revealed by an increase in emission as the quenching condition is relieved.

When calcein-loaded vesicle fraction 23 or 24 (Figure 2a)[13] is incubated at room temperature, pH 7.0, the fluorescence intensity remains constant over a period of 10 min. Acidification of the suspension to pH 6.5 then causes a rapid increase in emission intensity (Figure 4, curve a). Subsequent addition of the detergent Triton X-100 causes no further increase in fluorescence and thus confirms that the H^+-induced release of the dye from vesicles bearing surface-bound polyelectrolyte chains is both rapid and quantitative. The effectiveness of the polyelectrolyte is remarkable, given the modest concentration of polymer on the membrane surface (ca. 5% of the membrane by weight).

Figure 4 also shows the results of two control experiments. First, coincubation of EYPC and thiol-free PEAA, followed by passage of the mixture through the Sepharose column, afforded a vesicle suspension that was nearly pH-insensitive. Acidification of this suspension resulted in a fluorescence increase only 10% of that observed on subsequent addition of detergent (Figure 4, curve c). Chromatographic separation thus reduces the concentration of the polyelectrolyte to a level that is insufficient to cause membrane reorganization. In a second control experiment, thiol-free PEAA was incubated with the 9:1 mixture of EYPC and **5** and the mixture was chromatographed. In that case, the release of calcein on acidification was approximately 30% within 2 min and ca. 40% after 30 min (Figure 4, curve b). The presence of 10 mol % of **5** thus changes the behavior of the lipid membrane, either by increasing the adsorption of PEAA or by sensitizing the bilayer to reorganization by small amounts of adsorbed polymer. In any case, the results of the experiments summarized in Figure 4 demonstrate the effectiveness of a covalent surface conjugation procedure in the construction of pH-sensitive, semisynthetic phosphatidylcholine membranes.

(13) Similar experiments carried out on earlier vesicle fractions consistently resulted in partial, rather than quantitative, release of entrapped calcein upon acidification. We believe this to be a result of variations in vesicle structure (e.g., in the number of concentric bilayers), which would correlate with vesicle size and elution volume. We have not pursued this point. Selection of vesicle fraction 23 or 24 assured reproducible, rapid, and quantitative release of contents.

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Nitrogen Protonation of *N*-Nitrosodimethylamine†

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Abstract: Evidence for the presence of the $Me_2N(H)NO^+$ ion at kinetically significant concentrations in aqueous solutions of *N*-nitrosodimethylamine (NDMA) at pH $\leq$ 2 has been found. To obtain this evidence, the methyl group syn to the oxygen of NDMA was selectively deuteriated and the rate of $Z \rightleftarrows E$ equilibration in the resulting NDMA-d_3 was measured by nuclear magnetic resonance spectrometry as a function of pD. The reaction was first order in $[D^+]$, with the plot of observed first-order rate constants versus $[D^+]$ having a slope of $k/[D^+] = 10^{-2}$ M^{-1} s^{-1} at 3 °C and an ionic strength of 0.2 M. The observed rate data were used to estimate K_a for N-protonated NDMA as 10^{12}–10^{13} M by assuming that the rates of rotation about the N–N bond in $Me_2N(H)NO^+$ and of its deprotonation are similar to those for the isoelectronic N-protonated carboxamide function. The trisubstituted nitrogen of the nitrosamine thus appears to be several orders of magnitude less basic than that of the carboxamides.

N-Nitrosoammonium ions such as **2** (Scheme I) have been implicated in several important transformations, including diazotization and deamination of primary amines,[1,2] nitrosation[1,2] and transnitrosation[2,3] of secondary amines, and photolysis[4] as well as protolysis,[5,6] rearrangement,[7] and mass spectral fragmentation[8] of carcinogenic *N*-nitroso compounds, but little is known about the lifetimes of these ions in solution. Indeed, many of the above reactions are so rapid as to suggest that steady-state concentrations of **2** must be immeasurably small, with a wealth of published data firmly establishing the oxygen atom, rather than nitrogen, as the preferred site of stable electrophilic attachment to the potentially ambident nitrosamino function.[6,9-12] Thus, the O-protonated tautomer **3a** is the only conjugate acid of *N*-nitrosodimethylamine (**1a**) that is macroscopically observable by nuclear magnetic resonance (NMR) spectrometry in acidic solutions of **1a**.[6,9,12]

We now present evidence that reversible N-protonation of the simplest nitrosamine, **1a**, does occur to a detectable extent in dilute

† Dedicated to Prof. Dr. Rolf Preussmann on the occasion of his 60th birthday.
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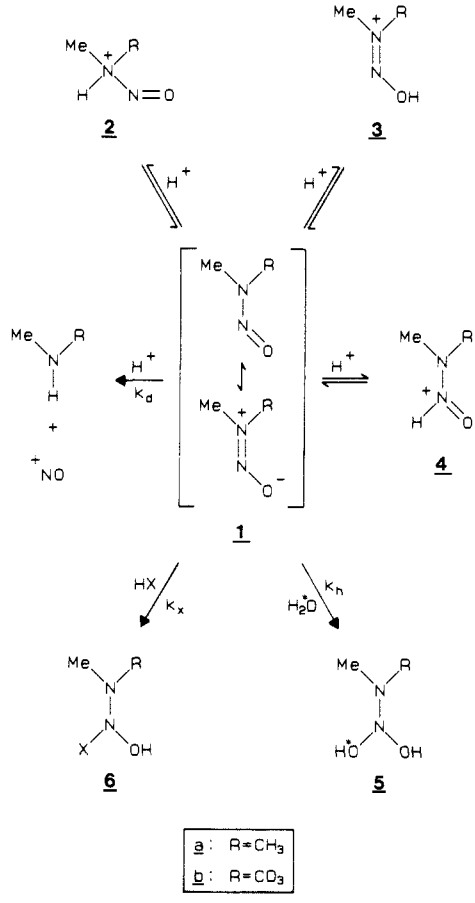
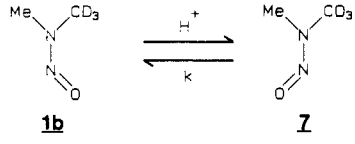
Scheme I. Possible Mechanisms for Isomerization of **1b**



aqueous acid and report kinetic data that permit the equilibrium constant for the process to be estimated.

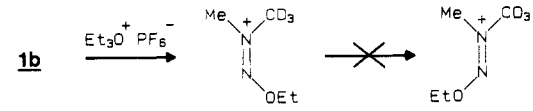
Results

The key finding came when an aqueous sample of the selectively deuteriated nitrosamine **1b** was acidified. It had been anticipated that the increase in the formal order of the N–N bond resulting from O-protonation (tautomer **3b**) would increase the barrier to rotation, preventing the conformation having the labeled methyl group syn to the oxygen from undergoing conversion to the *E* form. The opposite was observed, however, with rapid conformational equilibration (between **1b** and **7**, as shown in eq 1) being the outcome.



(1)

The assumption that the O-coordinated nitrosamine would be conformationally frozen was confirmed by treating **1b** with a Meerwein salt (eq 2). As predicted, the resulting *N,N*-di-



(2)

alkyl-*N'*-alkoxydiazenium ion **8** showed no evidence of conversion to *E* isomer **9** when it was dissolved in strongly acidified deuterium oxide.

Interconversion of **1b** and **7** was found to be first order in acid. A series of solutions of **1b** (0.40 mM) in D_2O containing DCl/KCl at a constant ionic strength of 0.2 M was prepared. The rate of isomerization in each was determined by following the decrease in intensity of the single proton magnetic resonance signal at δ 3.8 for **1b** (or, alternatively, the increase in signal at δ 3.1 for **7**) at nine different measured pD values between 1 and 2.5. First-order rate plots were linear throughout the observation periods employed. All runs were conducted at 3 °C to suppress the thermal equilibration of **1b** and **7** seen at 37 °C ($t_{1/2}$ 2 h).[13] A plot of k versus $[D^+]$ was linear (Figure 1), with regression analysis yielding a slope of 1.0×10^{-2} M^{-1} s^{-1} and $r = 0.991$.

Mechanisms that could potentially explain such facile *Z* to *E* interconversion without requiring reversible N-protonation of **1** can be excluded on the basis of the following experiments. Addition of water across the N=O bond (to produce an *N,N*-dihydroxyhydrazine **5**, whose reversion to starting material would yield **1b** and **7** with equal probability) could account for the observed equilibration, but such a mechanism would require that oxygen from the aqueous acid be exchanged with the oxygen of **1** at a rate comparable to that of the conformational equilibration. We prepared a sample of ^{18}O-labeled *N*-nitrosodimethylamine and exposed it to the above reaction conditions for up to 3 days without detecting loss of label by mass spectrometry. This rules out the participation in the equilibration reaction of a reversible hydration/dehydration mechanism.

Evidence against mechanisms involving nucleophilic attack on the nitroso nitrogen, such as the addition of a protic acid across the N–O double bond (to produce a species, **6**, in which rotation about the formal single bond between the nitrogens might be rapid enough to account for equilibration), was also obtained. Such mechanisms should be greatly favored by increases in anion nucleophilicity, but the k for equilibration in D_2SO_4 fit the line obtained for the DCl solutions (Figure 1). The observed specific acid catalysis by hydronium ion rules out participation by the counterion through rate-limiting nucleophilic attack at the central nitrogen atom.

Finally, significant involvement of a denitrosation–recombination pathway yielding free secondary amine and nitrosating agent as intermediates was excluded by running the equilibration of **1b** and **7** in the presence of unlabeled dimethylamine hydrochloride; no increase in the **1a**:**1b** ratio could be detected by single ion monitoring mass spectrometry at m/z 74 and 77, indicating that no transnitrosation occurs under these conditions.[14]

Discussion

Position of N-Protonation. Since k_h, k_x, and k_d (Scheme I) as well as the rate constant for thermal equilibration of **1b** and **7** are thus all too small to contribute significantly to the observed *Z* to *E* interconversion reactions summarized in Figure 1, and since the spectra of **8** indicate that O-coordinated ion **3b** should be conformationally stable, reversible N-protonation of the nitrosamino group appears to offer the only plausible explanation for the observed first-order dependence on hydrogen ion concentration. We believe the intermediacy of ion **2b**, with its obligatory N–N bond order of one, provides the most likely route to the rapid randomization of conformation observed in the conversion of **1b** to **7**. Alternatively, protonation at the nitroso nitrogen (as in **4**)

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(14) No reaction other than the equilibration of **1b** and **7** could be detected by NMR in any equilibration mixture, even after holding at 25 °C for 1 week.

could contribute to the results. Resonance stabilization should be less important in this ion than in the free nitrosamine because the canonical structure with the formal double bond between the nitrogens would place a full positive charge on each of the contiguous nitrogens. The resulting destabilization should tend to lower the bond order and hence increase the rate of rotation. The involvement of **4** appears unlikely, however, on the basis of recent ab initio calculations.[15] We conclude that *N*-nitrosodimethylamine is protonated to a kinetically significant extent at the amino nitrogen atom in aqueous solutions at pH's of ~2.5 or less.

Extent of N-Protonation. It should be possible to estimate the concentration of ion **2** present at equilibrium from the observed rate constants (k) plotted in Figure 1. Since no evidence of any chemical change other than the conformational equilibration shown in eq 1 was observed in any of the acidic nitrosamine solutions studied,[14] the overall transformation can be represented by eq 3,

$$\mathbf{1b} + H^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathbf{2b} \underset{k_r}{\overset{k_r}{\rightleftharpoons}} \mathbf{7}\cdot H^+ \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \mathbf{7} + H^+ \qquad (3)$$

where k_1, k_2, and k_r are the rate constants for protonation of **1**, deprotonation of **2**, and rotation about the N–N bond in the *N*-nitrosoammonium ion, respectively. The general expression for the approach to equilibrium in this system is given in eq 4

$$k = 2k_r k_1[H^+]/(k_2 + 2k_r) \qquad (4)$$

provided that the concentration of **1** is much larger than that of the N-protonated species (as it must be since the measured pK_a's for the more abundant O-protonated nitrosamine tautomers[12,16] are smaller than the pH's of the solutions used here).

These considerations suggest a value of 10^{12}–10^{13} M for the acid dissociation constant of **2** regardless of the relative sizes of k_r and k_2. Thus, if rotation about the N–N bond is much slower than deprotonation (i.e., if $k_2 \gg k_r$), eq 4 rearranges to $k/[H^+] = 2k_r/K_a$, where $K_a = k_2/k_1$. Since $k/[H^+]$ is the measured slope (10^{-2} M^{-1} s^{-1}) of the line in Figure 1, K_a can be calculated if the rotational barrier is known. If this barrier in **2** is assumed to be roughly similar to that of the isoelectronic N-protonated carboxamide function,[17] i.e., if $k_r \approx 3 \times 10^{10}$ s^{-1}, then $K_a \approx 6 \times 10^{12}$ M.

If $k_r \gg k_2$, on the other hand, eq 4 becomes $k/[H^+] = k_1 = 10^{-2}$ M^{-1} s^{-1}.[18] By substituting this value into the expression for the dissociation constant, $K_a = k_2/k_1$, the extent of N-protonation can be estimated from a determination of k_2. Assuming that the deprotonation rate for **2** approaches the limit of diffusion-controlled encounter with water, as is also apparently the case for the carboxamides[19] (i.e., assuming that $k_2 \approx 10^{10}$–10^{11} s^{-1}), then $K_a \approx 10^{12}$–10^{13} M.

The same quantitative conclusion is indicated even if rotation and deprotonation are comparable in rate. If $k_r = k_2/2$, for example, eq 4 rearranges to $k[H^+] = k_r/K_a$. Under these conditions, K_a is half the value calculated above for $k_2 \gg k_r$, i.e., $K_a \approx 3 \times 10^{12}$ M.

We conclude that the most reasonable first estimate for the equilibrium constant (K_a) for dissociation of **2** is in the range of 10^{12}–10^{13} M. It should be emphasized that this is at best a semiquantitative measurement. The assumption that k_r and k_2 for a nitrosamine are similar to those for the carboxamides ignores the effects of substituents on these rates, leading to errors that may well be quite large. The magnitude of several other potential sources of error is likely to be small by comparison, so these have also been neglected in the calculations. The impact of O-protonation on the rate equation has not been considered, for example, but the pK_a for this process is <1[12,16] and we were working in the pH range of 1–2.5. Thus, only a small proportion of the nitrosamine could have been removed from the conformational equilibrium by conversion to the rotationally unreactive conjugate acid **3**. Additionally, the effects of temperature on the rate constants involved have not been measured, and it is assumed that the thermal equilibration of **1b** and **7** is negligibly slow compared to the acid-catalyzed pathway. These considerations should not affect conclusions regarding rates and equilibria at the temperatures and pH values used here (for which the uncatalyzed pathway was demonstrated to make no significant contribution to the rates summarized in Figure 1), but they may have to be taken into account in extrapolating the present estimates to substantially different conditions. Furthermore, no attempt has been made to adjust for secondary kinetic and solvent isotope effects in extrapolating from these deuteriated media to normal aqueous solutions of the nitrosamine. However, any net difference between k^H and k^D attributable to these types of isotope effect[20] should also be much smaller than the errors arising from the assumed value of 10^{10}–10^{11} s^{-1} for k_2 or from the assumption of similarity between the k_r's for the N-protonated nitrosamines and carboxamides.

If K_a is indeed in the range of 10^{12}–10^{13} M, then the nitrogen of the nitrosamino group would appear to be several orders of magnitude less basic than that of the carboxamides, for which pK_a's for N-protonation of −6.8 to −8.4 have been reported.[21] If the rotational barriers in the N-protonated nitrosamines and carboxamides[17] are equal and deprotonation of each is diffusion controlled, one might predict that the difference in K_a is determined primarily by differences in the protonation rate constants (k_1). To a first approximation, this prediction is borne out by experiment since the rate constant for *N*-methylacetamide N-protonation is 3.8×10^2 M^{-1} s^{-1},[22] or 4–5 orders of magnitude larger than that suggested above for N-protonation of **1** ($k_1 = 10^{-2}$ M^{-1} s^{-1}). The nitrosamines and carboxamides can display similar acid–base reactivity in other important respects, however. The carboxamides have also been shown to undergo acid-catalyzed rotation about the *N*-acyl bond ascribed to analogous N-protonation,[22] and the pK_a's for the more abundant O-protonated tautomers are in the same range (−1.8 to +0.8 for the carboxamides[22,23] and +0.3 to +0.6 for the nitrosamines[12,16]).

Conclusions and Significance

N-Nitrosodialkylammonium ions have long been presumed to be transiently involved in the formation, protolytic cleavage, photolysis, transnitrosation, and gas-phase fragmentation reactions of the carcinogenic nitrosamines, but little information is available concerning their steady-state abundance. The present results indicate that such ions exist in noninfinitesimal concentrations at equilibrium in simple solutions of an *N*-nitrosodialkylamine in dilute aqueous acid. Our kinetic data suggest that the trisubstituted nitrogen atom of the nitrosamino group is protonated at least 4–5 orders of magnitude less rapidly and extensively than that of the carboxamido function, presumably reflecting much more efficient resonance delocalization of electron density away from nitrogen toward oxygen in the nitrosamines than in the carboxamides. However, it is possible that introduction of electron-withdrawing substituents at the α-carbon atom (as happens,

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(18) Protonation of the trisubstituted nitrogen has been reported to be rate-limiting in certain other transformations of *N*-nitroso compounds, including protolysis of *N*-nitrosoureas[7] and the transnitrosation reactions of *N*-nitrosarylamines.[2,3]

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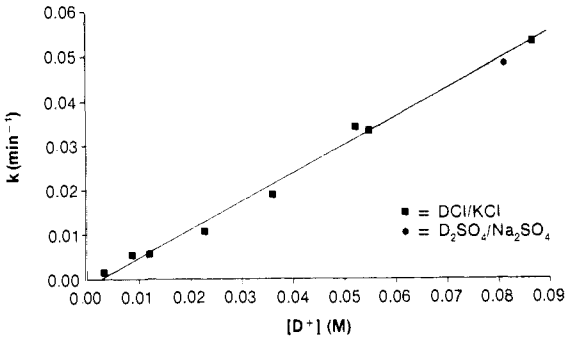

Figure 1. Dependence of observed rate constant k on hydrogen ion concentration for interconversion of **1b** and **7** at 3 °C and μ = 0.2 M.

for example, when a nitrosamine is α-hydroxylated during metabolic activation to the proximately carcinogenic form[24]) could partially reverse this effect. The finding that proton transfer can catalyze Z to E interconversion in nitrosamines bearing two different carbon substituents could help explain how reactions having a clear conformational requirement can be driven rapidly to completion despite large energy barriers for the normal (uncatalyzed) $Z \rightleftarrows E$ interconversion. Finally, it should be noted that *N*-nitrosodimethylamine (**1a**) is a potent experimental carcinogen[24] found in the urine of every person studied in a recent survey[25] and is therefore a suspected causative agent in human cancer. The availability of the new rate and equilibrium constant data reported above for the fundamental acid–base reactions of this compound could be of importance in seeking to refine our understanding of the biological as well as chemical behavior of the nitrosamines and their conjugate acids.

Experimental Section

Warning! The materials used are potentially explosive (the alkanediazotates[13]), poisonous (thallium compounds[26]), and/or carcinogenic (*N*-nitroso compounds[24]). These substances must be handled, stored, and discarded only with proper respect for their hazardous properties.

Reagents and Methods. All chemicals were obtained from Aldrich Chemical Co. except for the following. Anhydrous hydrogen chloride and dimethylamine gases were obtained from Matheson Gas Products. Iodomethane-d_3 and [^{18}O]water were from MSD Isotopes. Methylhydrazine was obtained from Kodak. The preparation of (*Z*)-*N*-ethoxy-*N'*-methyl-*N'*-(methyl-d_3)diazenium hexafluorophosphate (**8**) has been described previously.[13] ^{1}H NMR spectra were obtained at 300.074 MHz on a Nicolet NT-300 wide-bore spectrometer with a 5-mm fixed-tune probe locked on the deuterium of the solvent in use and referenced to tetramethylsilane. Mass spectral measurements of isotopic enrichments were obtained via gas chromatography–mass spectrometry (GC–MS) of dichloromethane extracts of the aqueous solutions with a DB Wax capillary column with selected ion monitoring of the relevant molecular ions on a VG 70-250 double-focusing mass spectrometer.

Preparation of (*Z*)-*N*-Nitroso-*N*-(methyl-d_3)methylamine (1b). The reaction of thallous ethoxide with methylhydrazine and *n*-butyl nitrite in diethyl ether was used to produce thallium(I) (*E*)-methanediazotate. Treatment of this material with iodomethane-d_3 at 0 °C led to the formation of **1b**. These procedures were conducted exactly as previously described[13] except that D_2O was used in the final workup. This resulted in the production of a solution of **1b** in D_2O that had a concentration of 4.0 M (by UV) and was used for all of the subsequent NMR experiments. The ^{1}H NMR of a freshly prepared solution exhibited a large singlet due to the *Z* isomer (δ 3.80) and a small singlet due to the *E* isomer (δ 3.15) as well as a small impurity as a singlet at δ 3.21. The solution was stored in the freezer at −15 °C until used.

Preparation of ^{18}O-Labeled *N*-Nitrosodimethylamine.[27] A solution of sodium nitrite (162 mg, 2.34 mmol) in [^{18}O]water (1.05 g, 98.3 atom % ^{18}O) was cooled in an ice bath, and a stream of anhydrous HCl was passed into this solution until the weight had increased by ca. 0.2 g with concurrent separation of a fine white precipitate. After being stirred at room temperature for 7 h under nitrogen, the mixture was again cooled on ice and a stream of anhydrous dimethylamine was bubbled through until the weight had increased by 0.25 g. The resulting brown solution was stirred overnight at room temperature under nitrogen and extracted with dichloromethane (2 × 2 mL). The extract was dried over magnesium sulfate to remove any remaining [^{18}O]water. The remaining organic solution was placed atop 1.0 mL of distilled water (normal ^{16}O/^{18}O content), and the dichloromethane was removed by careful distillation through a Vigreux column over several hours. The resulting aqueous solution was ca. 3 M in ^{18}O-labeled *N*-nitrosodimethylamine with an isotopic enrichment of 88 atom % (by mass spectrometry).

Kinetic Studies. The solutions in the pD range from 1.0 to 2.5 were prepared as follows. Stock solutions of 0.2 M DCl in D_2O and 0.2 M KCl in D_2O were cooled on ice. Appropriate aliquots of these solutions were measured into NMR tubes with a syringe such that the final volume for each was 1.0 mL regardless of pD. Just prior to each run, 100 μL of the D_2O solution of **1b** was added and the sample was inserted into the precooled (3 °C) NMR probe.

Data collection was begun as soon as possible thereafter, generally between 15 and 30 min after mixing. Spectra were obtained at intervals ranging from 2.5 (for the sample at pD 1.06) to 15.0 min (pD 2.48) over a period of ~3 half-lives for each sample. (At pD 1.06 this corresponded to 30 min, while at pD 2.48 the sample was monitored for 300 min.) After data collection the samples were warmed to room temperature and their actual pD values were obtained by using a standard pH probe and meter that had been calibrated with protiated buffers and then equilibrated in D_2O. The pD of each solution was taken as pD = pH + 0.4, as is common practice.[28] Solutions were then allowed to stand for 12 h and the infinity point spectra were run. No deviation from these infinity spectra occurred after the samples had been stored for up to 1 week.

Data treatment was as follows. For each spectrum, the areas under the singlets attributable to the *Z* isomer (**1b**) (δ 3.8) and to the *E* isomer (**7**) superimposed on a small (unidentified but unchanging) impurity at δ 3.2 were measured and normalized so that for a given pD their sum was invariant.[29] The values of the rate constants were obtained from these data in the usual manner via a linear regression analysis of the 12–20 data points available for each pD used. The values for $k \times 10^2$ (in min^{-1}) and the correlation coefficients obtained for the various solutions were as follows: pD 1.06, 5.23 and 0.977; pD 1.26, 3.30 and 0.991; pD 1.28, 3.38 and 0.995; pD 1.44, 1.87 and 0.999; pD 1.64, 1.06 and 0.999; pD 1.91, 0.558 and 0.998; pD 2.05, 0.534 and 0.992; pD 2.48, 0.156 and 0.996. These data were then used to prepare Figure 1. The measurement in D_2SO_4/Na_2SO_4 solution was performed in a similar fashion. The measured pD of this sample was 1.09 and the value of $k \times 10^2$ was 4.77 min^{-1} with a correlation coefficient of 0.964.

^{16}O/^{18}O Exchange Experiments. Solutions of ^{18}O-labeled *N*-nitrosodimethylamine in HCl/KCl/$H_2{}^{16}O$ having pH values of 1.1 and 2.5 were prepared by procedures analogous to those used for the NMR studies. A solution containing only KCl without acid was also used. These three solutions, as well as an unaltered sample of the aqueous solution from the previously described preparation, were stored at room temperature for 3 days and then checked for isotopic composition as described above. No detectable change occurred (i.e., the samples were still 88 atom % ^{18}O). The error in the mass spectral determinations (which were done in triplicate) was estimated at ±2%.

Attempted Equilibration of 1b with Excess Dimethylamine. A solution of **1b** in HCl/KCl at a pH of 1.1 was prepared. To this was added unlabeled dimethylamine hydrochloride (0.1 g). After being stirred at room temperature for 2 days, this sample of equilibrated **1b** + **7** was compared to the original stock solution of **1b** by GC–MS. Both proved to be >99% methyl-d_3 enriched, and there were no distinguishable differences between the samples.

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