographic investigations of the reduction of NO in aqueous solution on Hg or C electrodes reported half-waves between −0.5 and −1.10 V (23, 38) but none have directly assessed the reversibility or mechanism of reduction. Direct measurement of the one-electron reduction of NO requires a fast technique amenable to low NO concentrations, first achieved by Benderskii *et al*. (24) in a little-cited series of reports. Electron photoemission from Hg into a solution of $NO_2^-$ solution was used to form NO in low concentrations at the Hg electrode surface, and the resulting photocurrents were analyzed at varied potentials. By varying the frequency of the alternating photoexcitation, reduction currents were obtained attributable to short-lived intermediates of $NO_2^-$ decomposition, including NO. By this method, a reversible reduction of NO was reported at

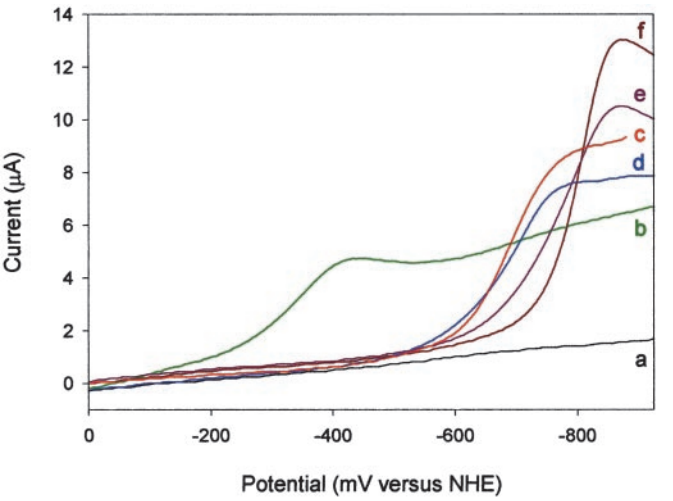


**Fig. 2.** Linear sweep voltammograms for pH-dependent reduction of NO. (*a*) Background. (*b*) Saturated $O_2$, about $3 \times 10^{-3}$ M ($O_2/O_2^-$, $E_{1/2}$ = −0.320 V). (*c–f*) Saturated NO, about $3 \times 10^{-3}$ M, in buffer at (*c*) pH 4, (*d*) pH 7, (*e*) pH 10, and (*f*) pH 13 ($NO/NO^-$, $E_{1/2}$ = −0.686, −0.684, −0.768, and −0.797 V, respectively).

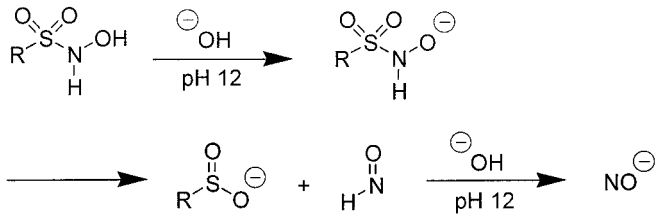


**Fig. 3.** Generation of nitroxyl anion ($NO^-$) from an alkyl sulfohydroxamic acid at pH 12.

−0.81 V (Table 1). Our electrochemical results described herein lend semiquantitative support to this highly negative reduction potential for NO.

**Chemical Reduction Experiments.** The reductive capacity of $NO^-$ was further characterized through chemical means. Alkyl and aryl sulfohydroxamic acids are relatively stable at physiological pH, but rapidly liberate HNO and/or $NO^-$ under basic conditions (Fig. 3) (39, 40).

$NO^-$ was generated from MSHA at high pH, and its redox chemistry was investigated by observing electron transfer from $NO^-$ to a series of viologen acceptors (Fig. 4). Although reduction of $MV^{2+}$ and $V1^{2+}$ were found to be facile, formation of the $V2^{\cdot+}$ chromophore at 516 nm upon reaction of $V2^{2+}$ with $NO^-$ was slow, indicating that the reduction potential of $NO^-$ is close to −0.7 V. The resultant viologen radical cations arise via reduction by $NO^-$, and not by the putative intermediate, hyponitrite ($N_2O_2^{2-}$), as the latter possesses a reduction potential of about +0.4 V versus NHE.

Conversely, reduction of methyl viologen $MV^{2+}$ was not observed upon reaction with HNO/$NO^-$ generated from a decomposing solution of Angeli's salt at pH 7. This observed difference in reactivity suggests that only nitroxyl (HNO) is generated from Angeli's salt at pH 7, whereas $^3NO^-$ is generated from MSHA at pH 12.

## Discussion

**Biological Implications.** It is well established that NO is capable of binding to a variety of proteins which otherwise bind and reduce $O_2$. For example, NO forms stable nitrosyl complexes with cytochrome *c* oxidase (16, 41) and cytochrome P450 (42–44), two primary $O_2$-binding and $O_2$-reducing proteins, and formation of these complexes has been implicated in the inhibition of their activity. Up to now, it has been difficult to reconcile the observation that NO was capable of binding at the $O_2$ binding site of these redox proteins and yet was not readily reduced, as (*i*) NO binds with high affinity to the $O_2$-reducing proteins; and (*ii*) based on its previously reported reduction potentials, the reduction of NO should be thermodynamically more facile than that of $O_2$.

From a physiological perspective, it is important that NO is resistant to reduction via the electron transport chain or, in general, via electron transfer processes compared with $O_2$. The substantial thermodynamic barrier predicted for the conversion

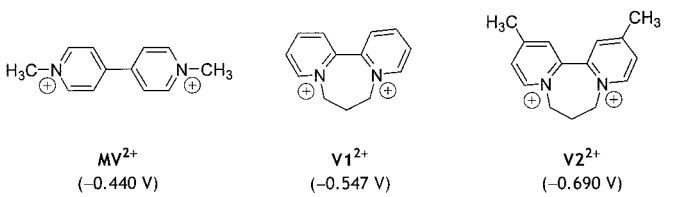


**Fig. 4.** Viologen electron acceptors. Experimental reduction potentials of each acceptor (versus NHE) are given in parentheses.

of NO to $NO^-$ suggests that, under physiological conditions, direct reduction of NO to $NO^-$ does not occur to a significant extent. Other mechanisms for *in vivo* generation of HNO and/or $NO^-$ in biological systems do exist (14, 45–47); however, direct one-electron reduction of NO to form $NO^-$ is improbable.

An important question now arises from these findings: does this thermodynamic barrier to reduction, compared with $O_2$, represent a chemically important aspect of NO biochemistry/physiology? Clearly this barrier to reduction may increase the lifetime of NO by eliminating reduction as a mechanism of NO degradation. There are now profound physiological implications of this resistance to reduction.

(*i*) Previous reports indicate that HNO is cytotoxic (8, 9). If the reduction of NO to HNO were facile, the utility of NO as a physiological messenger molecule would be greatly compromised and, indeed, organisms would not have evolved to use NO in so many diverse and important ways. (*ii*) A specific example involves the opposite effects of HNO and NO in ischemia-reperfusion injury (8). NO protects against postischemic injury, whereas exogenously delivered HNO greatly exacerbates injury. The barrier to reduction allows NO to exert its protective effects and prevent the endogenous formation of this potentially toxic reduced species. (*iii*) The ability of NO to regulate or inhibit mitochondrial activity depends on the stability of the cytochrome *c* oxidase-nitrosyl complex. Because NO is stable with respect to reduction and has high affinity for cytochrome *c* oxidase, it readily inhibits and regulates mitochondrial respiration by competition with $O_2$.

NO reduction to nitrous oxide ($N_2O$) can occur with cytochrome P450nor, and perhaps even with the heme protein cytochrome *c* oxidase, although this has been disputed (48). Our results make it very unlikely that this could occur by direct reduction of NO and subsequent reaction of HNO, namely 2 HNO → $N_2O$ + $H_2O$. The reduction of NO by cytochrome *c* oxidase appears to occur at only high concentrations of NO ($1–2 \times 10^{-3}$ M), and under these conditions the reduction likely occurs through a bis-NO-cytochrome *c* oxidase complex (49).[††] Instead, it is likely that a reduction of an $(NO)_2$ complex is occurring, rather than of a free NO or a monomeric adduct. Cytochrome P450nor, an enzyme specifically used by denitrifying fungi to convert $NO_3^-$ and $NO_2^-$ to $N_2O$, is also capable of reducing NO to $N_2O$ (50). In this reaction, a reduced iron-NO complex is proposed to react with another NO to generate $N_2O$. Thus, as with cytochrome *c* oxidase, it is unlikely that NO reduction to free $NO^-$ occurs directly. Generation of $N_2O$ requires the interaction of two NO molecules at the active site of the enzyme, an unlikely event given the low concentration of NO under physiological or even pathophysiological conditions. Thus, our contention that NO is much more difficult to reduce compared with $O_2$ pertains strictly to the monomeric species and remains consistent with other studies that likely involve reduction of multiassociated NO complexes.

**Consequences of NO Reduction Potentials on Nitroxyl Acidity.** Just as the reduction potentials for NO were originally derived from the literature pKa value of 4.7 for HNO, the pKa of HNO may be derived from the reduction potentials of NO. Stanbury (25) has estimated a value of −3.3 kcal/mol for the aqueous solvation energy of HNO; a similar value of −4.6 kcal/mol is predicted by our polarized continuum model calculations. From the respective gas phase $\Delta_f G^0$ values for HNO and NO of 29.4 and 20.7 kcal/mol and the aqueous solubility of NO of $1.9 \times 10^{-3}$ M/atm as provided in the Stanbury review, a pKa of 11.6 (±3.4) is derived for $^3NO^-$. From the singlet-triplet gap of 20.9 kcal/mol predicted for $NO^-$ (12), a pKa value of 23.0 (±3.4) is predicted for $^1NO^-$. These revised values based on reduction potentials are significantly higher than previous theoretical and experimental estimates, but are in accord with a recent theoretical prediction

by Dixon of a pKa range of 10 to 13 for $^3NO^-$ (D. A. Dixon, personal communication). Thus, HNO is now implicated to be the nearly exclusive species present in the acid/base equilibrium of $HNO/NO^-$ in biological systems.

It is important to note that the actual observed potential for reduction of NO to $^3NO^-$ will be a function of NO concentration, as well as of the pH of the solution in which $^3NO^-$ is generated. Our calculated reduction potential of −0.76 V corresponds to a standard state of 1 M NO. The concentration of NO under physiological conditions is substantially lower, typically within the nanomolar to micromolar range (51, 52). A decrease in the concentration of NO will give rise to a more negative reduction potential.

Protonation of $^3NO^-$ to HNO will be thermodynamically favorable in aqueous solution and result in a positive shift in NO reduction potential as the pH of the solution is lowered. This effect can be clearly seen in curves *c–f* in Fig. 2. From the pKa value of 11.6 for $^3NO^-$, a potential of about −0.5 V (1 M versus NHE) can be estimated for the reduction of NO to $^3NO^-$ and subsequent protonation to HNO at pH 7.2. Despite the favorable thermochemistry of protonation, direct reduction of NO to $^3NO^-$ under physiological conditions is still energetically prohibitive.

## Conclusion

Clearly, the reduction potential of NO is significantly more negative than has been widely reported. Our measured half-wave potential for the reduction of NO at pH 7 is −0.68 V (versus NHE), and our observation that only species with reduction potentials more positive than approximately −0.7 V are reduced by a decomposing solution of the $HNO/NO^-$ donor, MSHA, support our calculated value of −0.76 V for the reduction of free NO to $^3NO^-$. At significantly higher concentrations, reduction of dimers or higher-order adducts of NO becomes possible. The reduction potential of the metastable $(NO)_2$ is predicted to be quite favorable, +0.33 V (1 M versus NHE) (M.D.B. and K.N.H., unpublished results).

The establishment of the resistance of NO toward biological reduction represents a fundamental change in understanding of NO biochemistry. Although NO reduction was previously considered to be a probable biological occurrence, satisfactory explanations were lacking for the absence of NO reduction even when $O_2$ was reduced. We have established that NO is relatively inert to direct, one-electron reduction processes that can occur under physiological conditions. This process allows $O_2$ to be reduced exclusively in the presence of NO and represents a significant demarcation in the physiological chemistry of these two important diatomics. The high thermodynamic barrier for conversion of NO to HNO via a direct reduction pathway may allow for the distinct physiological effects observed for these redox-related species.

We are appreciative of Dr. David Dixon (Pacific Northwest National Laboratory) for sharing with us theoretical results before publication, and Prof. Peter Wardman (Gray Cancer Institute, Northwood, U.K.) for helpful discussions. We are grateful to the National Institutes of General Medical Sciences, National Institutes of Health (Grant GM59446 to K.N.H.), and the National Science Foundation (Grant CHE-0096380 to J.M.F. and E.F.; Grant CHE-0100774 to P.J.F.) for financial support of this research. M.D.B. acknowledges the support of the National Research Service Award, National Institutes of Health (Grant F32CA76770). W.L. was supported by a Seed Grant from the Chao Family Cancer Center, and C.S. was supported by a U.S. Public Health Service National Research Service Award (Grant GM08496).

1. Kerwin, J. F., Jr., Lancaster, J. R. & Feldman, P. L. (1995) *J. Med. Chem.* **38,** 4343–4362.
2. Brown, G. C. (1999) *Biochim. Biophys. Acta* **1411,** 351–369.
3. Cooke, J. P., Mont-Reynaud, R., Tsao, P. S. & Maxwell, A. J. (2000) in *Nitric Oxide Biology and Pathobiology*, ed. Ignarro, L. J. (Academic, San Diego), pp. 759–783 and references therein.
4. Heitmeyer, M. R. & Corbett, J. A. (2000) in *Nitric Oxide Biology and Pathobiology*, ed. Ignarro, L. J. (Academic, San Diego), pp. 785–810 and references therein.
5. Gonzales-Zulueta, M., Dawson, V. L. & Dawson, T. M. (2000) in *Nitric Oxide Biology and Pathobiology*, ed. Ignarro, L. J. (Academic, San Diego), pp. 695–710 and references therein.
6. Giulivi, C., Poderoso, J. J. & Boveris, A. (1998) *J. Biol. Chem.* **273,** 11038–11043.
7. Darley-Usmar, V. M., Patel, R. P., O'Donnell, V. B. & Freeman, B. A. (2000) in *Nitric Oxide Biology and Pathobiology*, ed. Ignarro, L. J. (Academic, San Diego), pp. 265–276 and references therein.
8. Ma, X. L., Cao, F., Liu, G. L., Lopez, B. L., Christopher, T. A., Fukuto, J. M., Wink, D. A. & Feelisch, M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 14617–14622.
9. Wink, D. A., Feelisch, M., Fukuto, J., Christodoulou, D., Jourd'heuil, D., Grisham, M., Vodovotz, V., Cook, J. A., Krishna, M., DeGraff, W., *et al.* (1998) *Arch. Biochem. Biophys.* **351,** 66–74.
10. Chazotte-Aubert, L., Oikawa, S., Gilibert, I., Bianchini, F., Kawanishi, S. & Ohshima, H. (1999) *J. Biol. Chem.* **274,** 20909–20915.
11. Miranda, K. M., Espey, M. G., Yamada, K., Krishna, M., Ludwick, N., Kim, S., Jourd'heuil, D., Grisham, M. B., Feelisch, M., Fukuto, J. M. & Wink, D. A. (2001) *J. Biol. Chem.* **276,** 1720–1727.
12. Bartberger, M. D., Fukuto, J. M. & Houk, K. N. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 2194–2198.
13. Doyle, M. P., Mahapatro, S. N., Broene, R. D. & Guy, J. K. (1988) *J. Am. Chem. Soc.* **110,** 593–599.
14. Wong, P. S.-Y., Hyun, J., Fukuto, J. M., Shiroda, F. N., DeMaster, E. G. & Nagasawa, H. T. (1998) *Biochemistry* **37,** 5362–5371.
15. Paolocci, N., Saavedra, W. F., Miranda, K. M., Martignani, C., Isoda, T., Hare, J. M., Espey, M. G., Fukuto, J. M., Feelisch, M., Wink, D. A. & Kass, D. A. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 10463–10468.
16. Hoshino, M., Ozawa, K. & Ford, P. C. (1993) *J. Am. Chem. Soc.* **115,** 9568–9575.
17. Wood, P. M. (1987) *Trends Biochem. Sci.* **12,** 250–251.
18. Sawyer, D. T. (1991) *Oxygen Chemistry* (Oxford Univ. Press, New York), pp. 19–51.
19. de Vooys, A. C. A., Koper, M. T. M., van Santen, R. A. & can Veen, J. A. R. (2001) *J. Catalysis* **202,** 387–394 and references therein.
20. da Cunha, M. C. P. M., De Souza, J. P. I. & Nart, F. C. (2000) *Langmuir* **16,** 771–777.
21. Hara, K., Kamata, M., Sonoyama, N. & Sakata, T. (1998) *J. Electroananal. Chem.* **451,** 181–186.
22. Ye, S. & Kita, H. (1993) *J. Electroanal. Chem.* **346,** 489–495.
23. Ehman, D. L. & Sawyer, D. T. (1968) *J. Electroanal. Chem.* **16,** 541–549.
24. Benderskii, V. A., Krivenko, A. G. & Ponomarev, E. A. (1989) *Soviet Electrochem.* **25,** 154–161.
25. Stanbury, D. M. (1989) *Adv. Inorg. Chem.* **33,** 69–138.
26. Grätzel, V. M., Taniguchi, S. & Henglein, A. (1970) *Ber. Bunsenges. Phys. Chem.* **74,** 1003–1010.
27. Montgomery, J. A., Frisch, M. J., Ochterski, J. W. & Petersson, G. A. (1999) *J. Chem. Phys.* **110,** 2822–2827.
28. Cossi, M., Barone, V., Cammi, R. & Tomasi, J. (1996) *Chem. Phys. Lett.* **255,** 327–335.
29. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Zakrzewski, V. G., Montgomery, J. A., Jr., Stratmann, R. E., Burant, J. C., *et al.* (1998) GAUSSIAN (Gaussian, Pittsburgh).
30. Conner, C. N., Donald, C. E., Hughes, M. N. & Sami, C. (1989) *Polyhedron* **8,** 2621–2622.
31. Linstrom, P. J. & Mallard, W. G., eds. (2001) *National Institute of Standards and Technology Chemistry WebBook: National Institute of Standards and Technology Standard Reference Database No. 69*, July 2001, http://webbook.nist.gov/chemistry.
32. Ram, M. S. & Stanbury, D. M. (1985) *Inorg. Chem.* **24,** 2954–2962.
33. Koppenol, W. H. & Rush, J. D. (1987) *J. Phys. Chem.* **91,** 4429–4430.
34. Schwarz, H. A. & Dodson, R. W. (1989) *J. Phys. Chem.* **93,** 409–414.
35. Pearson, R. G. (1986) *J. Am. Chem. Soc.* **108,** 6109–6114.
36. Koppenol, W. H. (1983) in *Oxy Radicals and Their Scavenger Systems—Vol. I: Molecular Aspects*, eds. Cohen, G. & Greenwald, R. A. (Elsevier, New York), pp. 274–277.
37. Seddon, W. A., Fletcher, J. W. & Sopchysyn, F. C. (1973) *Can. J. Chem.* **51,** 1123–1130.
38. Riccoboni, I. & Lanza, P. (1948) *Ric. Sci.* **18,** 1055.
39. Bonner, F. T. & Ko, Y. H. (1992) *Inorg. Chem.* **31,** 2514–2519.
40. King, S. B. & Nagasawa, H. T. (1999) *Methods Enzymol.* **301,** 211–220.

41. Brudvig, G. W., Stevens, T. H. & Chan, S. I. (1980) *Biochemistry* **19,** 5275–5285.
42. Ebel, R. E., O'Keefe, D. H. & Peterson, J. A. (1975) *FEBS Lett.* **55,** 198–201.
43. O'Keefe, D. H., Ebel, R. E. & Peterson, J. A. (1978) *J. Biol. Chem.* **253,** 3509–3516.
44. Hu, S. & Kincaid, J. R. (1991) *J. Am. Chem. Soc.* **113,** 9760–9766.
45. Hobbs, A. J., Fukuto, J. M. & Ignarro, L. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10992–10996.
46. Schmidt, H. H. H. W., Hofman, H., Schindler, U., Shutenko, Z. S., Cunningham, D. D. & Feelisch, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14492–14497.
47. Sharpe, M. A. & Cooper, C. E. (1998) *Biochem. J.* **332,** 9–19.
48. Stubauer, G., Giuffre, A., Brunori, M. & Sarti, P. (1998) *Biochem. Biophys. Res. Commun.* **245,** 459–465.
49. Zhao, X.-J., Sampath, V. & Caughey, W. S. (1994) *Biochem. Biophys. Res. Commun.* **204,** 537–543.
50. Shiro, Y., Fujii, M., Iizuka, T., Adachi, S., Tsukamoto, K., Nakahara, K. & Shoun, H. (1995) *J. Biol. Chem.* **270,** 1617–1623.
51. Tyler, D. D. (1975) *Biochem. J.* **147,** 493–504.
52. Moncada, S., Palmer, R. M. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.