Generation of Nitric Oxide by Enzymatic Oxidation of 
N-Hydroxy-N-nitrosaminoles*

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The nitric oxide (\(\text{N}=\text{O}\)) free radical exhibits potent cytotoxic, mutagenic and vasodilatory properties. We have examined the hypothesis that the hydroxynitrosamino functionality (\(\text{N}=\text{O}\)) which occurs naturally in antineoplastic and antihypertensive agents, will directly generate \(\text{N}=\text{O}\) following peroxidatic 1-electron oxidation.

Cupferron (\(\phi\text{N}=\text{O}\)) is indeed an excellent \((k > 10^7 \text{ M}^{-1} \text{s}^{-1})\) substrate for horseradish peroxidase. The products are \(\text{N}=\text{O}\) and nitrosobenzene (\(\phi\text{N}=\text{O}\)) which are generated and consumed as follows. First, cupferron is oxidized by the classical peroxidatic mechanism to form an unstable nitroxide free radical (\(2\phi\text{N}=\text{O}\) + \(\text{H}_2\text{O}_2\) \(\rightarrow\) \(2\phi\text{N}=\text{O}\) + \(2\text{OH}^-\)) which then forms \(\text{N}=\text{O}\) and \(\phi\text{N}=\text{O}\) spontaneously (\(\phi\text{N}=\text{O}\) → \(\text{N}=\text{O}\) + \(\phi\text{N}=\text{O}\)). The \(\text{N}=\text{O}\) then reacts with \(\phi\text{N}=\text{O}\) to reform cupferron (\(\phi\text{N}=\text{O}\) + \(2\text{N}=\text{O}\) + \(\text{H}_2\text{O}\) \(\rightarrow\) \(\phi\text{N}=\text{O}\) + \(\text{N}=\text{O}\) + \(\text{NO}_2\) + \(2\text{H}^+\)) or with the enzyme to generate the characteristic peroxidase–\(\text{N}=\text{O}\) chromophore. Simultaneously, in a competitive reaction with \(\text{O}_2\), the \(\text{N}=\text{O}\) is converted to \(\text{NO}_2\) (\(4\text{N}=\text{O}\) + \(\text{O}_2\) + \(2\text{H}_2\text{O}\) \(\rightarrow\) \(4\text{NO}_2\) + \(4\text{H}^+\)). The reactivity of hydroxynitrosamino compounds with horseradish peroxidase is in the order cupferron > hydroxynitrosaminomethane > alanosine.

These model reactions, involving direct oxidation of the hydroxynitrosamino moiety, comprise a novel pathway for the biological production of \(\text{N}=\text{O}\).

The \(\text{N}=\text{O}\) free radical is highly reactive and exhibits potent cytotoxic, mutagenic, and vasodilatory activities (13–16).

We have examined mechanisms by which nitro and nitroso compounds may be enzymatically converted to reactive products capable of covalently modifying cellular constituents (7–11). In particular, we have reported that enzymatic oxidation of the \(\alpha\)-amino group of alanosine to the carbonyl level permits decomposition of the hydroxynitrosamine moiety into nitric oxide in a reaction that resembles the \(\beta\)-decarboxylation of oxalacetate (Equation 1) (12). The nitric oxide free radical is generally stable under physiological conditions in the absence of enzyme action (1–4).

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The hemoprotein horseradish peroxidase is known to catalyze the 1-electron oxidation of compounds which resemble hydroxynitrosamines. These compounds include hydroxamic acids (17) and nitroalkane anions (18). We have thus examined alanosine and cupferron as substrates for horseradish peroxidase in order to test the feasibility of nitric oxide generation from hydroxynitrosamines by direct enzymatic oxidation of the hydroxynitrosamino group.

MATERIALS AND METHODS
L-Alanosine (NSC 153355) was a gift from D. A. Cooney, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute. Cupferron and nitrosobenzene were obtained from Aldrich. The crystalline sodium salt of N-hydroxy-N-nitrosamine (Aldrich) (19). Aqueous hydrogen peroxide was obtained from Fisher and standardized by polarographic measurement of the O₂ formed by the action of catalase. Nitric oxide was a Matheson product.

Horseradish peroxidase was the Type VI product offered by Sigma. D-Amino acid oxidase was isolated from hog kidneys (20) while other enzymes were Sigma products.

Electronic spectra were recorded with the Cary 15 instrument. Optical kinetic measurements were performed with a Gilford model 2200 recording spectrophotometer or a Durrum-Gibson stopped-flow apparatus. Oxygen concentrations were measured by means of a Clark-type polarographic electrode from Yellow Springs Instrument Co.

The concentration of O₂ in air-saturated solutions was taken to be 0.24 mM. Solutions were made anaerobic by flushing with 99.999% argon (Airco Industrial Gases). Peroxidase concentration was measured using ε₂₈₃ = 8.51 × 10⁵ M⁻¹ cm⁻¹ (21, 22). Inorganic nitrite was colorimetrically assayed by means of its diazotization of sulfanilamide followed by chronogenic diazo coupling to N-(1-naphthyl)ethylenediamine (23). All reaction mixtures were maintained at 25 °C.

RESULTS
Products of Enzymatic Oxidation of Cupferron—Peroxidase catalyzes the 2-electron reduction of H₂O₂ to water by a variety of substrates of the form AH₂. Generally, two molecules of reducing substrate sequentially contribute 1 electron each to the H₂O₂-oxidized enzyme as described under Equation 3 (24).

\[
Peroxidase + H₂O₂ \rightarrow \text{Compound I} \quad (3a)
\]

\[
\text{Compound I} + AH₂ \xrightarrow{k₁} \text{Compound II} + AH⁺ \quad (3b)
\]

\[
\text{Compound II} + AH₂ \xrightarrow{k₂} \text{peroxidase} + AH⁺ \quad (3c)
\]

The product AH⁺ may undergo various nonenzymatic reactions. For instance, we found that the propane-2-nitronate anion is oxidized by the enzyme to a radical which can dimerize or autoxidize (18). 2,3-Dinitro-2,3-dimethylbutane is the major product found under anaerobic conditions while acetone is the major product obtained in the presence of O₂ (18).

We report here that cupferron is a good substrate for horseradish peroxidase. As illustrated in Fig. 1, the organic moiety of cupferron is quantitatively recovered as nitrosobenzene after action of the enzyme in the presence of H₂O₂. With limiting H₂O₂, 2.0 mol of cupferron are oxidized per mol of H₂O₂ in either aerobic or anaerobic buffers (Figs. 2 and 3). It is interesting that the nitrosobenzene can be observed to slowly (and incompletely) revert back to cupferron in anaerobic but not in aerobic reaction mixtures (Fig. 2). Nitric oxide is scavenged by O₂ in the aerobic reaction mixtures (25).
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of the NO\textsubscript{2}\textsuperscript{-} assay reagents. Under the conditions described in Table I, 0.65 mol of NO\textsubscript{2}\textsuperscript{-} precursor was detected per mol of aerobically oxidized cupferron. The yield of NO\textsubscript{2}\textsuperscript{-} precursor was reduced by 69% when the reaction mixture was purged of volatile components by continuous flushing with argon. This observation indicates that the autoxidizable NO\textsubscript{2}\textsuperscript{-} precursor formed in the reaction mixture is volatile and supports our contention that the product in question is, in fact, nitric oxide. It was not possible to completely rid reaction mixtures of NO\textsubscript{2}\textsuperscript{-} precursors by purging with argon. However, NO\textsubscript{2}\textsuperscript{-} can arise anaerobically by several routes which include hydrolysis of the nitric oxide dimer and reaction of nitric oxide with nitrosobenzene (26).

Nitric oxide binds to hemoproteins perturbing their visible spectra (27, 28), and sufficient nitric oxide accumulates under anaerobic conditions after peroxidase-mediated oxidation of excess cupferron by H\textsubscript{2}O\textsubscript{2} that a modified peroxidase spectrum is observed. That spectrum, which is distinct from those of peroxidase Compounds 1 and II, is shown in Fig. 5 and is identical to the NO-peroxidase spectrum (28). Native peroxidase is regenerated from the modified enzyme upon either purging with argon or reaction with O\textsubscript{2}.

We conclude that the initial inorganic product of peroxidase-catalyzed oxidation of cupferron is nitric oxide on the basis of its stoichiometric requirements, volatility, autoxidizability to NO\textsubscript{2}\textsuperscript{-}, reaction with nitrosobenzene, and reversible binding to heme. Lawless and Hawley have previously reported that the cyclic voltammographic behavior of cupferron is also consistent with Equation 2 (26).

Experiments in which stoichiometric quantities of enzyme and substrate are reacted provide evidence that the enzyme generates nitrosobenzene and nitric oxide by oxidizing cupferron via the classic peroxidase mechanism (Equation 3).
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FIG. 5. Spectra of peroxidase species. Compound I (---) is generated from 5.0 μM native peroxidase (- - - -) upon reaction with 50 μM H2O2 in 200 mM potassium phosphate buffer at pH 7.0 and 25 °C. Addition of 100 μM cupferron to that reaction mixture then yields a new species (-----) which can be converted back to native peroxidase by flushing the reaction mixture with either argon or oxygen. Also shown is the spectrum of Compound II (----). This spectrum was obtained by reaction of 5.0 μM native peroxidase with 5.0 μM H2O2 followed by 2.5 μM ascorbic acid. The spectrum of the modified enzyme (-----) coincides with that of the nitric oxide-modified enzyme (28).

Involving only 1-electron substrate oxidation. Reaction of resting peroxidase with 1.0 eq of H2O2 generates the spectrum of Compound I. Since k1 is much larger than k0, addition of 1.0 eq of cupferron to Compound I cleanly generates the spectrum of Compound II (Fig. 5).

Kinetics of Cupferron Oxidation—In 200 mM potassium phosphate buffer containing 1.0 mM H2O2 at pH 7.0 and 25 °C, the initial rate of peroxidase-catalyzed oxidation of cupferron is linearly dependent upon cupferron concentration up to at least 5.0 mM and corresponds to a bimolecular rate constant of 9.8 × 10^7 M^-1 s^-1. Saturation kinetics are observed when H2O2 is the variable substrate. The K_m value for peroxide depends linearly upon cupferron concentration with a proportionality constant of 1.9 × 10^5 M^-1 s^-1. The steady-state kinetics at pH 7.0 are thus described by Equation 5 where \( \phi_r = 9.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \) and \( \phi_v = 5.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \).

The value of \( \phi_r^{-1} \) increases with decreasing pH (Fig. 6). At pH 4.9, which roughly corresponds to the pK value of 4.5 for cupferron ionization (26), the measured value of \( \phi_r^{-1} \) is about half that calculated by extrapolation of the linear portion of the plot shown in Fig. 6. This result is similar to that observed with propane-2-nitronate as the substrate (18).

The rates of reaction of cupferron with Compound I and with Compound II were measured directly in the stopped-flow apparatus by observing Compound I at 411 nm and Compound II at 388 nm. These processes are described by the bimolecular rate constants \( k_1 \) and \( k_2 \), respectively, in Equation 3. We found that \( k_1 = 1.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \) and \( k_2 = 4.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) at pH 7.0. In the simplest peroxidatic scheme (Equation 3) consistent with Equation 5, \( \phi_r^{-1} \) should be given by \( 2k_1k_2(k_1 + k_2)^{-1} \). The value of \( \phi_r^{-1} \) calculated in this way from the stopped-flow measurements of \( k_1 \) and \( k_2 \) at pH 7.0 is 6.7 × 10^6 M^-1 s^-1. This value is in good agreement with the value of 9.8 × 10^7 M^-1 s^-1 determined for \( \phi_r^{-1} \) from the steady-state velocity measurements. The kinetic data are thus adequately described by Equation 5 and are consistent with the mechanism given in Equation 3. Interestingly, the bimolecular rate constant \( k_1 \) for the reaction of cupferron with Compound I increases with decreasing pH so that the value exceeds 10^9 M^-1 s^-1 at pH 4.5 (Fig. 6). The value thus approaches the diffusion-controlled limit.

**Peroxidase-catalyzed Oxidation of Alanosine and N-Hydroxy-N-nitrosaminomethane**—Alanosine and N-hydroxy-N-nitrosaminomethane also serve as reductive substrates for horseradish peroxidase, but these alkane derivatives are substantially less reactive with the enzyme than the aromatic compound cupferron. The reactivities of the substrates with peroxidase Compounds I and II are compared in Table II. Reaction of substrate with Compound II as described by \( k_2 \) in Equation 3 is rate-limiting at pH 5.0. The values of \( k_2 \) for cupferron and hydroxynitrosaminomethane are, respectively, 1.8 × 10^7 and 2.7 × 10^7 times that for L-alanosine at that pH. A preference for compounds with hydrophobic side chains is generally seen in the reaction of horseradish peroxidase with other chemical classes of reductive substrates (17, 29).

The alkyl substrates are expected to be oxidized to nitrooxide radicals which rapidly decompose into nitric oxide and C-nitroso compounds as shown for cupferron in Equation 4. Unlike nitrosobenzene, these C-nitroso compounds would rapidly tautomerase to oximes. However, analysis of the products of oxidation of the less reactive substrates alanosine and hydroxynitrosaminomethane is complicated by competing reactions that were not kinetically important or else not accessible in the case of cupferron. These competing reactions include the slow peroxidase-catalyzed oxidation of NO2 and oximes by H2O2 (30, 31). Substantially lower yields of NO2 are obtained from these reactions than from the reactions with cupferron.
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Nitric oxide occurs in the atmosphere as a result of lightning and of artificial pollution. It is also generated by microorganisms. Its systemic pharmacological effects are not known since it rapidly exerts severe pulmonary toxicity when administered by the inhalation route. So-called "silica pneumonia" in humans is thus secondary to nitric oxide evolution by microorganisms fermenting agricultural produce in poorly ventilated places (34, 35).

It remains to be ascertained whether oxidation of hydroxynitrosamines to nitric oxide occurs when the compounds are administered to living organisms. However, peroxidases resembling that from horseradish are widely distributed in nature (30). Furthermore, other types of enzymes may prove to catalyze 1-electron oxidation of hydroxynitrosamines. For instance, hydroxynitrosamines bear chemical similarity to hydroxyurea, and that drug is thought to be clinically useful because of its 1-electron oxidation by ribonucleotide reductase (36).

Prostaglandin synthetase is another pharmacologically important enzyme which can catalyze 1-electron oxidation of the N-hydroxy group (37).

Further experiments are required to determine whether nitric oxide production has any role in the therapeutic or adverse effects of alanosine (38, 39) or the carcinogenicity of cupferron (40). In this regard, it is interesting that antineoplastic and carcinogenic nitrosoureas and nitrosamines are metabolized in part to nitric oxide. Nitric oxide is produced by reduction, rather than 1-electron oxidation, of those compounds and occurs by the nonenzymatic action of thiols (41) and by the action of enzymes such as hepatic NADPH-cytochrome P-450 reductase (42). It may prove possible to deliberately design hydroxynitrosamines to be selectively toxic to organisms or tissues possessing nitric oxide-yielding enzymes.

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Table II

| Reaction rates of hydroxynitrosamines with peroxidase Compounds I and II |
|----------------------------------------------------------|
| The rate constants for reaction with Compound I are given by \( k_i \) while those for Compound II are given by \( k_p \) as defined in Equation 3. Disappearance of Compound I was followed in the stopped-flow apparatus at 411 nm while Compound II was observed at 398 nm. Compound I was generated by reaction of 5.0 \( \mu \text{M} \) \( \text{H}_{2}\text{O}_2 \) with 5.0 \( \mu \text{M} \) peroxidase. The buffer was 100 mM potassium acetate at pH 5.0 while phosphate was employed at pH 7.0 and 25 °C. |
| pH 5.0 | pH 7.0 |
| \( \text{L-Alanosine} \) |
| \( k_i \left( \text{M}^{-1} \text{s}^{-1} \right) \) | \( <1.4 \times 10^2 \) |
| \( k_p \left( \text{M}^{-1} \text{s}^{-1} \right) \) | \( 1.6 \times 10 \) |
| \( \text{CH}_3\text{N(NO)} \left( \text{NO} \right) \left( \text{O} \right)^{–} \) |
| \( k_i \left( \text{M}^{-1} \text{s}^{-1} \right) \) | \( 1.0 \times 10^4 \) |
| \( k_p \left( \text{M}^{-1} \text{s}^{-1} \right) \) | \( 4.3 \times 10^6 \) |
| Cupferron |
| \( k_i \left( \text{M}^{-1} \text{s}^{-1} \right) \) | \( >1.0 \times 10^7 \) |
| \( k_p \left( \text{M}^{-1} \text{s}^{-1} \right) \) | \( 2.9 \times 10^8 \) |

Table III

| Yields of nitrite upon enzymatic oxidation of alkyl hydroxynitrosamines |
|---------------------------------------------------------------|
| Inorganic nitrite was colorimetrically assayed upon completion of the peroxidase-catalyzed reaction of \( \text{H}_2\text{O}_2 \) with each hydroxynitrosamine at the indicated initial concentrations in the presence of 0.24 mM \( \text{O}_2 \) and 5.0 \( \mu \text{M} \) peroxidase in 100 mM potassium acetate buffer at pH 5.0 and 25 °C. One reason that the yields are substantially less than the theoretical limit of 2.0 mol of \( \text{NO}_2 \)/mol of \( \text{H}_2\text{O}_2 \) and that the yields decrease with increasing amounts of \( \text{H}_2\text{O}_2 \) is that enzymatic oxidation of nitrite competes with enzymatic oxidation of the hydroxynitrosamines. |
| [\( \text{H}_2\text{O}_2 \)]=[S] | [\( \text{NO}_2 \)]=[H\(_2\text{O}_2 \)] |
| \( \text{L-Alanosine} \) (1.4 mm) | 0.014 | 0.57 |
| (1.4 mm) | 0.028 | 0.43 |
| (0.1 mm) | 0.056 | 0.32 |
| \( \text{CH}_3\text{N(NO)} \left( \text{NO} \right) \left( \text{O} \right)^{–} \) (1.0 mm) | 0.10 | 1.50 |
| (0.1 mm) | 0.80 | 1.00 |

are thus obtained during the oxidation of alanosine and hydroxynitrosaminemethane when compared to cupferron. The data in Table III illustrate that, as expected, the yield of \( \text{NO}_2 \) from alanosine or hydroxynitrosaminemethane amounts to much less than 2.0 mol of \( \text{NO}_2 \)/mol of \( \text{H}_2\text{O}_2 \) consumed and decreases further as the \( \text{H}_2\text{O}_2 \) concentration approaches that of the hydroxynitrosamine substrate.

Other Oxidants—We have previously reported that nitroalkane anions are reductive substrates of the (heme-free) flavoenzymes D-amino acid oxidase from hog kidney (20, 32) and glucose oxidase from \( \text{Aspergillus niger} \) (33). The nitroalkane anions are also nonenzymatically oxidized by \( \text{KMnO}_4 \) and \( \text{K}_2\text{Fe(CN)}_6 \) under mild conditions (18). All of our observations thus far with nitrosalkanes as substrates for D-amino acid oxidase can be satisfied by ionic mechanisms (20, 32) while free-radical generation is apparent in the other reactions. Of these potential oxidants, only permanganate appears to accept electrons from the hydroxynitrosamines. The hydroxynitrosamines also failed to serve as reductive substrates for lactoperoxidase (buttermilk) and the copper-dependent enzymes ascorbate oxidase (yellow summer squash), tyrosinase (mushroom), and dopamine \( \beta \)-hydroxylase (bovine adrenal medulla). These observations and the marked differences (Table II) in reactivity of the three hydroxynitrosamines as substrates for horseradish peroxidase emphasize the active catalytic role of the enzyme in the conversion of the thermally stable hydroxynitrosamines into reactive products.
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