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CHEMICAL OXIDATION OF *N*-HYDROXYGUANIDINE COMPOUNDS

RELEASE OF NITRIC OXIDE, NITROXYL AND POSSIBLE RELATIONSHIP TO THE MECHANISM OF BIOLOGICAL NITRIC OXIDE GENERATION

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Abstract—N^{ω}-Hydroxy-L-arginine was found to cause vasodilation in arginine-depleted rabbit aorta. It is, therefore, likely to be a biosynthetic intermediate in the conversion of arginine to nitric oxide in this tissue. *N*-Hydroxyalkylguanidine compounds, including N^{ω}-hydroxy-L-arginine, were oxidized with various oxidizing agents and examined for their ability to release nitric oxide. All oxidizing agents tested were capable of oxidizing the *N*-hydroxyguanidine function but only lead tetra-acetate ($Pb(OAc)_4$) and potassium ferricyanide/hydrogen peroxide (K_3FeCN_6/H_2O_2) were capable of generating significant amounts of nitric oxide. Oxidation with K_3FeCN_6, lead oxide (PbO_2) and silver carbonate (Ag_2CO_3) resulted instead in the release of nitrous oxide (N_2O) presumably through the initial release of nitroxyl (HNO).

The generation of nitric oxide (NO‡) in biological systems has been demonstrated to play an important role in a variety of biological functions. The mechanism of NO formation is, as yet, not entirely understood. It is known that NO biosynthesis is a result of an enzyme-catalyzed oxidation of a terminal guanidinium nitrogen of arginine [1, 2]. This process has a requirement for both NADPH and oxygen. This initial oxidation of arginine appears to be a mono-oxygenation process in that N^{ω}-hydroxy-L-arginine (NOHA) is a biosynthetic intermediate in macrophage [3] and in bovine intrapulmonary artery [4]. Stuehr and coworkers [3] have also demonstrated that in the macrophage conversion of NOHA to NO is an enzymatic process. NOHA is likely to also be a biosynthetic intermediate for NO in other systems as well. As the chemistry of *N*-hydroxyguanidine compounds is relatively unexplored, we have investigated the chemical oxidation of *N*-hydroxyguanidine compounds as a prerequisite to understanding possible enzymatic processes that convert NOHA to NO. Mechanistically distinct chemical oxidants were tested for their ability to release NO from *N*-hydroxyguanidine compounds, including NOHA, to determine the chemical conditions that must exist for NO generation. NO generation from N^{ω}-hydroxy-L-arginine and model compounds, N^1-hydroxy-N^2-butylguanidine (NHBG) and *N*-(*N*-hydroxyamidino)piperidine (NHAP), has been investigated (Fig. 1). The relevance of these mechanistically distinct chemical oxidations to biological NO liberation and endogenous biosynthesis is addressed.

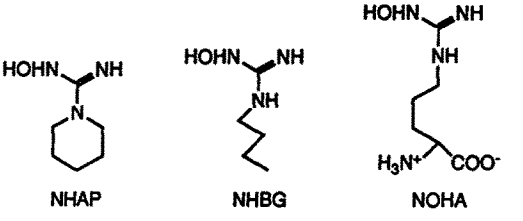

Fig. 1. Structures of compounds utilized in this study.

MATERIALS AND METHODS

Chemicals and solutions. NOHA was synthesized according to the method described earlier [5]. NHBG was synthesized by the general method of Bailey *et al.* [6]. Briefly, *n*-butyl amine was converted to the corresponding cyanamide using cyanogen bromide

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‡ Abbreviations: NO, nitric oxide; N_2O, nitrous oxide; HNO, nitroxyl/nitrosyl hydride; NHBG, N^1-hydroxy-N^2-butylguanidine; NHAP, *N*-(*N*-hydroxyamidino)piperidine; NOHA, N^{ω}-hydroxy-L-arginine; $Pb(OAc)_4$, lead tetra-acetate; K_3FeCN_6, potassium ferricyanide; PbO_2, lead oxide; K_3FeCN_6/H_2O_2, potassium ferricyanide/hydrogen peroxide; and Ag_2CO_3, silver carbonate.

followed by reaction with hydroxylamine. NHAP was synthesized by identical procedures from the commercially available *N*-cyanopiperidine (Aldrich Chemical Co., Milwaukee, WI). The products were characterized by proton nuclear magnetic resonance (NMR) spectroscopy using a Bruker AM360 instrument and by mass spectroscopy using a ZAB mass spectrometer (VG Analytical Inc., Manchester, UK). NHAP: NMR (deuterochloroform): δ 1.6 (broad s, 6H), 3.1 (broad singlet, 4H), 4.4 (broad s, 2H); mass spectrum: $m/z = 143$ (M^+, 15%), 84 (100%), exact mass measurement gives a molecular formula of $C_6H_{13}N_3O$ (143.1058622). NHBG; NMR (deuteroacetone): δ 0.9 (t, 3H), 1.3 (m, 2H), 1.45 (m, 2H), 2.9 (m, 2H), 4.0 (broad s, 1H), 4.5 (broad s, 2H); mass spectrum: $m/z = 131$ (M^+, 100%), 88 (40%), 72 (77%), exact mass measurement gives a molecular formula of $C_5H_{13}N_3O$ (131.1058622).

$Pb(OAc)_4$, PbO_2, and K_3FeCN_6 were purchased from the Aldrich Chemical Co. Ag_2CO_3 precipitated on celite was prepared by the method outlined in Fieser and Fieser [7]. L-Arginine, phenylephrine hydrochloride and acetylcholine were all purchased from the Sigma Chemical Co. (St. Louis, MO). N^G-Methyl-L-arginine was synthesized by the general method of Patthy *et al.* [8]. Krebs-bicarbonate solution consisted of 118 mM NaCl, 1.5 mM $CaCl_2$, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11 mM glucose and 0.023 mM EDTA.

Arterial smooth muscle relaxation. Rabbit aortic rings were prepared and changes in isometric force were measured as described previously [9]. All tissues were submaximally precontracted using phenylephrine before being challenged by the compounds. NOHA, NHAP and NHBG were added to the tissue baths after the tissues had been depleted of arginine by 24 hr of incubation in the tissue baths. Arginine depletion was confirmed by a loss or attenuation of acetylcholine response. The acetylcholine response was regained when arginine was added back to the tissue bath. In these arginine-depleted tissues, NOHA was able to elicit vasorelaxation, whereas NHAP and NHBG had no effect (see Fig. 2). After the NOHA-elicited relaxation, N^G-methyl-L-arginine was added to the tissue bath. The vasorelaxation caused by NOHA was shown to be reversible with the addition of this inhibitor.

In experiments involving evolved gases from the oxidation of the various *N*-hydroxyguanidine compounds, the headspace from the reaction vessel was swept and bubbled into the tissue bath using nitrogen (see Fig. 3). Fresh tissue was utilized in all cases.

Nitric oxide detection by chemiluminescence. NOHA, NHBG and NHAP were all oxidized by various oxidizing agents (see Table 1), and the evolution of nitric oxide was determined using an Antek 720 chemiluminescence detector. The reactions were carried out in a 200-mL three neck round bottom flask under nitrogen. All solvents were degassed prior to use by sparging with nitrogen for 15 min. Typically, the oxidant (approximately 0.05 mmol), in 2 mL of the appropriate solvent, was added to the flask followed by 5–10 min of a nitrogen sweep to remove oxygen. The *N*-hydroxyguanidine compounds (0.01 mmol), taken up in 2 mL of the appropriate degassed solvent, were then added to the oxidant through a rubber septum using a syringe. The reaction was allowed to proceed for 5 min under nitrogen before the headspace was swept into the detector with nitrogen at approximately 150 mL/min (see Fig. 3).

Nitrous oxide detection and characterization. N_2O was identified as a reaction product of the oxidation of NHAP and NHBG by K_3FeCN_6 and Ag_2CO_3 by GC and GC-mass spectral analysis. NHAP and NHBG (0.1 mmol) and Ag_2CO_3/celite (1 mmol) were placed in a 10-mL round bottom flask. The flasks were then flushed with nitrogen and sealed with a rubber septum. The reactions were started by the addition of 2 mL of degassed acetonitrile. The mixture was stirred for 5 min. A 10-mL sample of the headspace was then taken using a gas tight syringe and analyzed directly. The identical procedure was used for the oxidation of NHAP and NHBG by K_3FeCN_6 except that water was used as the solvent. N_2O detection was performed on a Carle gas chromatograph equipped with a 6 ft × ⅛ in. Porapak Q column and thermoconducticvity detector operating at room temperature and a flow rate of 20 mL/min. Tentative identification was obtained by comparison with the retention time of authentic N_2O. Further characterization of N_2O was performed on a Hewlett–Packard gas chromatography–mass spectroscopy (GC–MS) system consisting of an HP 5710A GC and an HP 5980A mass spectrometer on a 2 m × 1.9 mm Porapak Q column operating at 70° at a flow rate of 20 mL/min. The headspace of the reaction mixtures was injected directly on the column and the m/z mass fragment of 44 (M^+) monitored. The reactions generated a gas which responded to the selected ion (44) and with the identical GC retention time (4.5 min) as authentic N_2O.

Organic product determination for NHAP oxidation. The reaction product from the oxidation of NHAP was analyzed by GC and HPLC. GC analysis was performed on a Hewlett–Packard 5880 gas chromatograph equipped with a flame detector utilizing a Hewlett–Packard 25 m methyl silicone capillary column, 0.32 mm i.d., 0.52 mm film thickness with a flow rate of 3 mL/min, and an 8.3:1 split ratio. The GC was temperature programmed from 65° to 195° at a rate of 25°/min. Quantitation of *N*-cyanopiperidine was performed by GC utilizing a standard curve with 1-phenyl-2-butanone as an internal standard.

HPLC analysis was performed on a Rainin liquid chromatograph equipped with a 25 × 4.3 mm, 5 μm Microsorb C-18 reversed phase column and a Spectra-Physics 100 UV-vis detector operating at 210 nm. The following gradient was used to elute the reaction products; 40% acetonitrile:60% water to 100% acetonitrile over 8 min. Typically, the reaction mixture was rapidly filtered through a plug of glass wool and injected directly onto the column. In all instances, *N*-cyanopiperidine was the only product observed (as determined by retention time comparison with authentic standard).

RESULTS

The addition of NOHA directly to the tissue bath

after a 24-hr depletion of arginine in the rabbit aortic rings resulted in significant vasodilation (Fig. 2). The addition of NHAP or NHBG to the tissue bath gave no response. The vasorelaxation elicited by NOHA was reversed to a contractile response with the addition of a known inhibitor of NO synthesis, N^G-methyl-L-arginine. These results are consistent with previous work performed with bovine intrapulmonary artery indicating that NOHA is an intermediate in the synthesis of NO from arginine [4].

The *N*-hydroxyguanidine compounds NHBG and NHAP were oxidized with $Pb(OAc)_4$, K_3FeCN_6, PbO_2, K_3FeCN_6/H_2O_2, and Ag_2CO_3 under the conditions shown in Table 1. Due to solubility or solvent incompatibility problems, NOHA was oxidized only with K_3FeCN_6, K_3FeCN_6/H_2O_2 and $Pb(OAc)_4$. The release of NO from these reactions was monitored by direct chemiluminescence detection and by bioassay using rabbit aortic rings (Fig. 3). The chemiluminescence detector indicated that NO was released only from the oxidations performed with $Pb(OAc)_4$ and K_3FeCN_6/H_2O_2. Oxidations with K_3FeCN_6, PbO_2 and Ag_2CO_3 did not release NO. Careful analysis of the NHAP reactions, however, revealed that oxidation had occurred in all instances. NHAP was chosen for detailed study because of the stability and ease of analysis of the potential products. The analysis of the reaction products of the other substrates posed major analytical and synthetic challenges. The only identifiable organic reaction product from NHAP was the corresponding *N*-cyanopiperidine. None of the urea product, 1-piperidinecarboxamide, was ever found. The yield of *N*-cyanopiperidine ranged from 40 to 60% (Table 1). Also, GC analysis of the reactions with NHBG showed that no butylurea was generated under any of the reaction conditions. These urea products correspond to citrulline, the final product besides NO of the enzyme-catalyzed oxidation of arginine.

Bioassay results corroborate the results found with the chemiluminescence detector (Fig. 2, Table 1); of the oxidants tried, only $Pb(OAc)_4$ was able to oxidize the *N*-hydroxyguanidine compounds with release of NO as demonstrated by vasodilation. The gaseous products resulting from oxidation of NHAP with the other oxidants (Table 1) had no effect on rabbit aortic rings even though the primary organic product, *N*-cyanopiperidine, was the same as found in the $Pb(OAc)_4$-mediated oxidations. Preliminary analysis of the headspace of K_3FeCN_6- and Ag_2CO_3-mediated oxidation of NHAP by GC and GC–MS revealed that the primary volatile product was not NO but rather N_2O. Analysis of the headspace of the $Pb(OAc)_4$-mediated oxidation of NHAP showed no observable N_2O formation. (It is possible that N_2O was formed, but at levels below the limits of detectability of the GC assay.)

Attempted identification of intermediates in the oxidation of NHAP by any of the oxidants by TLC, GC or HPLC was unsuccessful. The only compound seen under these assay conditions was *N*-cyanopiperidine. Thus, any intermediates, if formed, were apparently unstable under the reaction or assay conditions.

DISCUSSION

The N-hydroxylation of arginine has been proposed as a crucial biosynthetic step in biological NO formation since NOHA has been shown unequivocally to be a biosynthetic intermediate in macrophage [3], and is consistent with being an intermediate in bovine intrapulmonary artery [4]. The results reported here with rabbit aorta are in agreement with the notion that NOHA is a biosynthetic intermediate since it also causes vasodilation in arginine-depleted tissues in a manner similar to arginine itself. Thus, the enzyme must N-hydroxylate arginine to generate NOHA as a first step in the biosynthesis of NO. The subsequent reaction or reactions that lead to NO release involve a formal three electron oxidation of NOHA and a carbon–nitrogen bond cleavage. Marletta and coworkers [2] have postulated a biosynthetic pathway in the macrophage which involves both NOHA and the corresponding nitroso compound as intermediates (Fig. 4) [The mechanism proposed by Marletta and coworkers cannot be entirely correct, however, as Kwon and coworkers [10] demonstrated that the oxygen incorporated into citrulline (the other reaction product besides NO) comes from oxygen and not water, as required of the Marletta mechanism.] The chemical properties of the proposed nitroso intermediate (the two electron oxidation product of NOHA) have yet to be explored. As an approach to elucidating the possible mechanisms by which NO can be released from *N*-hydroxyguanidine compounds (or the putative nitroso intermediate), oxidizing agents with varying proposed mechanisms of oxidation were tested for their ability to release NO. These oxidants are not intended to mimic the exact nature of the biochemically generated oxidant but rather to establish the chemical conditions conducive to NO generation. Although the proposed nitroso intermediate was not observed in these studies, it is likely that it was formed from the *N*-hydroxyguanidine compounds and underwent further reaction. Thus, the ability of the various oxidants to generate NO from *N*-hydroxyguanidines is likely to be a reflection of the reactivity of the nitroso intermediate.

The results of this study indicate that: (1) nitric oxide can be released from the oxidation of *N*-hydroxyguanidine compounds, presumably via a nitroso intermediate, but only under certain, specific oxidative conditions, (2) HNO can also be generated from the putative nitroso intermediate under other, specific oxidizing conditions, (3) the differences in the mechanism of oxidation by the various oxidants likely account for the differences in the nature of the released nitrogen oxide product (NO or HNO), (4) the organic product of the oxidation in aqueous solution is the cyanamide, or possibly the tautomeric carbodiimide in the case of NOHA or NHBG, and not the corresponding urea, and finally (5) the biological generation of NO from arginine likely involves a very specific oxidative interaction with an oxidative enzyme. These five points will now be addressed individually below.

NO *generation.* Interestingly, the only oxidants among those used that were capable of releasing NO from NOHA, NHAP or NHBG were $Pb(OAc)_4$ and

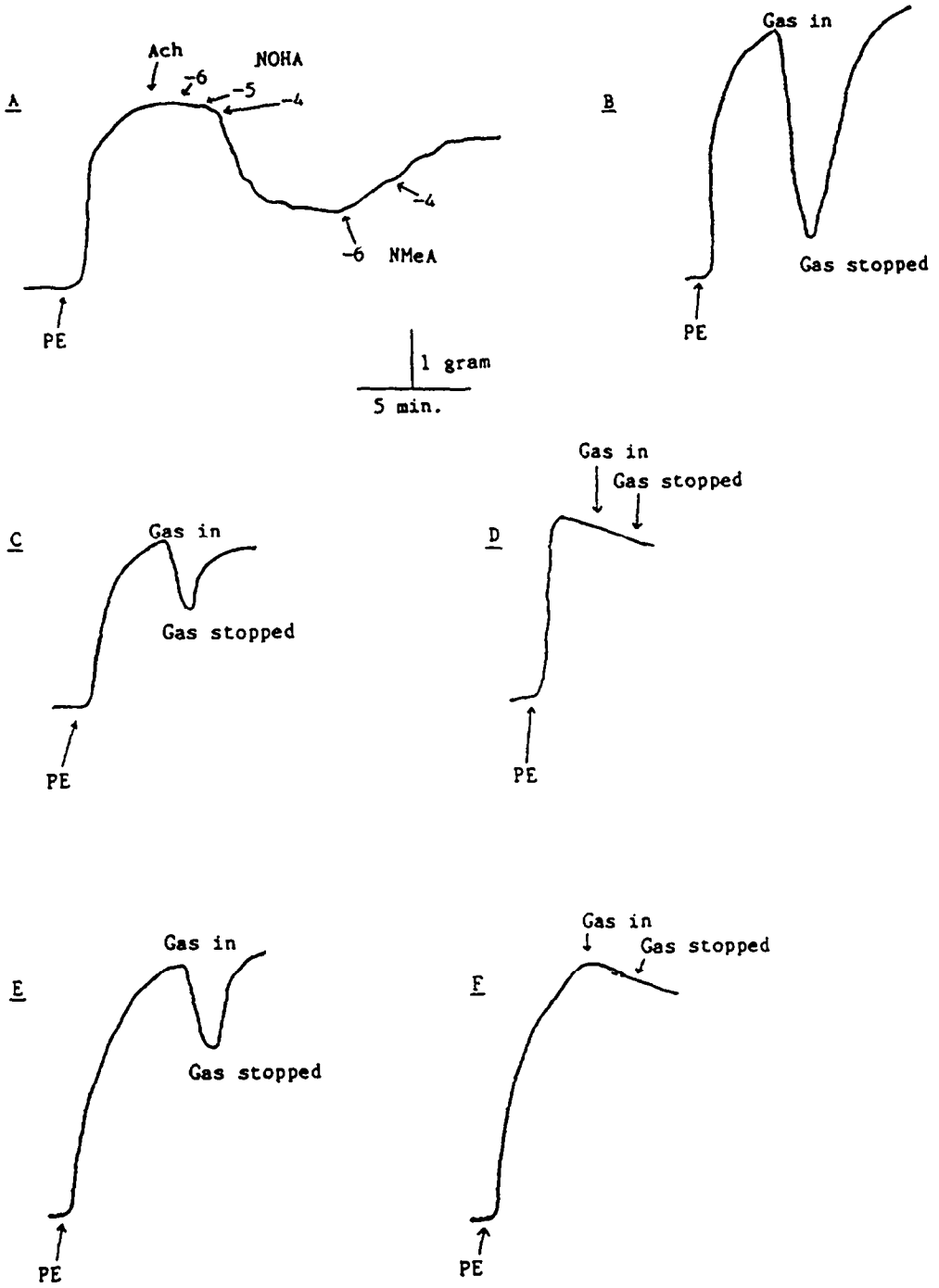

Fig. 2. Representative biological response traces of rabbit aortic rings precontracted with phenylephrine (PE) to (A) NOHA addition after arginine depletion, as shown by lack of response to acetylcholine (Ach), and N^G-methyl-L-arginine addition (NMeA), concentrations are expressed as the base powers of 10 and represent final bath concentrations, (B) evolved gases from the oxidation of NOHA by $Pb(OAc)_4$ in CH_3OH, (C) the oxidation of NHAP by $Pb(OAc)_4$ in acetonitrile, (D) the oxidation of NHAP by Ag_2CO_3 in acetonitrile, (E) the oxidation of NHBG by $Pb(OAc)_4$ in acetonitrile, and (F) the oxidation of NHBG by Ag_2CO_3 in acetonitrile. Results of NHAP or NHBG oxidation with K_3FeCN_6 (in water) or PbO_2 (in acetonitrile) gave results similar to panels D and F. PE was added to a final concentration of 0.001 mM in all cases to precontract tissues.

Table 1. Oxidation of *N*-hydroxyguanidine compounds by various oxidizing agents and the generation of nitric oxide and nitrous oxide*

Substrate	Oxidant	Solvent	NO†	N_2O‡	Vasorelaxing§	% CNP‖
NOHA	$Pb(OAc)_4$	MeOH¶	Yes	—**	Yes	
NOHA	K_3FeCN_6	H_2O	No	—	—	
NOHA	K_3FeCN_6/H_2O_2††	H_2O	Yes	—	—	
NHBG	$Pb(OAc)_4$	CH_3CN	Yes	Yes	Yes	
NHBG	K_3FeCN_6	H_2O	No	Yes	—	
NHBG	K_3FeCN_6/H_2O_2	H_2O	Yes	No	—	
NHBG	Ag_2CO_3	CH_3CN	No	Yes	No	
NHBG	PbO_2	CH_3CN	No	Yes	No	
NHAP	$Pb(OAc)_4$	CH_3CN	Yes	No	Yes	63
NHAP	K_3FeCN_6	H_2O	No	Yes	—	42
NHAP	K_3FeCN_6/H_2O_2	H_2O	Yes	No	—	52
NHAP	Ag_2CO_3	CH_3CN	No	Yes	No	49
NHAP	PbO_2	CH_3CN	No	Yes	No	—

* All reactions were carried out under nitrogen at room temperature for approximately 5 min.

† NO release was monitored by NO chemiluminescence detection. The instrument was able to detect >1% total conversion of the *N*-hydroxyguanidine to NO. Rough estimates of total conversion ranged from 25–45% based on detector response to authentic NO standards.

‡ N_2O generation was determined by GC. The exact values for the amounts of N_2O formed have not yet been determined. A positive response means only that the peak height was above the minimum detectable limit. Mass spectral response of authentic N_2O, however, indicates that conversion to N_2O was similar to that of NO.

§ As determined in rabbit aortic rings precontracted with phenylephrine.

‖ Yield of *N*-cyanopiperidine based on NHAP.

¶ $Pb(OAc)_4$ reacted with MeOH but was still able to oxidize NOHA at a significant enough rate to generate NO before it was completely destroyed by solvent.

** Not determined.

†† Hydrogen peroxide was added as a 30% solution (100 μL) in water.

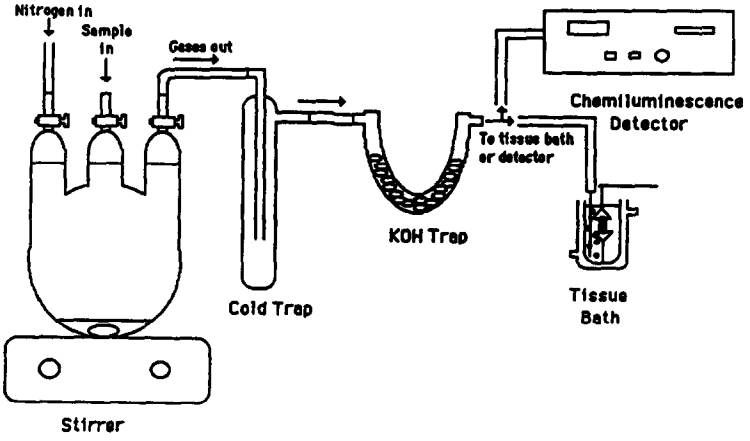

Fig. 3. Schematic of apparatus used to analyze evolved gases from the oxidation of *N*-hydroxyguanidine compounds.

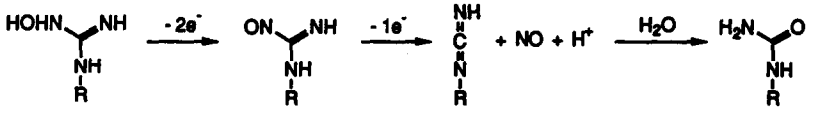

Fig. 4. Previously proposed mechanism of NO production from *N*-hydroxyguanidine compounds [2].

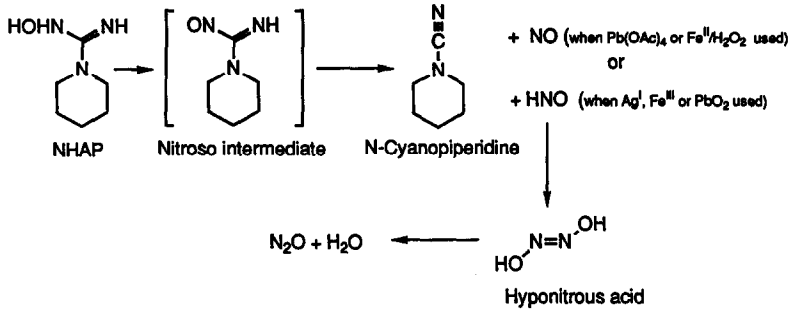

Fig. 5. Oxidation of *N*-hydroxyguanidine compounds to generate NO and N_2O.

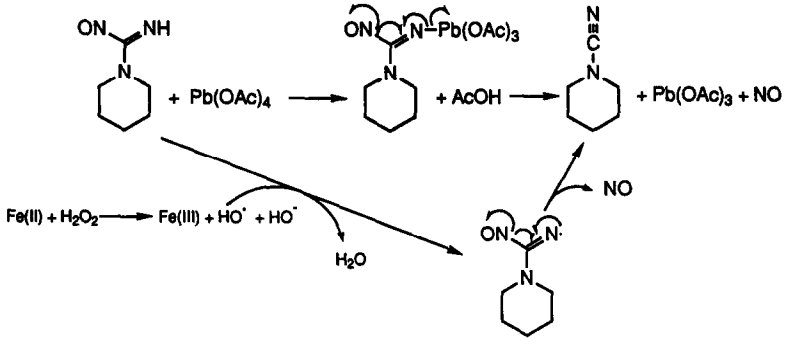

Fig. 6. Proposed mechanism of $Pb(OAc)_4$ and K_3FeCN_6/H_2O_2 oxidation of nitroso intermediate to generate NO.

K_3FeCN_6/H_2O_2. K_3FeCN_6, Ag_2CO_3 and PbO_2 did not generate detectable amounts of NO and their reaction was studied in greater detail with NHAP. Analysis of the reaction products of NHAP and these agents revealed that *N*-cyanopiperidine was formed with no other detectable organic products. *N*-cyanopiperidine was also the sole product from $Pb(OAc)_4$- and K_3FeCN_6/H_2O_2-mediated oxidations of NHAP. The yield of this product (Table 1) varied from 40 to 60%, depending on the oxidant, and thus represented a major reaction product. Thus, all the oxidants were capable of oxidizing the *N*-hydroxyguanidine function to the same final organic product but only $Pb(OAc)_4$ and K_3FeCN_6/H_2O_2 were capable of NO release.

HNO *generation.* Since oxidation had occurred in all instances and yet only $Pb(OAc)_4$- and K_3FeCN_6/H_2O_2-mediated oxidations were capable of releasing NO, the identity of the oxidized nitrogen species generated by Ag_2CO_3, K_3FeCN_6, and PbO_2 oxidants was sought. It is likely that the initial oxidation product in all cases was the nitroso intermediate (Fig. 5); in fact, K_3FeCN_6 and Ag_2CO_3 are typical oxidants used for the conversion of *N*-hydroxy compounds to the corresponding nitroso compounds [11, 12]. *N*-Cyanopiperidine formation from this species would require the loss of NO and a hydrogen atom (or the equivalent) and for this reason, liberation of nitroxyl, or nitrosyl hydride (HNO), was considered. This metastable species is known to rapidly dimerize to hyponitrous acid and then decompose to give N_2O and water [13] (Fig. 5). Headspace analysis of reaction mixtures of Ag_2CO_3-, K_3FeCN_6- and PbO_2-mediated oxidation of NHAP by GC and GC–MS revealed that, indeed, N_2O had been evolved. Thus, it appears that the nitroso intermediate, when formed under these conditions, decomposes to either NO (in the presence of $Pb(OAc)_4$ or K_3FeCN_6/H_2O_2) or HNO (in the presence of K_3FeCN_6, Ag_2CO_3 or PbO_2).

NO *versus* HNO *generation as a function of oxidant.* The ability of $Pb(OAc)_4$ and K_3FeCN_6/H_2O_2 to generate NO from *N*-hydroxyguanidine compounds compared to the other oxidants is likely due to the mechanism by which they oxidize substrates. $Pb(OAc)_4$ oxidations commonly occur through radical-mediated processes [14]. It is possible that a nitroso intermediate reacts with $Pb(OAc)_4$ to generate an N-Pb species (and acetic acid) which could decompose to give NO and a reduced lead species (Fig. 6). Oxidation of the *N*-hydroxyguanidine function by K_3FeCN_6/H_2O_2 would also generate the nitroso intermediate and an Fe(II) salt. This Fe(II) species would then react with hydrogen peroxide, generating hydroxyl radicals by the Fenton reaction. These radicals could react with the nitroso

intermediate by abstracting a hydrogen atom and liberating NO (outlined in Fig. 6). The other oxidants would not be expected to participate in these types of reactions. Apparently, it is the ability of $Pb(OAc)_4$ and K_3FeCN_6/H_2O_2 to "quench" the hydrogen atom which allows NO release. Otherwise, it appears that the nitroso compound decomposes to release HNO rather than NO. It should be noted that HNO release from organic molecules has some precedence in that it has been postulated to occur as a step in the conversion of *aci*-nitroalkanes to the corresponding aldehydes or ketones (the Nef reaction) [15, 16].

Lack of urea formation. One significant finding of this work was that the oxidations of NHAP and NHBG by ferricyanide, performed in aqueous solutions, did not generate any of the corresponding ureas (products equivalent to citrulline, 1-piperidinecarboxamide and butylurea, respectively). Although oxidation occurred, the organic reaction products did not react with water to form the urea compounds. Though cyanamides (the NHAP product) and carbodiimides (the presumed NHBG product) are known to react with water to generate ureas, the rate must be slow under the conditions of the reaction. The apparent slow reaction of these products with water indicates that the final citrulline forming reaction is possibly an enzyme-catalyzed oxidation of a carbodiimide or cyanamide intermediate involving an active oxygen species generated from dioxygen (perhaps superoxide or an enzyme bound equivalent).

Relevance to biological NO *generation.* It appears that NO is generated from arginine by a single protein [17, 18]. This protein is able to monohydroxylate a terminal guanidinium nitrogen by an NADPH and O_2-dependent process. The monohydroxylated species, NOHA, must than be further oxidized before NO release. The enzyme must not only perform the initial monohydroxylation of arginine, but also catalyze the oxidation of NOHA prior to NO release. Enzymatic involvement in this step has been demonstrated in the macrophage system [3]. As previously proposed by Marletta *et al.* [2], the most likely initial product of NOHA oxidation is the corresponding nitroso intermediate (Fig. 5). The properties of such a compound are currently unknown. However, the results presented herein indicate that such a nitroso intermediate is capable of releasing either NO or HNO depending on the oxidizing conditions. Apparently, some oxidants can prevent HNO release by specifically reacting with the terminal *N*-hydrogen atom and thus allow NO release. Although the exact nature of the biochemical species involved in NOHA oxidation is not known, the results of this study indicate that both NO and HNO are possible oxidative products and therefore, the biological oxidant must react in a way as to preclude HNO release. The organic product after NO release would be either a cyanamide or a carbodiimide. As these species apparently do not react rapidly with water, it is likely that the same enzyme involved in the previous reactions also catalyzes the reaction which results in the final urea product.

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