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Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine

(nitric oxide synthase/oxidative metabolism)

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ABSTRACT **Nitric oxide (NO) in oxygen-containing aqueous solution has a short half-life that is often attributed to a rapid oxidation to both NO_2^- and NO_3^-. The chemical fate of NO in aqueous solution is often assumed to be the same as that in air, where NO is oxidized to NO_2 followed by dimerization to N_2O_4. Water then reacts with N_2O_4 to form both NO_2^- and NO_3^-. We report here that NO in aqueous solution containing oxygen is oxidized primarily to NO_2^- with little or no formation of NO_3^-. In the presence of oxyhemoglobin or oxymyoglobin, however, NO and NO_2^- were oxidized completely to NO_3^-. Methemoglobin was inactive in this regard. The unpurified cytosolic fraction from rat cerebellum, which contains constitutive NO synthase activity, catalyzed the conversion of L-arginine primarily to NO_3^- (NO_2^-/NO_3^- ratio = 0.25). After chromatography on DEAE-Sephacel or affinity chromatography using 2′,5′-ADP-Sepharose 4B, active fractions containing NO synthase activity catalyzed the conversion of L-arginine primarily to NO_2^- (NO_2^-/NO_3^- ratio = 5.6) or only to NO_2^-, respectively. Unpurified cytosol from activated rat alveolar macrophages catalyzed the conversion of L-arginine to NO_2^- without formation of NO_3^-. Addition of 30 μM oxyhemoglobin to all enzyme reaction mixtures resulted in the formation primarily of NO_3^- (NO_2^-/NO_3^- ratio = 0.09 to 0.20). Cyanide ion, which displaces NO_2^- from its binding sites on oxyhemoglobin, inhibited the formation of NO_3^-, thereby allowing NO_2^- to accumulate. These observations indicate clearly that the primary decomposition product of NO in aerobic aqueous solution is NO_2^- and that further oxidation to NO_3^- requires the presence of additional oxidizing species such as oxyhemoproteins.**

Sufficient evidence has been amassed to indicate a wide biological role for endogenous nitric oxide (NO) in modulating physiological and pathophysiological processes (1). NO is synthesized in various cell types by a family of isoforms of NO synthase (2). Some isoforms are constitutive and activated by calcium, whereas other isoforms are inducible and regulated by transcriptional mechanisms. Both isoforms catalyze the same complex oxidation of L-arginine to NO plus L-citrulline (3–6). The mechanism of catalysis of NO synthase is similar to that for the cytochrome P450 monooxygenases in that molecular oxygen is incorporated into the substrate by reactions involving NADPH, flavins, and heme (7).

To appreciate the diverse biological actions of NO, it is essential to understand not only the biosynthesis but also the metabolism of NO and the chemistry of NO in aqueous solution. NO endogenously synthesized by vascular endothelial cells has a short biological half-life of 5 sec or less (8, 9). Similar concentrations (0.01–1 μM) of authentic NO in pure aqueous solution, however, display half-lives of 500 sec or longer (10). This means that, in the presence of biological tissues, NO is rapidly converted to a less-active or inactive product. The chemical lability of NO in cells and tissues has been attributed to a rapid oxidation to both NO_2^- and NO_3^- (11–14). The common belief that NO is oxidatively metabolized to both NO_2^- and NO_3^- derives largely from experiments with intact cells, tissues, and whole animals rather than pure aqueous systems. For example, macrophages that have been activated in culture to induce NO synthase activity generate both NO_2^- and NO_3^- (15). Moreover, endogenous NO_2^- production in whole animals cannot be observed by assaying plasma or urine because of the nearly complete oxidation of NO or NO_2^- to NO_3^- (12). NO gas reacts with oxygen to form NO_2 gas, which dimerizes to N_2O_4. N_2O_4 dismutates spontaneously in water to form NO_2^- (as HNO_2) and NO_3^- (as HNO_3) (16). The assumption is commonly made that NO in an aqueous solution containing oxygen generates NO_2^- and NO_3^-. This assumption is inconsistent with chemical studies showing that pure aqueous solutions of NO generate primarily NO_2^- (10, 17, 18).

The objective of the present study was to examine some of the chemical properties of NO in aqueous solution with regard to factors involving the oxidative formation of NO_2^- and NO_3^-. To this end authentic NO and NO generated from L-arginine by constitutive and inducible NO synthase isoforms were compared. Moreover, the influence of added oxyhemoproteins on the oxidation of authentic and L-arginine-derived NO was determined as oxyhemoproteins are known to catalyze the oxidation of NO and NO_2^- to NO_3^- (19–21).

MATERIALS AND METHODS

Reagents. Hemoglobin (human), myoglobin (equine), methemoglobin (human), bacterial lipopolysaccharide (phenol-extracted *Escherichia coli* serotype 0128:B12), minimal essential medium (MEM), L-arginine, L-citrulline, NADPH, FAD, calmodulin, and the remainder of the reagents employed for the NO synthase assays (22) were purchased from Sigma. Fungibact was from Irvine Scientific and interferon γ (rat, recombinant) was from GIBCO/BRL. Dowex AG50W-X8 (H^+ form), 100–200 mesh, and Dowex AG 1-X8 (acetate form), 100–200, mesh were obtained from Bio-Rad. DEAE-Sephacel and 2′,5′-ADP-Sepharose 4B were obtained from Pharmacia. Vanadium(III) chloride was purchased from Aldrich. Sodium nitrite, sodium nitrate, and potassium cyanide were obtained from Fisher. NO gas (99%) and NO_2 gas (99%) were obtained from Matheson. Aquasol-2 was purchased from DuPont, and

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Abbreviation: NO, nitric oxide.

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L-[2,3,4,5-^{3}H]arginine hydrochloride (77 Ci/mmol; 1 Ci = 37 GBq) was from Amersham.

Determination of NO_2^- and NO_3^-. The concentrations of NO_2^- and NO_3^- were determined by chemiluminescence as described (22). Samples containing NO_2^- and NO_3^- were first reduced to NO, which was quantified by a chemiluminescence detector after reaction with ozone. Refluxing 1% potassium iodide in glacial acetic acid was used to determine NO_2^- concentrations. NO_2^- is quantitatively reduced to NO in this solution. NO_3^- cannot be detected by this method because it cannot be reduced to NO. Both NO_2^- and NO_3^-, however, are quantitatively reduced to NO in refluxing acidic vanadium(III). Refluxing 0.1 M vanadium(III) chloride in 2 M HCL was used to determine total NO_2^- plus NO_3^- (NO_X^-) concentrations. Values for NO_3^- were calculated by subtracting NO_2^- from NO_X^- values. Fig. 1 illustrates the selectivity of refluxing acidic iodide for NO_2^- and the linearity of the standard curves for NO_2^-, NO_3^-, and combinations of the two over the concentration range used in this study.

Chemical Reactions Involving NO, NO_2, and NO_2^-. Individual experimental details are described in the figure legends. NO gas was purified just before use (23) and handled with nitrogen-flushed gas-tight Teflon-sealed Hamilton microliter syringes. NO_2 gas was used as obtained commercially (99%) and was handled carefully in a fume hood with gas-tight stainless-steel Hamilton microliter syringes. Oxyhemoglobin and oxymyoglobin were prepared from dithionite-reduced hemoproteins in oxygenated 50 mM sodium phosphate (pH 7.4) as described (24).

NO Synthase Assay. NO synthase activity was measured by monitoring the formation of both NO and L-citrulline as described (22). The source of constitutive NO synthase was the cytosolic fraction from rat cerebellum obtained by centrifugation of 25% (wt/vol) homogenates at 100,000 × *g* for 60 min at 4°C. The composition of the homogenizing buffer was 50 mM Tris·HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, and 2 μM leupeptin. The source of inducible NO synthase was the cytosolic fraction from cytokine-activated rat alveolar macrophages obtained by centrifugation of 1% homogenates at 100,000 × *g* for 60 min at 4°C. A rat alveolar macrophage cell line, NR8383 (kindly provided by R. J. Helmke, Department of Pediatrics, University of Texas Health Sciences Center, San Antonio), was used as described (25). Cells were incubated at 10^6 cells per ml in MEM containing 7.5% (wt/vol) sodium bicarbonate, 1% Fungibact, 2 mM L-glutamine, and 2% (vol/vol) fetal calf serum. NO synthase was induced by addition of bacterial lipopolysaccharide (35 ng per 10^6 cells) plus interferon γ (500 units per 10^6 cells) and incubation of the cells for 18 hr. Cells were collected, washed twice with phosphate-buffered saline, and 1% homogenates were made as described above.

Purification of Constitutive NO Synthase. Crude cytosolic fraction obtained from rat cerebellum was chromatographed on a column (0.7 cm in diameter) of DEAE-Sephacel (2-ml bed volume) equilibrated in homogenizing buffer to remove contaminating hemoglobin (26). Cytosol (5 ml) was cycled through the resin five times and the resin was washed with 20 ml of homogenizing buffer. NO synthase was eluted with buffer containing 300 mM NaCl and appeared in the second 1-ml fraction. Contaminating hemoglobin was eluted in the initial wash cycle. Constitutive NO synthase from rat cerebellum cytosol was also partially purified by affinity chromatography on a column (0.7 cm in diameter) of 2′,5′-ADP-Sepharose 4B (1-ml bed volume) as described (27). Purified preparations had ≈150-fold greater specific activity than the starting unpurified cytosol.

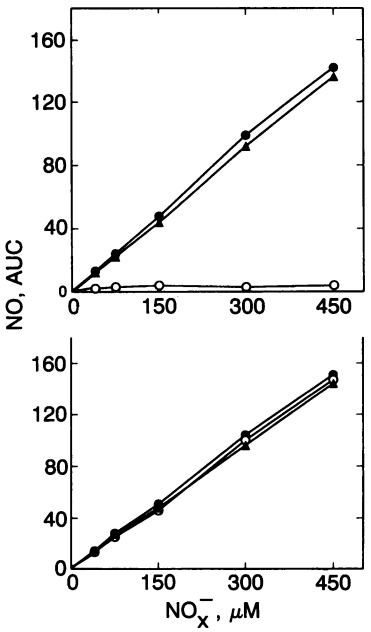

FIG. 1. Standard curves for concentrations of NO_2^-, NO_3^-, and NO_2^- plus NO_3^- as determined by chemiluminescence. Data are expressed as area under the curve (AUC) in relative units as analyzed with a Hewlett–Packard HP 3396 series II integrator. ●, NO_2^-; ○, NO_3^-; ▲, NO_2^- plus NO_3^-. (*Upper*) Refluxing acidic potassium iodide. (*Lower*) Refluxing acidic vanadium(III). NO_X^- refers to either NO_2^- or NO_3^-. (*Upper*) Combinations of NO_2^- ($NaNO_2$) plus NO_3^- ($NaNO_3$) were prepared by mixing together equimolar concentrations such that final concentrations of each component were those indicated on the *x* axis. (*Lower*) However, equal volumes of $NaNO_2$ and $NaNO_3$ solutions each at the concentrations indicated on the *x* axis were combined to yield a total NO_X^- concentration equal to the same value.

RESULTS

Oxidation of NO in Aqueous Solution. The principal oxidation product of NO in aerated or oxygenated sodium phosphate buffer (pH 7.4) was NO_2^- (Fig. 2). A reaction time of 30 min was more than sufficient to allow the complete oxidation of NO to NO_2^-, as the half-life of 300 μM NO in air-saturated

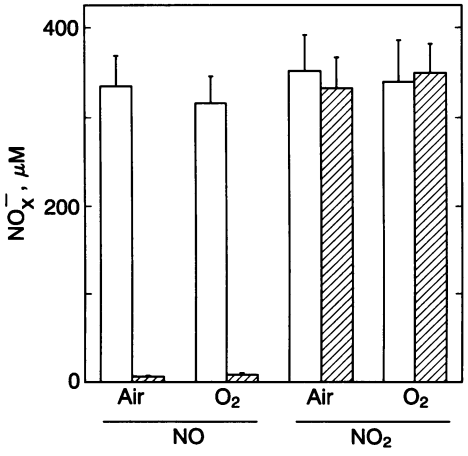

FIG. 2. Oxidation of NO and NO_2 in aqueous solution containing air or oxygen. Data are expressed as the concentration of NO_X^-. Bars: open, NO_2^-; hatched, NO_3^-. NO-saturated water under nitrogen (100 μl) was delivered into 2.4 ml of 50 mM sodium phosphate (pH 7.4) that had been equilibrated at 22°C with either air or oxygen, and samples were incubated at 22°C for 30 min. NO_2 gas (25 μl) was carefully and slowly delivered as a fine stream of bubbles into the bottom of a tube containing 2.5 ml of 50 mM sodium phosphate (pH 7.4) that had been equilibrated at 22°C with either air or oxygen. Values represent the mean ± SEM of duplicate determinations from three experiments.

aqueous solution is <1 sec (10). Additional experiments in which reaction times were varied from 15 min to 24 hr revealed that NO_2^- was still the principal oxidation product of NO. Little or no NO_3^- was detected. The reaction of NO_2 gas in aqueous solution was much different than that of NO in that NO_2 reacted with the water to yield equimolar quantities of NO_2^- and NO_3^- (Fig. 2).

Reactions of NO in Aqueous Solution with Hemoproteins. An aliquot of aqueous saturated NO solution under nitrogen was delivered into a reaction vessel containing 0.33 mM oxyhemoglobin (1.33 mM monomer concentration) in 50 mM sodium phosphate buffer (pH 7.4) at 22°C and exposed to air. The initial concentration of NO in solution was ≈300 μM. Aliquots of reaction mixture were removed every 30 min and assayed for NO_2^- and NO_3^-. The concentration of NO_2^- declined to nearly undetectable levels after 3 hr, whereas the concentration of NO_X^- remained constant (Fig. 3). Thus, the concentration of NO_3^- increased at the same rate that NO_2^- disappeared. The half-life of NO_2^- in the presence of oxyhemoglobin at 22°C under the defined experimental conditions was estimated to be 75 min. A similar reaction conducted at 37°C yielded a half-life of NO_2^- of ≈45 min (Fig. 4). Oxymyoglobin, at a concentration of heme equivalent to that of oxyhemoglobin, also catalyzed the complete oxidation of NO to NO_3^-. Although only the values for NO_2^- are illustrated in Fig. 4, NO_X^- determinations and NO_3^- calculations were made as well, and the formation of NO_3^- always paralleled the disappearance of NO_2^-. The reaction of NO with oxymyoglobin appeared to be autocatalytic in that the initial rate of oxidation was slow and increased with time. This observation has been made previously and is not well understood (21). Methemoglobin failed to catalyze the oxidation of NO to NO_3^-.

As the half-life of relatively high concentrations (300 μM) of NO in aqueous solution is <1 sec, it is possible that much or most of the NO in the experiments described above was in the form of NO_2^- prior to reaction with oxyhemoproteins to form NO_3^-. Accordingly, sodium nitrite ($Na^+NO_2^-$) was tested for its reactivity with hemoproteins at 37°C. The reactions of NO_2^- with oxyhemoglobin and oxymyoglobin were nearly identical to the reactions of NO with the oxyhemoproteins (Fig. 4). Methemoglobin failed to catalyze the oxidation of NO_2^- to NO_3^-. Potassium cyanide (3–10 mM) completely prevented the oxyhemoglobin-catalyzed oxidation of NO_2^- to NO_3^-.

Chemical Properties of the NO Reaction Product of NO Synthase. The NO reaction product formed from L-arginine by unpurified and partially purified constitutive NO synthase prepared from rat cerebellum cytosol was analyzed for NO_2^- and NO_3^- (Fig. 5). When expressed as total NO_X^- formed, the

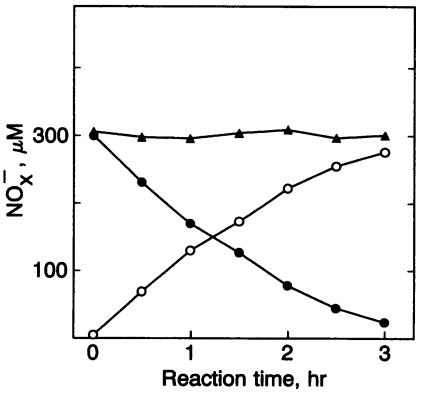

FIG. 3. Influence of oxyhemoglobin on the oxidation of NO. Data are expressed as the concentration of NO_X^-. NO-saturated water under nitrogen (100 μl) was delivered into 2.4 ml of 50 mM sodium phosphate (pH 7.4) containing 0.33 mM oxyhemoglobin at 22°C and exposed to air. Aliquots (100 μl) of the reaction mixture were removed at 30-min intervals and assayed for NO_2^- (●), NO_3^- (○), and NO_X^- (▲). The data illustrated are from one representative experiment of a total of six experiments.

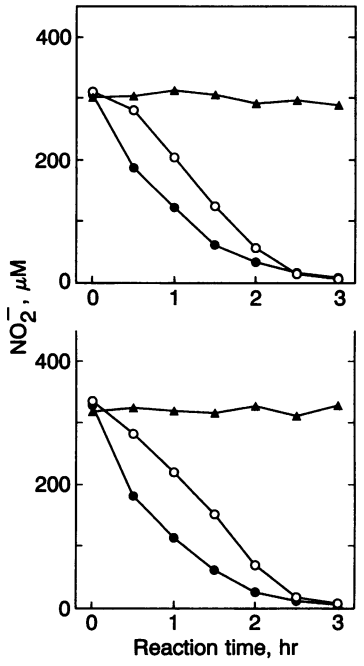

FIG. 4. Influence of oxyhemoglobin, oxymyoglobin, and methemoglobin on the oxidation of NO and NO_2^-. (*Upper*) NO-saturated water under nitrogen (100 μl) was delivered into 2.4 ml of 50 mM sodium phosphate (pH 7.4) containing 0.33 mM oxyhemoglobin (●), 1.3 mM oxymyoglobin (○), or 0.33 mM methemoglobin (▲) at 37°C. Aliquots (100 μl) of the reaction mixtures were removed at 30-min intervals and assayed for NO_2^-. (*Lower*) $NaNO_2$ (100 μl; 8 mM) instead of NO was delivered into 2.4 ml of the three reaction mixtures as described above. The data illustrated are from one representative experiment of a total of four experiments involving NO and four experiments involving $NaNO_2$.

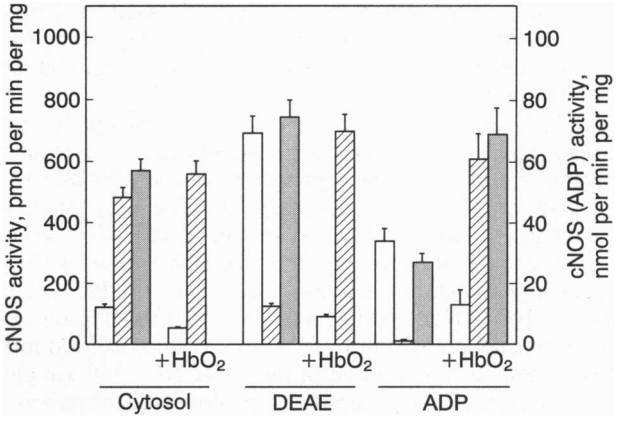

FIG. 5. Influence of partial enzyme purification and oxyhemoglobin on the oxidation of NO formed from L-arginine by constitutive NO synthase (cNOS) from rat cerebellum. The *y* axis on the left signifies the specific activity of NO synthase in the cytosol and after DEAE-Sephacel chromatography (DEAE). The *y* axis on the right signifies the specific activity of NO synthase purified by affinity chromatography (ADP). Enzymatic reactions were conducted at 37°C for 30 min in 50 mM Tris·HCl (pH 7.4) containing 1 mM L-arginine, 2 mM NADPH, 25 μM tetrahydrobiopterin, 100 μM FAD, 5 μg of calmodulin, 2 mM $CaCl_2$, enzyme fraction containing 0.4–0.8 mg of protein (4 μg of protein for ADP-purified enzyme), and 30 μM oxyhemoglobin (HbO_2) where indicated. Reaction products were assayed for NO_2^- (open bars), NO_X^- (hatched bars), and L-citrulline (shaded bars). Data represent the mean ± SEM of duplicate or quadruplicate determinations from three experiments.

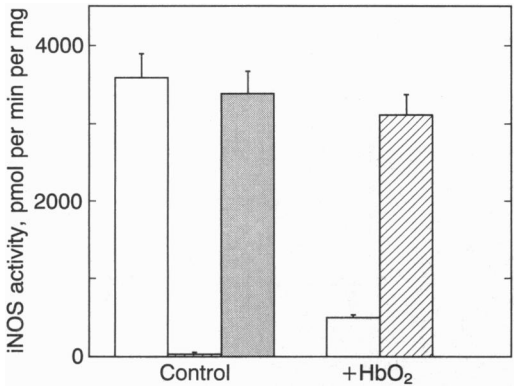

FIG. 6. Oxidation of NO formed from L-arginine by inducible NO synthase (iNOS) from activated rat alveolar macrophages. Enzymatic reactions were conducted at 37°C for 30 min in 50 mM Tris·HCl (pH 7.4) containing 1 mM L-arginine, 2 mM NADPH, 25 μM tetrahydrobiopterin, 100 μM FAD, enzyme fraction containing 0.28 mg of protein, and 30 μM oxyhemoglobin (HbO_2) where indicated. Reaction mixtures were assayed for NO_2^- (open bars), NO_3^- (hatched bars), and L-citrulline (shaded bars). Data represent the mean ± SEM of duplicate or quadruplicate determinations from three experiments.

concentration of NO formed during the incubation period was ≈200 μM, which is similar to the concentration of authentic NO and NO_2^- employed in the above studies. The major NO reaction product generated in the enzymatic mixtures containing unpurified cytosol fractions was NO_3^-. The molar ratio of NO_2^-/NO_3^- was 0.25. The cytosol fraction was chromatographed on a column of DEAE-Sephacel to remove proteins including hemoglobin and other hemoproteins. Fractions containing partially purified NO synthase were assayed and analyzed for NO_2^- and NO_3^- (Fig. 5). Unlike the unpurified cytosol, partially purified fractions generated primarily NO_2^- (NO_2^-/NO_3^- ratio = 5.6). Enzymatic reactions with partially purified NO synthase conducted in the presence of added 30 μM oxyhemoglobin, however, generated primarily NO_3^- (NO_2^-/NO_3^- ratio = 0.13). NO synthase purified by affinity chromatography on columns of 2',5'-ADP-Sepharose 4B generated only NO_2^-, whereas the major enzymatic product generated in the presence of added 30 μM oxyhemoglobin was NO_3^- (Fig. 5).

The NO reaction product formed from L-arginine by unpurified inducible NO synthase prepared from the cytosol of activated rat alveolar macrophages (cell line NR8383) was analyzed for NO_2^- and NO_3^- (Fig. 6). The only reaction product generated in enzymatic mixtures was NO_2^-. When enzymatic reactions were conducted in the presence of added 30 μM oxyhemoglobin, however, the major product generated was NO_3^- (NO_2^-/NO_3^- ratio = 0.17). The addition of 3 mM or 10 mM potassium cyanide to enzyme reaction mixtures containing constitutive or inducible NO synthase plus 30 μM oxyhemoglobin completely prevented the formation of NO_3^-, thereby allowing NO_2^- to accumulate.

DISCUSSION

The present data indicate that NO in aqueous solution containing oxygen has a different chemical fate than NO in a gaseous mixture containing oxygen. NO gas reacts with oxygen to form NO_2 gas, which dimerizes to N_2O_4. Although several different mechanisms are possible, this is a termolecular reaction and can be written as follows (28):

$$NO + O_2 \rightleftharpoons OONO$$

$$OONO + NO \rightleftharpoons ONOONO \rightarrow 2NO_2 \rightarrow O_2NNO_2\ (N_2O_4)$$

N_2O_4 dismutates spontaneously in water and buffer at pH 7.4 to yield both NO_2^- and NO_3^- by the following reaction (16):

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$$

Accordingly, both NO_2^- and NO_3^- can be formed from NO gas in the presence of oxygen and water.

NO in oxygen-containing aqueous solution, however, did not yield significant quantities of NO_3^-. In contrast, the delivery of NO_2 gas into reaction vessels containing phosphate buffer yielded equimolar quantities of NO_2^- and NO_3^-. This means that in the experiments with NO solution either NO_2 was not formed or NO_2 did not accumulate in quantities sufficient to dimerize to N_2O_4 and react with water to yield both NO_2^- and NO_3^-. The oxidation of NO to NO_2^- in aqueous solution can be represented as follows (10, 17):

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+$$

The precise mechanism of the above chemical reaction is not well understood but could be represented in the following manner:

$$2NO + O_2 \rightarrow 2NO_2$$

$$2NO_2 + 2NO \rightleftharpoons 2N_2O_3$$

$$2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+$$

The chemical reaction proposed above would necessitate a rapid series of reactions immediately after the formation of NO_2 to maintain the concentration of NO_2 low enough so as not to form appreciable quantities of N_2O_4, which would otherwise proceed to form NO_3^- along with NO_2^-. Nitrous anhydride (N_2O_3) reacts rapidly with water to yield NO_2^- (17).

To appreciate and understand the significance of the short biological half-life of endogenously synthesized NO, it is essential to understand the chemical half-life of NO. The autooxidation of NO in oxygen-containing aqueous solution follows second-order kinetics (10). That is, the rate of NO oxidation is proportional to the square of the NO concentration. This means that NO concentrations of ≈300 μM in the presence of oxygen have a half-life of <1 sec. Although the steady-state concentration of NO is difficult to determine, the quantities of authentic NO and NO generated by NO synthase in the present study were equivalent to concentrations ranging from 125 μM to 350 μM. Much lower NO concentrations of 0.05–1 μM that are biologically active possess half-lives ranging from 500 sec to several hours. This is true for NO in pure aqueous solutions but is not true for low concentrations of authentic NO or endogenous NO in the presence of biological tissues, where the half-life of NO is 3–5 sec (8, 9). Numerous chemical interactions in cells or tissues could account for the short biological half-life of NO including reactions with oxygen, superoxide anion, other oxygen-derived radicals, and oxyhemoproteins (8).

Reactions between excess NO or NO_2^- and lower concentrations of oxyhemoproteins have been studied with the objective of monitoring the conversion of oxyhemoprotein to methemoglobin or metmyoglobin (16, 17, 19). The objective of the present study, however, was to determine the influence of excess oxyhemoprotein on rates of oxidation of NO or NO_2^- to NO_3^-. Methemoglobin or metmyoglobin were not measured in the present experiments but are presumably the coproducts of oxidation (29). Oxyhemoglobin and oxymyoglobin catalyzed the complete conversion of NO or NO_2^- to NO_3^- at 37°C. Methemoglobin failed to catalyze the formation of appreciable quantities of NO_3^-.

In comparing the conversion of NO to NO_3^- with the conversion of NO_2^- to NO_3^- in the presence of oxyhemopro-

teins, we noted that the rates of conversion to NO_3^- were similar. This observation is attributed to the rapid reaction between the relatively high concentrations (300 μM) of NO and oxygen to yield NO_2^- before the NO can react appreciably with oxyhemoglobin to yield NO_3^-. High concentrations of NO are well-known to possess very short half-lives in the presence of oxygen in that the chemical half-life of NO varies inversely with the square of its concentration (10). Thus, the apparent formation of NO_3^- from NO in the presence of oxyhemoglobin (Fig. 3) is attributed to the reaction between NO_2^- and oxyhemoglobin to yield NO_3^-.

The mechanism by which oxyhemoproteins catalyze the oxidation of NO and NO_2^- to NO_3^- is not well understood, and various mechanisms have been proposed (16, 17, 19). Part of the problem lies in the dependency of the reaction on numerous factors including concentration of NO or NO_2^-, temperature, pH, and the autocatalytic nature of the reaction at low NO concentrations. Two common equations describing the stoichiometry of the reaction between NO_2^- and oxyhemoglobin or oxymyoglobin are as follows (20, 21):

$$2Fe^{2+}O_2 + 3NO_2^- + 2H^+ \rightleftharpoons 2Fe^{3+} + 3NO_3^- + H_2O$$

$$4Fe^{2+}O_2 + 4NO_2^- + 4H^+ \rightleftharpoons 4Fe^{3+} + 4NO_3^- + O_2 + 2H_2O$$

The oxidation of NO_2^- to NO_3^- by oxyhemoproteins can be inhibited by excess cyanide ion, which displaces NO_2^- from its binding site on the heme iron (20). This earlier observation was confirmed in the present study, where 3–10 mM potassium cyanide abolished the oxidation of 300 μM NO_2^- to NO_3^- in the presence of 0.33 mM oxyhemoglobin.

One objective of this study was to compare authentic and endogenously synthesized NO with regard to oxidative metabolism. Unpurified cytosolic fractions prepared from rat cerebellum catalyzed the conversion of L-arginine primarily to NO_3^-, and this was attributed to the presence of contaminating hemoglobin and perhaps other hemoproteins that catalyze the oxidation of NO_2^- to NO_3^-. Chromatography on DEAE-Sephacel to remove most of the contaminating hemoglobin (24) or affinity chromatography on 2′,5′-ADP-Sepharose 4B to partially purify the NO synthase (25) yielded fractions that catalyzed the conversion of L-arginine primarily or only to NO_2^-. The cytosolic fraction prepared from a homogeneous cell line of activated rat alveolar macrophages, which was rich in induced NO synthase activity but free of contaminating hemoglobin, catalyzed the conversion of L-arginine to NO_2^- but not to NO_3^-. Consistent with the view that contaminating hemoglobin in unpurified rat cerebellum fractions was responsible for NO_3^- formation, the addition of 30 μM oxyhemoglobin back to enzyme reaction mixtures containing partially purified constitutive NO synthase or unpurified inducible NO synthase resulted in the formation primarily of NO_3^-. The finding that cyanide, which binds to heme iron and thereby blocks NO_2^- binding (17), completely prevented the formation of NO_3^- in crude enzyme reaction mixtures in the absence or presence of added oxyhemoglobin argues strongly that contaminating hemoproteins were largely responsible for any NO_3^- formation from L-arginine by NO synthase.

In conclusion, the present study indicates clearly that comparable concentrations of authentic NO and L-arginine-derived NO catalyzed by NO synthase behave similarly in aqueous solution containing oxygen. The principal spontaneous oxidation product of NO in aqueous solution in the absence of contaminating biological constituents such as hemoproteins is NO_2^-. Neither the constitutive nor the inducible isoform of NO synthase catalyzes the oxidation of L-arginine to NO_3^-, and L-arginine-derived NO is not oxidized to NO_3^- unless contaminating hemoproteins are present.

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