Kinetics of N-Nitrosation in Oxygenated Nitric Oxide Solutions at Physiological pH: Role of Nitrous Anhydride and Effects of Phosphate and Chloride

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Abstract: The kinetics of N-nitrosation in oxygenated nitric oxide (NO) solutions at physiological pH are important because of the cytotoxic, mutagenic, and carcinogenic effects of NO and its derivatives. N-nitrosation of morpholine in the presence of NO and O_2 at pH 7.4 was investigated using a novel reactor in which NO, nitrite (NO_2^-), and *N*-nitrosomorpholine (NMor) were continuously monitored. A nitrogen balance showed that NO_2^- and NMor were the principal nitrogen products derived from NO. Phosphate and chloride ions were shown to inhibit N-nitrosation of morpholine, whereas nitrate, nitrite, thiocyanate, and perchlorate had little or no effect. The effects of phosphate, in particular, were substantial: 0.05 M phosphate caused a 20-fold reduction in NMor formation. All data were consistent with N_2O_3 being the principal nitrosating agent at physiological pH. A scheme in which phosphate and chloride react with N_2O_3 to form nitrosyl compounds, which are then rapidly hydrolyzed to NO_2^-, explains the inhibitory effects of these anions. Rate constants for the reactions of morpholine, phosphate, and chloride with N_2O_3 were estimated at 25 and 37 °C. Relations were derived to predict the concentrations of N_2O_3 and the various nitrosyl complexes, based on pseudo-steady-state assumptions applied to these and other species present in small amounts.

Introduction

Nitric oxide (NO) is synthesized in the body by a wide variety of cell types, including macrophages, endothelial cells, neutrophils, neurons, and hepatocytes. Blood pressure regulation, inhibition of platelet aggregation, and neurotransmission are among the important physiological functions of NO.[1] When present at sufficient concentrations, however, NO and its nitrogen oxide derivatives have cytotoxic and mutagenic effects.[2,3] Nitrous anhydride (N_2O_3), produced from the reaction of NO with molecular oxygen, may damage DNA through direct nitrosation of primary amines on DNA bases. In addition, N_2O_3 may indirectly damage DNA via nitrosation of various secondary amines to form *N*-nitrosamines, which can then be metabolized to form strongly alkylating electrophiles that react with DNA.[2] Nitrous anhydride may also react with essential sulfhydryl groups of various proteins.[4]

Concern over the endogenous formation of carcinogenic nitrosamines and nitrosamides, by the reaction of nitrite with various amines or amides in the stomach, has motivated the extensive study of N-nitrosation under very acidic conditions. Nitrosamines have been shown to result from the reaction of secondary amines either with N_2O_3 or with various nitrosyl compounds, including NOSCN and NOCl.[5-7] However, nitrosation reactions have received much less attention at physiological pH, and the roles of N_2O_3 and nitrosyl compounds are not well established under these conditions. In particular, it was suggested on the basis of kinetic data that N-nitrosation of sodium azide in oxygenated NO solutions does not involve N_2O_3, although the reactive intermediate was not identified.[8] Clearly, it is important to identify the key nitrosating agent(s) present in physiological solutions.

The experiments reported here were designed to examine the role of N_2O_3 in N-nitrosation at physiological pH, as well as the effects of various anions. Nitrosation of morpholine was studied in a novel reactor permitting continuous measurements of NO, nitrite (NO_2^-), and *N*-nitrosomorpholine (NMor) concentrations. The nitrosating species were generated from the reaction of NO with O_2. A detailed analysis of the kinetics of NMor formation suggests that N_2O_3 is indeed the likely intermediate at physiological pH. A very unexpected result was the strong inhibition of N-nitrosation by phosphate and (to a lesser extent) chloride. A kinetic scheme is presented which explains these observations, and which also is consistent with previous findings at acidic pH.

Materials and Methods

Reagents. Nitric oxide was passed through a column of 4–8 mesh soda lime to remove NO_x impurities. Argon, after passage through an oxygen trap, was mixed with NO using controlled gas flow meters

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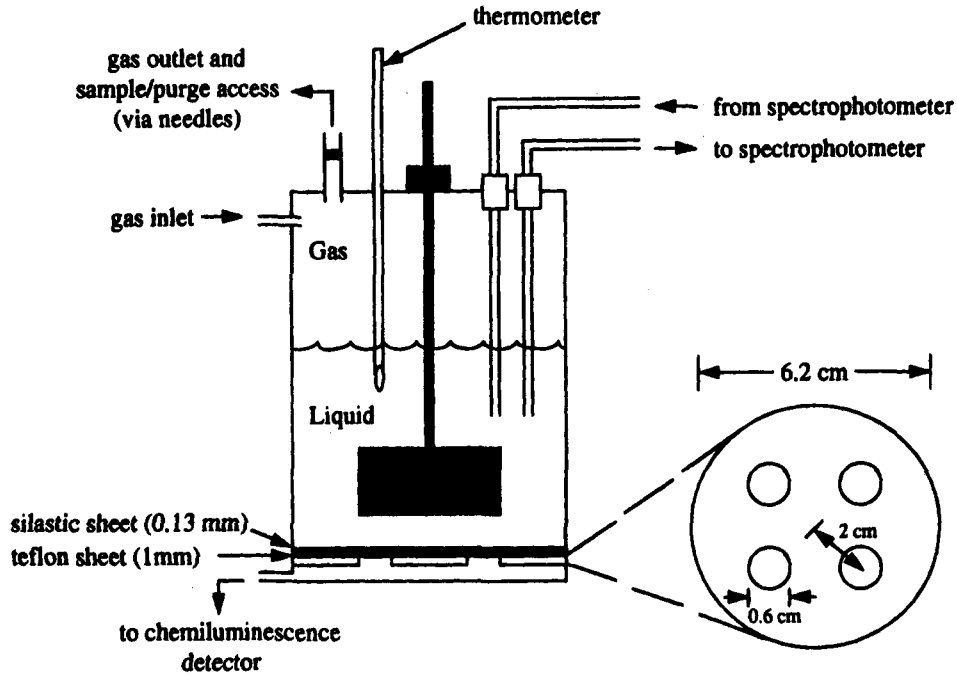

Figure 1. Schematic of apparatus used to study N-nitrosation of morpholine. The reactor was a modified, 200 mL stirred ultrafiltration cell. Continuous entry of NO into a chemiluminescence detector was via a composite membrane at the base of the reactor, consisting of a poly-(dimethylsiloxane) (Silastic) membrane laminated to a Teflon sheet. The flow loop led to a spectrophotometer for continuous monitoring of nitrite and nitrosomorpholine. Reprinted with permission from ref 9. Copyright 1994 American Chemical Society.

(Porter Instrument Co., Hatfield, PA) to obtain the desired NO gas concentration. Buffers containing 0.002, 0.01, or 0.05 M phosphate were prepared by adding equimolar amounts of Na_2HPO_4 and NaH_2PO_4 to deionized water. Solutions containing 0.04 M nitrate, nitrite, chloride, perchlorate, or thiocyanate were prepared by adding the respective sodium salts to the 0.01 M phosphate buffer. Morpholine (Aldrich Chemical Co., Milwaukee, WI) and *N*-nitrosomorpholine (Sigma Chemical Co., St. Louis, MO) were used for the N-nitrosation studies.

Reactor. The reactor was a modified 200 mL stirred ultrafiltration cell (Amicon, Danvers, MA, model 8200) as shown schematically in Figure 1. A composite membrane (6.2 cm diameter) consisting of a 1 mm thick Teflon sheet laminated with a 0.13 mm thick poly-(dimethylsiloxane) sheet (Mempro, Troy, NY) was fitted to the base of the stirred cell for NO detection. The Teflon layer, with four symmetrically positioned holes (0.6 cm diameter), limited the amount of NO which could diffuse across the composite membrane and into the chemiluminescence detector. The stirrer was replaced with that from a CYTOSTIR bioreactor (Kontes, Vineland, NJ). Gas inlet and outlet ports, two ports for a flow loop connected to a spectrophotometer (for NO_2^- and NMor measurements), and a thermometer were added. A 20 gauge hypodermic needle was inserted into the gas outlet port for gas purging and NO introduction and removed during the reaction. The reactor was either at ambient temperature (25 ± 1 °C) or placed in a heated water bath (37 ± 1 °C). The mass transfer characteristics of this reactor, including rates of NO and O_2 transport across the gas–liquid interface and rates of NO transfer through the membrane at the base, have been described previously, together with the differential mass balance equations which relate concentration changes to rates of reaction and mass transfer.[9]

Nitric Oxide Analysis. The external side of the composite membrane was exposed to the high vacuum of a chemiluminescence detector (Thermedics Detection Inc., Woburn, MA, model TEA-502) for NO monitoring. The response time of the detector, governed by the time required for NO transport through the composite membrane, was ~2 s, similar to the response time using mass spectrometry.[10] The minimum aqueous NO concentration measurable by the detector was estimated at 0.01 μM (signal-to-noise ratio of 3), and the output of the detector was linear up to concentrations of at least 40 μM. For calibration of the detector, dissolved NO concentrations were calculated from known values of the NO gas concentration and solubility.[11]

Nitrite and *N*-Nitrosomorpholine Analysis. The aqueous solution was continuously circulated at 45 mL/min through the 1/8 in. diameter flow loop (volume of ~8 mL) and into a 10 mm spectrophotometer flow cell (Shimadzu Model UV160u) using a pulseless pump (Cole Parmer, Chicago, IL, models 000–305 and 184–000). Absorbances at 250 nm were linearly proportional to the NMor concentration with no interference from NO_2^-. The nitrite ion concentration was proportional to the absorbance measured at 209 nm, although absorption of NMor at 209 nm necessitated a correction of −0.27 μM NO_2^-/μM NMor. Both NMor and NO_2^- had minimum detectable limits of ~1 μM.

N-Nitrosation of Morpholine. Morpholine at 300–2500 μM was added to 150 mL of buffer in the reactor, stirring initiated at 100 rpm, and recirculation begun through the flow loop. After recording the pH, removal of O_2 was initiated by bubbling the solution with Ar for at least 40 min, after which a mixture of NO/Ar was bubbled into the solution for at least 30 min to obtain the desired aqueous NO concentration. The flow rates used in both cases were 350 sccm at 25 °C and 300 sccm at 37 °C. Bubbling of the NO/Ar mixture was then terminated, and residual NO in the head space was removed by introducing Ar via the gas inlet for 1.5 min, again at flow rates of 350 and 300 sccm at 25 and 37 °C, respectively. A 21% O_2, balance N_2, mixture was then introduced through the gas inlet at the same flow rate. Diffusion of O_2 into the aqueous phase initiated the oxidation of NO, which at this time was approximately 28 μM at both 25 and 37 °C. The reaction was allowed to proceed for at least 30 min, during which NO and NMor concentrations were monitored continuously. Upon completion of the reaction, removal of O_2 was again initiated and the reaction protocol was repeated. This time the concentrations of NO and NO_2^- were monitored, and the pH was measured at the end. The buffers contained phosphate alone (at 0.002, 0.01, or 0.05 M) or 0.04 M $NaNO_3$, $NaNO_2$, NaSCN, $NaClO_4$, or NaCl added to 0.01 M phosphate.

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Results

Morpholine Concentration and pH. The total morpholine concentration remained essentially constant during all experiments, due to the negligible amounts converted to NMor (<1.5%). Of greater interest than total morpholine is the uncharged form, which is the substrate for nitrosation. Denoting total morpholine as Mor and the unprotonated form as Mor°, the respective concentrations are related by

$$[\text{Mor}^\circ] = \frac{[\text{Mor}]}{1 + 10^{pK-pH}} \quad (1)$$

where the pK at 25 °C is 8.5 for morpholine.[12] Thus, at pH 7.4, 7.4% of total morpholine is available for N-nitrosation; the presence of significant amounts of Mor° makes morpholine a suitable amine to study at physiological pH. The initial pH (after addition of morpholine to the buffer) actually ranged from 7.27 to 7.55, depending on the particular combination of phosphate and morpholine concentrations employed. The pH was very nearly constant during a given experiment, so that [Mor°] was also virtually constant.

Effects of Phosphate on N-Nitrosation. As discussed later in more detail, the simplest assumption is that N_2O_3 is formed in solution by oxidation of NO, and that there are no effects of anions on the levels of N_2O_3 or on the rate of nitrosation of morpholine. According to this view, N_2O_3 either reacts with morpholine to form NMor or is hydrolyzed to form NO_2^-; ultimately, the nitrogen of NO is converted either to NMor or to NO_2^-. The kinetic analysis discussed later implies then that for all times

$$\frac{\Delta[\text{NMor}]}{\Delta[NO_2^-] - \Delta[\text{NMor}]} = k^*[\text{Mor}^\circ] \quad (2)$$

where Δ[NMor] and $\Delta[NO_2^-]$ represent the changes in the concentrations of these species during the reaction (i.e., following the introduction of O_2) and k^* is a constant. This constant is proportional to the rate constant for morpholine nitrosation by N_2O_3, divided by the rate constant for N_2O_3 hydrolysis (see below). It should be noted that prior to the deliberate introduction of O_2, some NO_2^- and NMor were detected in all of the experiments, reaching concentrations of $\leq$60 and $\leq$10 μM, respectively. The initial NO_2^- and NMor appeared to result from an air leak in the part of the flow loop within the spectrophotometer which we were unable to eliminate. The air leak led to a baseline O_2 concentration estimated at ~2 μM (1% of air saturation), which allowed some reaction of NO and O_2 during the time it took NO to reach its initial steady state concentration. However, the existence of this small air leak did not affect the analysis, other than making it necessary to use concentration changes, rather than absolute concentrations, in eq 2.

Figure 2 shows the results at various phosphate concentrations, at both 25 and 37 °C, plotted in the manner suggested by eq 2. As predicted, the results at all phosphate concentrations and both temperatures are well represented by straight lines. However, the slopes of these lines (the values of k^*) depend not only on temperature, as expected, but also on the phosphate concentration. The marked decrease in slope with increasing phosphate concentration indicates that phosphate inhibits nitrosamine formation, an unexpected result. The data do not reveal which of the specific forms of phosphate was inhibitory, because the $HPO_4^{2-}/H_2PO_4^-$ ratio was approximately the same

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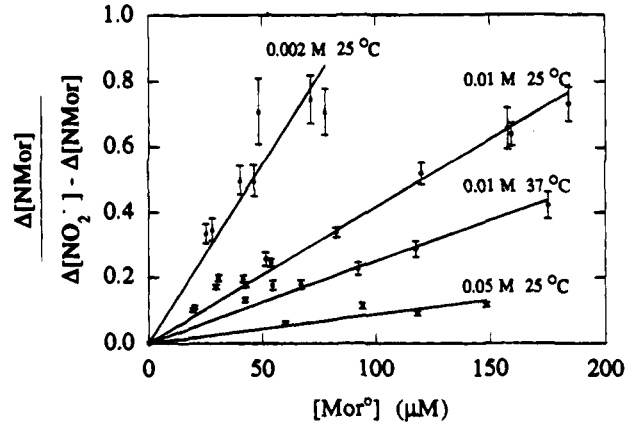

Figure 2. Effect of total phosphate concentration and temperature on N-nitrosomorpholine (NMor) formation. The concentration of the unprotonated form of morpholine (Mor°) remained essentially constant throughout each experiment. The symbols show the mean ± standard deviation for reaction times ranging from 3 to 30 min. Also shown are the best-fit lines (least squares). The numbers of experiments were 7, 13, and 4 at 0.002, 0.01, and 0.05 M phosphate, respectively, at 25 °C and 6 at 37 °C.

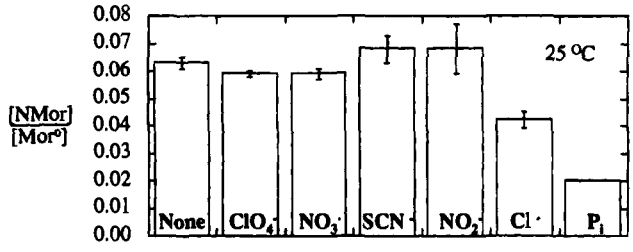

Figure 3. Effects of various anions on N-nitrosomorpholine (NMor) formation at 25 °C, after 25–30 min of reaction. Each anion shown was added at a concentration of 0.04 M to 0.01 M phosphate; none means 0.01 M phosphate alone, and P_i denotes added phosphate, for a total concentration of 0.05 M. The mean value ± standard deviation is shown for 3–5 experiments with each anion.

in all experiments. When the slopes were calculated using the average data between 3 and 30 min, values of k^* at 25 °C for 0.002, 0.01, and 0.05 M phosphate were found to be 11 000 ± 700, 4200 ± 90, and 880 ± 90 M^{-1}, respectively. At 37 °C, k^* was 2500 ± 80 M^{-1} for 0.01 M phosphate, indicating that increasing the temperature enhanced the inhibitory effect of phosphate.

Effects of Other Anions on N-Nitrosation. The effects of ClO_4^-, NO_3^-, SCN^-, NO_2^-, and Cl^- on NMor formation at 25 °C are illustrated in Figure 3. The concentration of Nmor attained at reaction times of 25–30 min is expressed relative to the concentration of the neutral form of morpholine, denoted as Mor°. Recall that in each case the other salts are added at 0.04 M to 0.01 M phosphate; "none" means 0.01 M phosphate alone and "P_i" refers to added (inorganic) phosphate, or a total phosphate concentration of 0.05 M. As seen by comparison with the 0.01 M phosphate control (none), there was little or no effect of ClO_4^-, NO_3^-, SCN^-, or NO_2^- on NMor formation. Chloride had a significant inhibitory effect, although not as strong as that of phosphate. The inhibitory effects of Cl^- at 25 and 37 °C are illustrated further in Figure 4, which is another plot based on eq 2. As with phosphate, increasing the temperature enhanced the inhibitory effect of Cl^- on N-nitrosation. Values of k^* for 0.01 M phosphate and 0.04 M Cl^- at 25 and 37 °C were 2300 ± 20 and 1500 ± 40 M^{-1}, respectively.

Nitrogen Balance. To account for all of the nitrogen introduced into the reactor as NO, it was necessary to consider

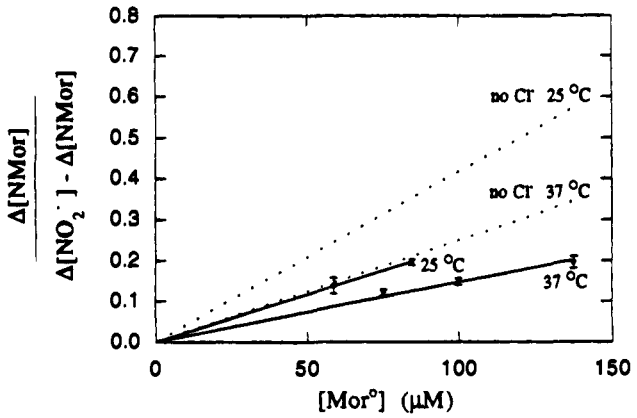

Figure 4. Effect of 0.04 M Cl^- on N-nitrosomorpholine (NMor) formation at 25 and 37 °C, in the presence of 0.01 M phosphate. The mean value ± standard deviation is shown for reaction times ranging from 3 to 30 min. The solid lines are fitted to the Cl^- data; the dashed lines show results with no Cl^- (phosphate only).

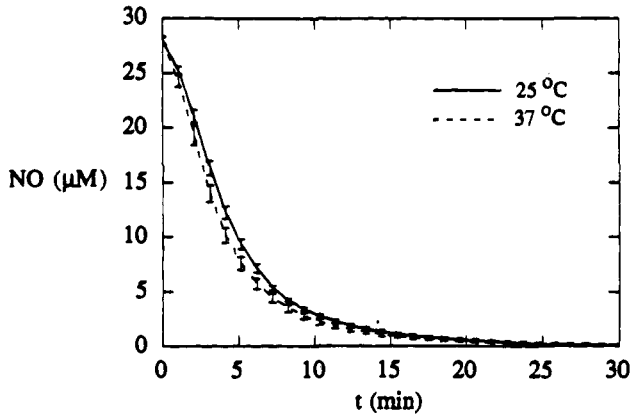

Figure 5. Change in NO concentration with time, during the reaction of NO with O_2 in the presence of morpholine at 25 and 37 °C. The symbols show the mean ± standard deviation for eight experiments at 25 °C and seven experiments at 37 °C. The curves represent the predictions from the kinetic model reported previously,[9] modified as described in the text.

the amount of NO lost from the aqueous phase through physical processes (NO mass transfer into the gas phase and detector), and thus unavailable for reaction. The predicted change in NO concentration due to physical processes, denoted as $[NO]_{lost}$, is given by

$$[NO]_{lost} = (k_L a/V)_{NO} \int_0^{t^*} [NO]\, dt \qquad (3)$$

where $(k_L a/V)_{NO}$ is the volumetric mass transfer coefficient characterizing the transport of NO into the gas phase and into the detection system. Values for $(k_L a/V)_{NO}$ of 7.5×10^{-4} s^{-1} and 10.2×10^{-4} s^{-1} at 25 and 37 °C, respectively, were measured previously for this reactor.[9]

The decay of the aqueous NO concentration after the initiation of the reaction is shown in Figure 5. None of the anions showed any noticeable effect on the NO concentration profile, so that all results at a given temperature have been pooled. The initial NO concentration was ~28 μM at both 25 and 37 °C, but the initial rate of decay was slightly faster at the higher temperature. From eq 3, the area under either curve in Figure 5 from $t = 0$ to some time $t = t^*$, multiplied by the volumetric mass transfer coefficient, equals the amount of NO lost through physical processes alone up to time t^*. Continuous measurements of NO, NMor, and NO_2^- concentrations showed that for any choice of t^* from 3 to 30 min, the sum of these concentrations and $[NO]_{lost}$ was within experimental uncertainty of the initial NO concentration. For example, at $t^* = 20$ min, the deviations from the initial NO concentration were 1.6% and 6.0% at 25 and 37 °C, respectively. Because all of the nitrogen could be accounted for in this manner, we conclude that NO, NMor, and NO_2^- were the only major nitrogen species.

Discussion

Reaction Scheme. The hypothesis we initially sought to test was that the rate of nitrosation of morpholine (or some other secondary amine, R_2NH) at near-neutral pH could be explained by a reaction scheme written as follows:[9,13,14]

$$2NO + O_2 \xrightarrow{k_1} 2NO_2 \qquad (4)$$

$$NO + NO_2 \underset{k_3}{\overset{k_2}{\rightleftharpoons}} N_2O_3 \qquad (5)$$

$$N_2O_3 + H_2O \underset{k_5}{\overset{k_4}{\rightleftharpoons}} 2HNO_2 \rightleftharpoons 2NO_2^- + 2H^+ \qquad (6)$$

$$N_2O_3 + R_2NH \xrightarrow{k_6} R_2NNO + NO_2^- + H^+ \qquad (7)$$

It is worth noting that reaction 4 represents at least two elementary steps. Reactions 4–7 are the same as those used previously to describe nitrosation of amines by N_2O_3 under acidic conditions.[5,6] The distinctive characteristic of the present experimental conditions is that at physiological pH essentially all of the HNO_2 is completely dissociated to form NO_2^- (the pK_a is 3.36 for nitrous acid[15]). This drives reaction 6 far to the right, making NO_2^- a sink for N_2O_3 formed from the oxidation of NO (reactions 4 and 5). Consequently, hydrolysis of N_2O_3 (reaction 6) and N-nitrosation of amines by N_2O_3 (reaction 7) act as competing, irreversible processes. In contrast, at very acidic pH (e.g., pH 2), the HNO_2/NO_2^- ratio favors nitrous acid, allowing NO_2^- to provide a *source* of HNO_2 and N_2O_3. Indeed, nitrosation activity has typically been generated at acidic pH simply by the addition of nitrite salts, rather than by addition of NO.

Reactions 4–7 provide a reasonable starting point, but the somewhat surprising finding that phosphate and chloride inhibit nitrosation of morpholine indicates that this scheme is somehow incomplete. It is especially interesting that Cl^- is a promoter of N-nitrosation at acidic pH,[7] but an inhibitor at neutral pH. Moreover, SCN^- is a strong promoter under acidic conditions, but had little or no effect on NMor formation in the present studies. The most straightforward explanation for the effects of the various anions (denoted as X^-) relates to the formation of nitrosyl compounds (XNO). The enhancement of the N-nitrosation of amines by SCN^-, Cl^-, and certain other anions at acidic pH has been explained by recognizing that the nitrosyl compounds themselves can act as nitrosating agents:[7,16,17]

$$XNO + H_2O \underset{k_8}{\overset{k_7}{\rightleftharpoons}} HNO_2 + H^+ + X^- \rightleftharpoons NO_2^- + 2H^+ + X^- \qquad (8)$$

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$$XNO + R_2NH \xrightarrow{k_9} R_2NNO + H^+ + X^- \quad (9)$$

Thus, if there is sufficient HNO_2 present, the reverse of reaction 8 can form XNO, which can then nitrosate amines as in reaction 9. As X^- is conserved in such a sequence, it acts as a homogeneous catalyst. However, these reactions still do not account for the inhibitory effects of an anion. In addition, the formation of XNO from HNO_2 and X^- (reverse of reaction 8) should be almost nonexistent at physiological pH, due again to the essentially complete ionization of HNO_2 to NO_2^-. This requires that if XNO is involved at near-neutral pH, it must be formed from some other reaction.

The additional reaction needed, which provides another pathway for forming XNO, is

$$N_2O_3 + X^- \xrightarrow{k_{10}} XNO + NO_2^- \quad (10)$$

Reaction 10, which seems not to have been mentioned in previous work on the biological effects of NO, is similar to the reaction of N_2O_3 with ascorbate.[18] The inhibitory effects of certain anions on N-nitrosation at physiological pH can now be explained as follows. If N-nitrosation with XNO (reaction 9) is slow compared to XNO hydrolysis (reaction 8), then XNO will not enhance N-nitrosation. Moreover, all of the N_2O_3 that reacts with X^- (reaction 10) will be converted to NO_2^- through reactions and 8 and 10. Thus, any anion which behaves in this manner will scavenge some of the N_2O_3, thereby depressing the rate of N-nitrosation. This inhibition of N-nitrosation due to N_2O_3 scavenging would not necessarily be seen under acidic conditions, because NO_2^- can then be converted back to HNO_2, making the reverse directions of reactions 6 and 8 significant. The manner in which a given anion might enhance N-nitrosation under acidic conditions, involving reactions 8 and 9, has already been discussed.

Kinetics at Physiological pH. A detailed analysis of the reaction kinetics is needed to check the consistency of the reaction scheme described above, and to estimate specific rate constants from the data. The following analysis applies to solutions containing one amine but (possibly) several anions, at near-neutral pH. Pseudo-steady state approximations are used for NO_2, N_2O_3, and XNO, which are present only in trace amounts, as confirmed by the overall nitrogen balance. Neglecting any physical losses of these species, the respective conservation equations are

$$\frac{d[NO_2]}{dt} = 2k_1[NO]^2[O_2] - k_2[NO][NO_2] + k_3[N_2O_3] \cong 0 \quad (11)$$

$$\frac{d[N_2O_3]}{dt} = k_2[NO][NO_2] - (k_3 + k_4)[N_2O_3] + k_5[HNO_2]^2 - k_6[N_2O_3][R_2NH] - \sum k_{10}[N_2O_3][X^-] \cong 0 \quad (12)$$

$$\frac{d[XNO]}{dt} = -k_7[XNO] + k_8[HNO_2][H^+][X^-] - k_9[XNO][R_2NH] + k_{10}[N_2O_3][X^-] \cong 0 \quad (13)$$

where the summation in eq 12 (and subsequent equations) is over all anions. Solving these algebraic equations for $[N_2O_3]$ and [XNO], while neglecting the terms involving $[HNO_2]$, we obtain

$$[N_2O_3] \cong \frac{2k_1[NO]^2[O_2]}{k_4 + k_6[R_2NH] + \sum k_{10}[X^-]} \quad (14)$$

$$[XNO] \cong \frac{k_{10}[X^-][N_2O_3]}{k_7 + \sum k_9[R_2NH]} \quad (15)$$

Neglecting the terms involving nitrous acid limits the applicability of eqs 14 and 15; specifically, it is necessary that $k_5[HNO_2]^2$ be much smaller than the numerator in eq 14, and that $k_8[HNO_2][H^+][X^-]$ be much smaller than the numerator in eq 15. As discussed later, these conditions were satisfied by the experiments reported here.

The rate of formation of the nitrosamine is given by

$$\frac{d[R_2NNO]}{dt} = (k_6[N_2O_3] + \sum k_9[XNO])[R_2NH] \quad (16)$$

Assuming that the only significant nitrosating agent is N_2O_3, as appears to have been the case in the present studies, eq 16 reduces to

$$\frac{d[R_2NNO]}{dt} \cong k_6[N_2O_3][R_2NH] \quad (17)$$

The rate of change in the nitrite concentration is obtained by writing a conservation equation for the sum of NO_2^- and HNO_2, analogous to eqs 11–13 but without the pseudo-steady state assumption. Again when all terms involving $[HNO_2]$ are neglected, the result is

$$\frac{d[NO_2^-]}{dt} \cong (2k_4 + k_6[R_2NH] + \sum k_{10}[X^-])[N_2O_3] + \sum k_7[XNO] \quad (18)$$

Using eq 15 to evaluate [XNO] in eq 18, and assuming that hydrolysis of XNO is much faster than N-nitrosation by XNO (i.e., $k_7 \gg k_9[R_2NH]$), we obtain

$$\frac{d[NO_2^-]}{dt} \cong (2k_4 + k_6[R_2NH] + 2\sum k_{10}[X^-])[N_2O_3] \quad (19)$$

Equation 19 should be applicable to phosphate and chloride in the present experiments.

To obtain a relation of the same form as eq 2, we combine eqs 17 and 19 to give

$$\frac{d[NO_2^-]}{d[R_2NNO]} \cong \frac{2k_4 + k_6[R_2NH] + 2\sum k_{10}[X^-]}{k_6[R_2NH]} \quad (20)$$

The right-hand side of eq 20 was very nearly constant during the experiments reported here, because total morpholine concentration, pH, and anion concentrations were all approximately constant. Accordingly, integration of eq 20 followed by some rearrangement yields

$$\frac{\Delta[R_2NNO]}{\Delta[NO_2^-] - \Delta[R_2NNO]} \equiv k^*[R_2NH] \quad (21)$$

$$k^* = \frac{1}{2}\left(\frac{k_6}{k_4 + \sum k_{10}[X^-]}\right) \quad (22)$$

It can be seen from eqs 21 and 22 that the lumped "constant" k^* which appears in eq 2 is constant only in the absence of

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Table 1. Rate Constants

rate constant	form of rate expression	value of rate constant 25 °C	37 °C	units	ref
k_1	$R_{NO_2} = 2k_1[NO]^2[O_2]$	2.1	2.4	10^6 M^{-2} s^{-1}	8, 9. 13, 14
k_2	$R_{N_2O_3} = k_2[NO][NO_2]$	1.1[a]		10^9 M^{-1} s^{-1}	20
k_3	$R_{NO} = k_3[N_2O_3]$	4.3[a]		10^6 s^{-1}	19, 20
k_4	$R_{HNO_2} = 2k_4[N_2O_3]$	1.6		10^3 s^{-1}	19
k_5	$R_{N_2O_3} = k_5[HNO_2]^2$	5.6	14.1[b]	M^{-1} s^{-1}	20
k_6/k_4	$R_{NMor} = k_6[N_2O_3][Mor^\circ]$	4.0	3.1[c]	10^4 M^{-1}	this work[7]
k_{10}^P/k_4	$R_{NOP_i} = k_{10}^P[N_2O_3][P_i]$	4.0[d]	5.2[d]	10^2 M^{-1}	this work
k_{10}^{Cl}/k_4	$R_{NOCl} = k_{10}^{Cl}[N_2O_3][Cl^-]$	9.0[d]	10.3[d]	10^1 M^{-1}	this work

[a] Value reported at 20 °C. [b] From average activation energy cited in ref 20. [c] From eq 26, with an activation energy for k_N of 10 kcal/mol (see ref 7). [d] From eqs 21 and 24, using the data in Figures 2 and 4.

participating anions. For anions which react with N_2O_3 (reaction 10), k^* will depend inversely on the anion concentration, as observed for phosphate and chloride.

Evaluation of Rate Constants. Applying eq 22 to a solution at physiological pH where the only anions are phosphate and chloride, we obtain

$$k^* = \frac{1}{2}\left(\frac{k_6/k_4}{1 + (k_{10}^P/k_4)[P_i] + (k_{10}^{Cl}/k_4)[Cl^-]}\right) \quad (23)$$

where P_i refers to total inorganic phosphate. Rearrangement of eq 23 gives

$$\frac{1}{2k^*[P_i]} = \left(\frac{k_{10}^P}{k_6}\right) + \left(\frac{k_4}{k_6}\right)\frac{1}{[P_i]} + \left(\frac{k_{10}^{Cl}}{k_6}\right)\frac{[Cl^-]}{[P_i]} \quad (24)$$

Linear regression of $1/(2k^*[P_i])$ vs $1/[P_i]$, using the data for phosphate alone, yielded $k_{10}^P/k_6 = (1.00 \pm 0.05) \times 10^{-2}$ and $k_4/k_6 = (2.5 \pm 0.3) \times 10^{-5}$ M at 25 °C. With $k_4 = 1600$ s^{-1} at 25 °C,[19] we obtained $k_6 = 6.4 \times 10^7$ M^{-1} s^{-1} for morpholine and $k_{10}^P = 6.4 \times 10^5$ M^{-1} s^{-1} at 25 °C. Using the data for solutions containing both chloride and phosphate, we calculated that $k_{10}^{Cl} = 1.4 \times 10^5$ M^{-1} s^{-1} at 25 °C. The values of these and other rate constants needed for reactions 4−10, at both 25 and 37 °C, are summarized in Table 1. Due to the wide range of values for k_4 reported at 25 °C[20-22] and the absence of a literature value for k_4 at 37 °C, the values of k_6, k_{10}^P, and k_{10}^{Cl} are expressed as ratios to that of k_4, as determined from eq 24.

A kinetic model was developed to compute concentrations of the various species as functions of time, based on the rate constants given in Table 1. Except for the inclusion of eqs 14, 17, and 19, the model was the same as that reported previously.[9] The values of [NO] computed with the model, shown by the solid curves in Figure 5, were in excellent agreement with the data over the entire 30 min period. These simulations were insensitive to the assumed anion concentrations, in accord with the experimental observations. Figure 6 shows a comparison of the measured and calculated values of $[NO_2^-]$ and [NMor] for representative runs at two different initial concentrations of morpholine. Again, the agreement between the model and data was quite satisfactory.

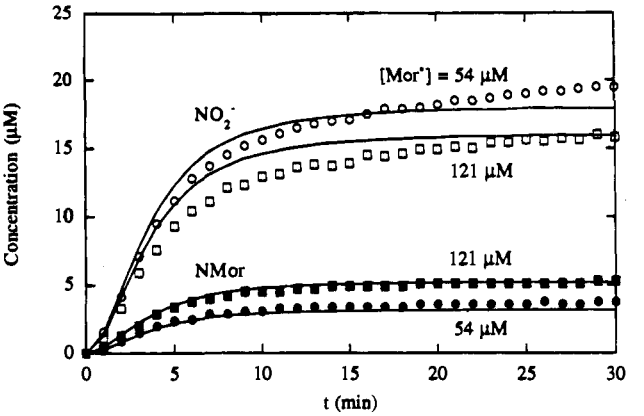

Figure 6. Change in *N*-nitrosomorpholine (NMor) and nitrite concentrations with time, for an initial NO concentration of 28 μM in 0.01 M phosphate buffer at 25 °C. Results are shown for two different initial concentrations of the uncharged form of morpholine (Mor°). For clarity, the continuously measured concentrations are shown by discrete symbols. The solid curves were calculated using the kinetic model.

Validity of Approximations. The validity of the approximations used in the derivations of eqs 14, 15, and 17−19 was assessed using the rate constants in Table 1 at 25 °C, assuming phosphate and chloride concentrations of 0.01 and 0.04 M, respectively. The O_2 concentration in all experiments was 240 μM. Throughout 30 min of all experiments, the minimum concentration of NO was approximately 0.1 μM, whereas the maximum concentrations of HNO_2 and Mor° were approximately 2 nM and 200 μM, respectively. As noted in the derivation of eq 14, it was assumed that $k_5[HNO_2]^2/(2k_1[NO]^2[O_2]) \ll 1$; using the concentrations stated above, the maximum value of this ratio was calculated to be 2×10^{-6}. Similarly, eq 15 applied to Cl^- requires that $k_8^{Cl}[HNO_2][H^+]/(k_{10}^{Cl}[N_2O_3]) \ll 1$ at pH 7.4. When $[N_2O_3]$ was calculated from eq 14 and $k_8^{Cl} = 975$ M^{-2} s^{-1} (reported at 0 °C[18]) was used as an approximation, this ratio was found not to exceed 1.4×10^{-3}. The corresponding value for phosphate will be even smaller because $k_{10}^P > k_{10}^{Cl}$, and because little or no XNO formation via reaction 8 was observed for phosphate (i.e., $k_8^P \ll k_8^{Cl}$).[7] Therefore, eq 15 is valid for both phosphate and chloride.

Equations 17 and 19 require that $k_9[XNO]/(k_6[N_2O_3]) \ll 1$ and $k_9[R_2NH]/k_7 \ll 1$, respectively. These relations are evidently valid for phosphate, because at acidic pH phosphate did not significantly affect nitrosation;[7] that is, k_9 for phosphate is negligible. For chloride, however, estimates of k_7^{Cl} and k_9^{Cl} are needed. The value of k_7^{Cl} is calculated to be about 1×10^6 s^{-1}, obtained by using $k_8^{Cl}/k_7^{Cl} = 1.0 \times 10^{-3}$ M^{-2} at 25 °C[17] together with the aforementioned value of k_8^{Cl} at 0 °C. Obtaining an estimate of k_9^{Cl} is somewhat more difficult. Data at acidic pH with bromide indicate that $k_8^{Br}k_9^{Br}/k_7^{Br}$ is approximately 2.2×10^6 M^{-3} s^{-1} at 25 °C.[7] Moreover, $k_8^{Cl}k_9^{Cl}/k_7^{Cl} \ll k_8^{Br}k_9^{Br}/k_7^{Br}$. Thus, we infer that $k_9^{Cl} \ll 2 \times 10^9$ M^{-1} s^{-1}. It follows that, for morpholine, $k_9^{Cl}[NOCl]/(k_6[N_2O_3]) \ll 0.2$ and $k_9^{Cl}[Mor^\circ]/k_7^{Cl} \ll 0.4$, thereby satisfying the requirements of eqs 17 and 19. Considering the various terms containing $[HNO_2]$ which were neglected in obtaining eq 18, we found that the ratio of any of these terms to the quantities retained in the equation did not exceed 1×10^{-4}.

An assumption made in eq 11 is that physical losses of NO_2 were negligible. On the basis of measured mass transfer rates for NO, the escape of NO_2 should be primarily across the gas−liquid interface, rather than through the membrane to the chemiluminescence detector.[9] Mass transfer of NO_2 across the gas−liquid interface would be represented by including a term $-(k_La/V)_{NO_2}[NO_2]$ on the right-hand side of eq 11. It follows

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that, for physical losses of NO_2 to be negligible compared to reactive losses, it is sufficient that $(k_L a/V)_{NO_2}/(k_2[NO]) \ll 1$. Using k_2 from Table 1 and assuming that the mass transfer coefficient for NO_2 was similar to that measured for NO, physical losses of NO_2 will be negligible provided that [NO] $\gg 1 \times 10^{-12}$ M. This was certainly the case for this study. Physical losses of N_2O_3 and NOX are also presumed to be negligible.

Comparison with Results at Acidic Conditions. At pH 2.3–4.0, in the presence of a nonparticipating anion (ClO_4^-), the rate of N-nitrosation of morpholine was expressed by Fan and Tannenbaum[7] as

$$\frac{d[R_2NNO]}{dt} = k_N[HNO_2]^2[R_2NH] \qquad (25)$$

where k_N is a lumped rate constant. This lumped constant can be expressed in terms of the present rate constants by adding eqs 11 and 12, and using the result to relate $[HNO_2]$ to $[N_2O_3]$. For the conditions of the Fan and Tannenbaum study, $k_6[R_2NH] \ll k_4$. With no participating anions, the k_{10} term from eq 12 is absent. Using a kinetic model which describes nitrosation kinetics under acidic conditions,[19] we infer also that $2k_1[NO]^2[O_2] \ll k_5[HNO_2]^2$. The sum of eqs 11 and 12 then gives $k_4[N_2O_3] \cong k_5[HNO_2]^2$. Finally, a comparison of eqs 17 and 25 implies that

$$k_N/k_5 = k_6/k_4 \qquad (26)$$

Using reported values[7,20] at 25 °C of $k_N = 2.3 \times 10^5$ M^{-2} s^{-1} and $k_5 = 5.6$ M^{-1} s^{-1}, eq 26 implies that $k_6/k_4 = 4.1 \times 10^4$ M^{-1} for morpholine under acidic conditions. This is in excellent agreement with the present findings at physiological pH ($k_6/k_4 = 4.0 \times 10^4$ M^{-1}). The consistency of these values provides strong support for the view that N_2O_3 is an intermediate involved in N-nitrosation at both acidic and physiological pH.

Nitrosation of Azide. The role of N_2O_3 as an intermediate in N-nitrosation at physiological pH has been questioned on the basis of an observed decrease in the rate of azide nitrosation relative to the rate of N_2O_3 hydrolysis at physiological pH, as compared to acidic conditions.[8,21] The calculated decrease in k_6/k_4 in the more recent study[21] was 20-fold, although it could range from 10- to 40-fold, depending on the value of k_4, which has a reported range[20,22,23] of 530–2000 s^{-1}. Of importance is that the solutions used in the recent azide study[21] contained 0.01 M phosphate. According to eq 23, that level of phosphate would be sufficient to reduce the apparent rate constant for azide N-nitrosation by 5-fold. Thus, the finding that phosphate inhibits N-nitrosation at physiological pH explains much of the apparent discrepancy. Moreover, the second-order dependence of $[N_2O_3]$ on [NO] (eq 14) is consistent with that of the unidentified intermediate discussed in the earlier azide study.[8]

Conclusions. The present finding that N-nitrosation of morpholine is inhibited by phosphate and chloride at physiological pH, together with previous observations, suggests the reaction network depicted in Figure 7. In the presence of NO and O_2, N_2O_3 is generated. Aside from its reaction with amines

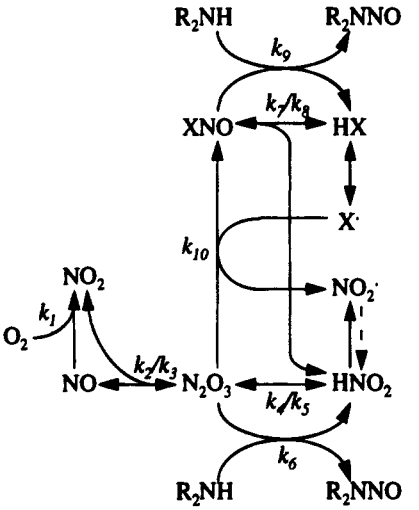

Figure 7. A schematic of the principal reactions involving nitrogen oxide species, various anions (X^-), and secondary amines (R_2NH) in aqueous solutions. The dashed arrow is significant only under acidic conditions. The rate constants shown are discussed in the text and in Table 1.

or other organic substrates, the disposition of N_2O_3 is affected by two major processes, hydrolysis to HNO_2 and reaction with any of several anions (X^-) to form nitrosyl compounds (XNO). The nitrosyl compounds in turn can react with amines or be hydrolyzed to HNO_2. At physiological pH, any HNO_2 formed is almost entirely converted to NO_2^-, thereby tending to favor hydrolysis of the nitrosating agents (N_2O_3 or XNO) over nitrosation of amines. With certain anions (phosphate and Cl^-), the hydrolysis of XNO is evidently much faster than the nitrosation of amines by XNO. Such anions will merely act as scavengers of N_2O_3, thereby lowering the rate of N-nitrosation. Other anions (SCN^-, NO_3^-, and ClO_4^-) appear to be relatively unreactive with N_2O_3, and have little or no effect on the rate of N-nitrosation at near-neutral pH. (It is possible that the reactivity of, say, SCN^- with N_2O_3 is not negligible, but that it was masked under our experimental conditions. This would have occurred if the ratio of NO_2^- to R_2NNO formed via NOSCN happened to be the same as in the absence of SCN^-.) Under sufficiently acidic conditions (pH < ~4), NO_2^- no longer acts as an irreversible sink for HNO_2. As indicated by the dashed line in Figure 7, any NO_2^- present can then be converted to HNO_2, XNO, and N_2O_3. Thus, N_2O_3 levels and N-nitrosation rates at acidic pH may be unaffected by certain anions; this is the finding for phosphate.[7] For those anions whose nitrosyl forms exhibit sufficient reactivity with amines (e.g., SCN^- and, to a lesser extent, Cl^-), nitrosamine formation at acidic pH may be enhanced by XNO. Accordingly, an anion such as Cl^-, which has a nitrosyl form that reacts both with N_2O_3 and with amines, can either inhibit or promote nitrosamine formation, depending on the pH.

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