1642 J. Org. Chem. 1994, 59, 1642-1654

Mechanistic Studies by Electron Paramagnetic Resonance Spectroscopy on the Formation of 2-(*N*-Chloroimino)-5,5-dimethylpyrrolidine-1-oxyl Radical from 5,5-Dimethyl-1-pyrroline 1-Oxide and Hypochlorite-Treated Ammoni

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Received July 26, 1993•

Mixtures of ammonium ion (NH_4^+) or ammonia (NH_3), hypochlorous acid (HOCl), and 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) gave the radical 2-(*N*-chloroimino)-5,5-dimethylpyrrolidine-1-oxyl (1). The first step in the formation of 1 was the reaction of HOCl and NH_4^+ to provide ammonia chloramines: monochloramine (NH_2Cl), dichloramine ($NHCl_2$), and trichloramine (NCl_3). Chloramine composition and the formation of 1 were dependent on pH, the ratio of NH_4^+ to HOCl, and, at acidic pH, on the concentration of chloride in the medium. Conditions were chosen to isolate the individual chloramines in solution for further study. NCl_3 and DMPO gave 1; however, $NHCl_2$ and NH_2Cl required additional oxidants such as HOCl or PbO_2 to produce 1. Studies with ^{15}N-labeled chloramines confirmed that $NHCl_2$ and NH_2Cl reacted with DMPO to form *N*-chloro intermediates that yielded 1 upon subsequent oxidation. Light had no effect on the formation of 1, and UV irradiation did not enhance the EPR signal intensity but caused rapid decay, indicating that radical intermediates of ammonia chloramines were not involved. The mechanism of formation of 1 appeared to involve temporary attachment of chloramine Cl to the nitroxide oxygen of DMPO which activated its β-carbon for nucleophilic addition of the chloramine N. Subsequent *N*-chlorination and/or dehydrochlorination, depending on the reactive chloramine, would then provide 1. However, nucleophilic addition of H_2O to the activated β-carbon of DMPO was competitive because 5,5-dimethyl-2-hydroxypyrrolidine-1-oxyl (DMPO-OH) or 5,5-dimethyl-2-pyrrolidone-1-oxyl (DMPOX) radicals were sometimes observed as minor products along with 1. Analogous chloroimine radicals were not obtained from the reaction of ammonia chloramines with 3,3,4,4-tetramethyl-1-pyrroline 1-oxide (M_4PO) and *N*-*tert*-butyl-α-phenylnitrone (PBN), although their 2-oxo nitroxyl derivatives and hydroxyl adducts were formed as radical products suggesting that nucleophilic addition of H_2O was dominant with these nitroxides.

Introduction

Neutrophils are a specialized group of white blood cells that provide a first line of defense against invading microorganisms.[1] When activated by bacteria or other appropriate stimuli, neutrophils produce hypochlorous acid (HOCl),[2] an important microbicidal compound.[3] Reaction of HOCl with nitrogen compounds furnishes NCl derivatives that are relatively long-lived in cells.[4] Ammonium ion (NH_4^+) or ammonia, a product of various metabolic deamination reactions, can combine with HOCl to give ammonia chloramines which are lipophilic and able to penetrate cell membranes and cause oxidative damage.[4b,5]

Ammonia chloramines also display microbicidal properties,[6] and they are often employed in disinfecting municipal water supplies in a process called chloramination.[6a,7] In chloramination, chlorine and ammonia are added to the water. The more common method of disinfecting water is by chlorination, where only chlorine is added.[8] However, chlorine reacts with organic compounds that may be present in surface waters to give chlorinated byproducts, including chloroform, which are carcinogenic.[8,9] Recent studies have shown that there is a positive association of bladder and rectal cancer in humans with the consumption of chlorinated byproducts

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• Abstract published in *Advance ACS Abstracts*, March 1, 1994.

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0022-3263/94/1959-1642$04.50/0

in drinking water.[10] In view of this cancer risk, chloramination offers an advantage because ammonia chloramines are less reactive than chlorine with organic compounds and thus produce only low levels of chlorinated byproducts.[6a] On the other hand, chloramines are less effective than chlorine as microbicidal agents.[6a,7,8b,11] The chemical mechanisms that underly the cytocidal properties of HOCl and chloramines are, therefore, of practical as well as theoretical interest.

We have previously demonstrated that HOCl-treated NH_4^+ reacts with 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) to give a radical product, 2-(*N*-chloroimino)-5,5-dimethylpyrrolidine-1-oxyl (**1**).[12] DMPO is frequently used as a spin trap in the study of radical species produced in activated neutrophils and various chemical systems.[13] The formation and EPR detection of **1** may be useful as a sensitive and convenient method for analyzing HOCl, NH_4^+, and ammonia chloramines in biological systems and in water disinfection. In the present study, we examine the influence of various reaction conditions such as pH, nitrogen-to-chlorine ratio, and chloride concentration on the formation of **1** and propose a mechanism for its generation from NH_4^+, HOCl, and DMPO.

Experimental Section

Materials and general procedures, except where otherwise indicated, were identical to those described previously.[12] Hypochlorous anhydride, Cl_2O, was prepared by the method of Cady.[14] HOCl was freshly prepared by mixing stock Cl_2O with water[12] and assayed by UV-iodometry.[15] 3,3,4,4-Tetramethyl-1-pyrroline 1-oxide (M_4PO), *N-tert*-butyl-α-phenylnitrone (PBN), and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were obtained from Aldrich or Sigma. 1-Hydroxy-5,5-dimethyl-2-pyrrolidone (DMPOXH) was prepared from 2-nitropropane and methyl acrylate by the method of Bonnet et al.[16] 2-Amino-5,5-dimethylpyrroline 1-oxide was prepared from 2-nitropropane and acrylonitrile.[12,17] Four buffer systems were used: 0.2 M KH_2PO_4–H_3PO_4, pH 3.0 (buffer A); 0.2 M CH_3CO_2Na–CH_3CO_2H, pH 5.4 (buffer B); 0.2 M K_2HPO_4–KH_2PO_4, pH 7.0 (buffer C); and 0.1 M $Na_2B_4O_7$, pH 9.1 (buffer D).

EPR spectra were recorded at ambient temperature in a flat quartz cell with an IBM Instruments Model ER-200D spectrometer equipped with a dual cavity and operated at a frequency of 9.79 GHz, with field modulation of 100 kHz, microwave power of 20 mW, receiver gain of 2.52×10^4, and a time constant of 0.164 s. Computer simulation and integration of EPR spectra were performed using software supplied with the spectrometer. For UV irradiation of EPR samples within the resonant cavity, a PEK 110 mercury-arc high-pressure lamp operating at 100 W and rated at 14×10^4 candle cm^{-2} was used; the lamp was placed 45 cm from the EPR cell. UV spectra were obtained at ambient temperature with a Hewlett-Packard Model 8452A diode array spectrophotometer and 10-, 5-, 2-, or 1-mm light-path quartz cuvettes. pH values of EPR samples were determined with an Orion Model 399A pH meter equipped with a miniature glass electrode from Microelectrodes, Inc. Reported values are final pH readings of reaction mixtures unless stated otherwise. All additions to reaction mixtures and pH adjustments of buffer systems were conducted with vortex mixing. Analytical thin-layer chromatography (TLC) was performed on 100-μm silica gel plates (Kodak Chromagram). The KI–starch solution used in TLC studies was freshly prepared by dissolving starch (0.5% w/v) and KI (1% w/v) in water.

EPR Examination of Reaction Mixtures. HOCl (25 μL, 1 μmol) was added to a solution of $NH_4H_2PO_4$ (50 μL, 10 μmol) in buffer A (40 mM, final volume 250 μL) in a 1.5-mL polypropylene centrifuge tube. The reaction mixture was stirred in a vortex and allowed to stand for 1 min. DMPO (2 μL, 18 μmol) was added to the solution and the mixture stirred briefly. The reaction progress was timed from the addition of DMPO. The reaction mixture was immediately transferred to a flat EPR cell and placed in the cavity of the spectrometer, and the spectrum was recorded. Parallel reaction mixtures, without and with DMPO, were also examined by UV spectroscopy.

The above general procedure was repeated with the following variations: (1) Concentrations of HOCl (0.8–6.4 mM), $NH_4H_2PO_4$ (2–40 mM), and DMPO (18–359 mM) were varied; (2) Before the addition of HOCl, the initial solution was supplemented with various concentrations of KCl (0.2–20 mM); (3) Buffer A was replaced by C and D, and the concentration of $NH_4H_2PO_4$ was varied (0.8–40 mM); (4) Spectra were recorded: (a) in the dark, (b) in the presence of ambient fluorescent light, and (c) with UV irradiation; 0.6 μmol of HOCl was used; (5) Buffer A was replaced by D, and spectra were recorded as in procedure 4; (6) Buffer A was replaced by D, and DMPO replaced by M_4PO (40 μL, 20 μmol); 0.5 μmol of $NH_4H_2PO_4$ was used; (7) DMPO was replaced by M_4PO (50 μL, 50 μmol), and $NH_4H_2PO_4$ by NH_4Cl (50 μL, 10 μmol); 3.5 and 0.7 μmol of HOCl were used; (8) DMPO was replaced by PBN (150 μL, 7.5 μmol); 1 and 4 μmol of $NH_4H_2PO_4$ were used; and (9) Buffer A was replaced by D, and DMPO was replaced by PBN (150 μL, 7.5 μmol); 0.4 μmol of $NH_4H_2PO_4$ was used.

Estimation of Radical Concentrations. To determine radical concentration, the area of the absorption curve of the EPR signal was measured and compared with that of 20 μM TEMPOL, a stable nitroxyl radical. To estimate the concentration of a given radical from its EPR peak height, correlation charts of peak height and signal area were used.

Reaction of DMPO with NCl_3 and $^{15}NCl_3$. DMPO (2 μL, 18 μmol) was added to a solution of NH_4Cl (25 μL, 5 μmol) in

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buffer A (40 mM, final volume 125 μL) in a 1.5-mL polypropylene centrifuge tube. The reaction mixture was stirred in a vortex and kept aside for 1 min. In a second tube, HOCl (25 μL, 1 μmol) was added to a solution of $^{15}NH_4Cl$ (25 μL, 5 μmol) in buffer A (40 mM, final volume 125 μL) and the mixture stirred and allowed to stand for 1 min. The contents of the first tube were immediately transferred to the second tube and the mixture was stirred briefly. The reaction progress was timed from the addition of the contents of the first tube to the second. The reaction mixture was transferred to a flat EPR cell and placed in the cavity of the spectrometer, and the spectrum was recorded. This experiment was also performed by interchanging NH_4Cl and $^{15}NH_4Cl$ in the reaction sequence. Spectrophotometric analysis of parallel reaction mixtures showed that the reaction product of NH_4Cl and HOCl in buffer A was NCl_3 (λ_{max} 336 nm),[18] free of $NHCl_2$ and NH_2Cl.

Reaction of DMPO with $NHCl_2$ and $^{15}NHCl_2$. A stock solution of $NHCl_2$ in buffer B (40 mM) was prepared as follows. HOCl (625 μL, 50 μmol) was added to a solution of $NH_4H_2PO_4$ or NH_4Cl (300 μL, 60 μmol) in buffer B (40 mM, final volume 5 mL, pH 4.7) in a stoppered test tube covered with Al foil. The reaction mixture was stirred well and kept at 4 °C for 18–24 h. Spectrophotometric analysis showed that this solution contained $NHCl_2$ (λ_{max} 294–296 nm)[18] and was free of NH_2Cl and NCl_3. The concentration of $NHCl_2$ was estimated as 4 mM by using the reported value[18c] for the extinction coefficient of $NHCl_2$ (ϵ_{294} = 280 L mol^{-1} cm^{-1}). $NHCl_2$ solutions prepared in this manner were relatively stable (less than 0.5% decomposition per hour at room temperature). A stock solution of 3.4 mM $^{15}NHCl_2$ was similarly prepared using $^{15}NH_4Cl$ and HOCl.

DMPO (2 μL, 18 μmol) was added to a solution of $NHCl_2$ (150 μL, 0.6 μmol) or $^{15}NHCl_2$ (150 μL, 0.5 μmol) in buffer B (40 mM, final volume 250 μL), and the EPR spectrum of the reaction mixture recorded. The reaction progress was timed from the addition of DMPO and the first scan made at 0.6 min. In separate experiments, the reaction mixture was supplemented with HOCl (7.5 μL, 0.6 μmol) 0.6 min following the addition of DMPO and the EPR recorded; parallel reaction mixtures, without and with supplementary HOCl, were also examined spectrophotometrically. In addition, in place of supplementary HOCl, excess PbO_2 (ca. 30 mg) was added to a similar reaction mixture of DMPO and $NHCl_2$ in buffer B and stirred for 5 s. The mixture was transferred to an all-plastic syringe and passed through a 13-mm 0.45 μm filter (Gelman Acrodisc), and the filtrate was scanned by EPR.

DMPO (2 μL, 18 μmol) was added to a solution of $^{15}NHCl_2$ (150 μL, 0.5 μmol) in buffer B (40 mM, final volume 250 μL) with magnetic stirring in a 2-mL conical Reactivial (Pierce). After 0.6 min, $NHCl_2$ (150 μL, 0.6 μmol) and HOCl (150 μL, 0.6 μmol, in 40 mM buffer B) were added simultaneously to the reaction mixture using two identical motor-driven pipettes. EPR spectra were then recorded. In a separate experiment, DMPO (2 μL, 18 μmol) was added to a mixture of $NHCl_2$ (75 μL, 0.3 μmol) and $^{15}NHCl_2$ (75 μL, 0.25 μmol) in buffer B (40 mM). After 0.6 min, HOCl (7.5 μL, 0.6 μmol, final volume 250 μL) was added and the EPR spectra were recorded.

Reaction of DMPO with NH_2Cl and $^{15}NH_2Cl$. HOCl (12.5 μL, 1 μmol) was added to $NH_4H_2PO_4$ (10 μL, 2 μmol) in buffer D (40 mM, final volume 250 μL) and the reaction mixture mixed and allowed to stand for 1 min. DMPO (2 μL, 18 μmol) was added to the solution, and EPR scanning was begun at 0.6 min. In a separate experiment, the reaction mixture was supplemented with HOCl (7.5 μL, 0.6 μmol) 0.6 min following the addition of DMPO, and the EPR spectra were recorded. In addition, in place of supplementary HOCl, excess PbO_2 (ca. 30 mg) was added to a similar reaction mixture of $NH_4H_2PO_4$, HOCl, and DMPO in buffer D and stirred for 5 s. The mixture was filtered as above and the filtrate scanned by EPR. UV spectral analysis of parallel reaction mixtures containing $NH_4H_2PO_4$ and HOCl showed that these solutions contained NH_2Cl (λ_{max} 244 nm)[18] and were free of $NHCl_2$ and NCl_3.

NaOCl (5 μL, 0.5 μmol) was added to NH_4Cl (5 μL, 1 μmol) in buffer D (40 mM, final volume 250 μL) in a 2-mL Reactivial with magnetic stirring. After 1 min, DMPO (2 μL, 18 μmol) was added and EPR scanning begun 0.7 min later. This experiment was repeated with the following modifications: (a) 0.7 min after the addition of DMPO, the reaction mixture was supplemented with NaOCl (5 μL, 0.5 μmol) and the EPR spectra were recorded; (b) 0.7 min after the addition of DMPO, a 250-μL aliquot of a 1-min aged solution of $^{15}NH_4Cl$ (5 μL, 1 μmol) and NaOCl (25 μL, 2.5 μmol) in buffer D (40 mM, final volume 500 μL) was added and the EPR spectra of the final mixture were recorded; (c) NH_4Cl and $^{15}NH_4Cl$ were interchanged in the above reaction sequence and the EPR spectra recorded.

Reaction of DMPO with NCl_3 in CCl_4. A solution of NCl_3 in CCl_4 was prepared as follows. Ice-cold NaOCl (25 μL, 10 μmol) was added to NH_4Cl (100 μL, 100 μmol) in 0.4 M KH_2PO_4–H_3PO_4 (pH 3, final volume 500 μL) at 0 °C in a polypropylene centrifuge tube, and the reaction mixture was stirred and allowed to stand for 1 min. The reaction mixture was extracted with CCl_4 (500 μL) and the organic layer separated by centrifugation and dried (Na_2SO_4). The concentration of NCl_3 in the organic extract was estimated as 2 mM using the reported value[19] for the extinction coefficient of NCl_3 in CCl_4 (ϵ_{344} 315 l mol^{-1} cm^{-1}).

A solution of DMPO (2 μL, 0.2 μmol) and NCl_3 (50 μL, 0.1 μmol) in CCl_4 was spotted (2 μL) on a TLC plate and the plate developed (10% EtOAc in $CHCl_3$) and visualized (KI–starch) as described previously.[12] A solution of DMPO (40 μL, 8 μmol) and NCl_3 (50 μL, 0.1 μmol) in CCl_4 (final volume 250 μL) was also analyzed by EPR. The EPR spectrum was compared with that obtained for a CCl_4 extract (dried over Na_2SO_4) of the reaction mixture of 2-amino-5,5-dimethylpyrroline 1-oxide (5 μL, 1 μmol) and NaOCl (10 μL, 1 μmol) in buffer A (40 mM, final volume 250 μL).

Results and Discussion

We previously showed that 2-(*N*-chloroimino)-5,5-dimethylpyrrolidine-1-oxyl radical (**1**) was formed in an aqueous solution of NH_4^+ (or NH_3), HOCl, and DMPO (**2**) and that N and Cl of the chloroimine group of **1** were derived from NH_4^+ and HOCl, respectively.[12] Although there is no apparent reaction of NH_4^+ and DMPO,[12,20] HOCl reacts with NH_4^+ and DMPO to produce chloramines (NH_2Cl, $NHCl_2$, and NCl_3)[21] and DMPOX (**3**),[22] respectively. Three possible mechanisms involving these reactive species may be postulated for the formation of **1**: (a) reaction of the chloramines with DMPOX, (b) addition of NH_3 to the 2-position of DMPO following activation of DMPO by HOCl in a manner analogous to the formation of DMPOX from aqueous HOCl and DMPO,[22b,23] and (c) reaction of the chloramines with DMPO.

The possibility of DMPOX as an intermediate was considered on the basis that some carbonyl compounds form imines with NH_3 or NH_2Cl which then react with HOCl or NH_2Cl to give chloroimines.[24] This possibility was tested using synthetic DMPOX. 1-Hydroxy-5,5-dimethyl-2-pyrrolidone (DMPOXH) (**4**) was prepared

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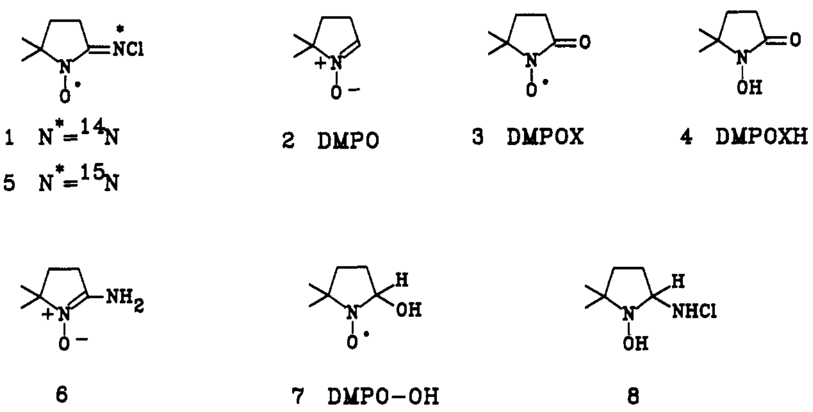
Chart 1



from 2-nitropropane and methyl acrylate following a literature procedure.[16] Treatment of 4 with PbO_2,[16b,25] HOCl, or HOCl-treated NH_4^+ provided DMPOX, which was identified by its characteristic EPR spectrum.[13d,16b] The addition of DMPOX, prepared by oxidation of 4 with PbO_2, to HOCl-treated $NH_4H_2PO_4$ at pH 2.5, 6.7, and 9.1 did not produce the EPR spectrum of the chloroimine nitroxyl 1,[12] and the signal of DMPOX remained virtually unchanged. Likewise, the addition of $NH_4H_2PO_4$ to DMPOX obtained from the reaction of DMPO with HOCl also produced no EPR-detectable change. These results confirmed that DMPOXH and/or DMPOX were not involved as intermediates in the formation of 1.

To determine whether N from NH_3 was incorporated into DMPO either directly (mechanism b above) or via a chloramine (mechanism c above), an experiment was performed using NH_4Cl and $^{15}NH_4Cl$. When a solution of DMPO and $^{15}NH_4Cl$ was mixed with HOCl-treated NH_4Cl at pH 3, an EPR spectrum that corresponded to 1[12] (Figure 1A) was found. However, when a solution of DMPO and NH_4Cl was mixed with HOCl-treated $^{15}NH_4Cl$, the EPR spectrum (Figure 1B) corresponded to that of 5,[12] the 2-[^{15}N]-isomer of 1. These results indicate that, at least at pH 3, N from a chloramine rather than from NH_3 was incorporated into the DMPO adduct. It also follows that the reaction of chloramines with DMPO was faster than the exchange of active Cl between chloramines and NH_3 at pH 3. The spectra were recorded over a period of 22 min during which the EPR signal of 5 (Figure 1B) completely disappeared with gradual development of a weak signal of DMPOX. The absence of EPR lines corresponding to the [^{15}N]-isomer in Figure 1A or to the [^{14}N]-isomer in Figure 1B, even under prolonged conditions, suggests that formation of the chloroimine nitroxyl radical is irreversible.

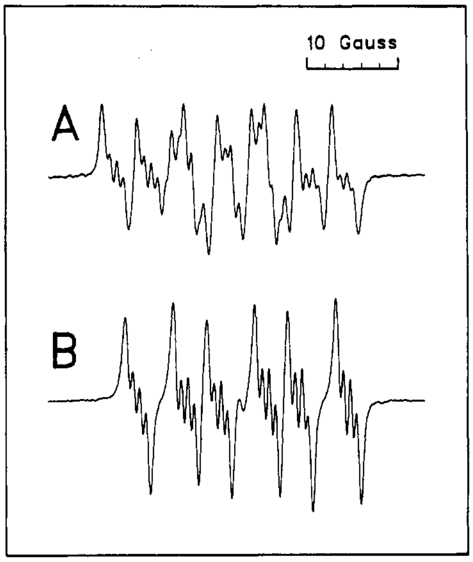


Figure 1. (A) EPR spectrum of 1 obtained by mixing 144 mM DMPO and 40 mM $^{15}NH_4Cl$ in 40 mM KH_2PO_4–H_3PO_4 (pH 3) with an equal volume of 40 mM NH_4Cl and 8 mM HOCl in 40 mM KH_2PO_4–H_3PO_4 (pH 3). Recording of the spectrum began 1.1 min after mixing the two solutions: A_{14N} = 8.9 G, $A_{14N\beta}$ = 3.71 G, A_{35Cl} = 0.78 G, A_{37Cl} = 0.7 G. (B) EPR spectrum of 5 obtained by exchanging NH_4Cl and $^{15}NH_4Cl$ in the sequence described in A. Recording of the spectrum began 0.8 min after mixing the two solutions: A_{14N} = 8.9 G, $A_{15N\beta}$ = 5.25 G, A_{35Cl} = 0.78 G, A_{37Cl} = 0.7 G.

Thus far, we have shown that a reaction of ammonia chloramines and DMPO is involved in the production of 1 from NH_4^+, HOCl, and DMPO. As mentioned above, three chloramines are formed from the reaction of NH_4^+ (or NH_3) and HOCl:[18,21]

$$NH_3 + HOCl \rightarrow NH_2Cl + H_2O$$

$$NH_2Cl + HOCl \rightarrow NHCl_2 + H_2O$$

$$NHCl_2 + HOCl \rightarrow NCl_3 + H_2O$$

The relative distribution of these chloramines depends on reaction conditions such as the ratio ($R_{N/Cl}$) of initial NH_4^+ and HOCl concentrations ($R_{N/Cl} = [NH_4^+]_0/[HOCl]_0$), pH,[18c,21,26] and Cl^- ion concentration.[27] For example, low $R_{N/Cl}$ under acidic or neutral conditions favors formation of the higher chloramines $NHCl_2$ and NCl_3. At high pH values (≥9), only NH_2Cl is observed and the higher chloramines are unstable. As the pH is decreased, NH_2Cl undergoes dismutation to higher chloramines, and this dismutation is catalyzed by Cl^- ions under acidic conditions. Thus, the composition of chloramines in a given mixture can be varied by appropriately controlling reaction

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(24) (a) Hauser, C. R. *J. Am. Chem. Soc.* **1930**, *52*, 1108–1111. (b) Hauser, C. R.; Gillaspie, A. G.; LeMaistre, J. W. *J. Am. Chem. Soc.* **1935**, *57*, 567–570. (c) Theilacker, W.; Wegner, E. *Angew. Chem.* **1960**, *72*, 127–131. (d) Curtin, D. Y.; Grubbs, E. J.; McCarty, C. G. *J. Am. Chem. Soc.* **1966**, *88*, 2775–2786. (e) Lacey, M. J. *Aust. J. Chem.* **1970**, *23*, 841–842. (f) Boosen, K.-J. *Helv. Chim. Acta* **1977**, *60*, 1256–1261.

(25) Aurich, H. G.; Trosken, J. *Leibigs Ann. Chem.* **1971**, *745*, 159–163.

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(27) (a) Morris, J. C. In *Principles and Applications of Water Chemistry*; Faust, S. D., Hunter, J. V., Eds.; John Wiley: New York, 1967; pp 23–53. (b) Hand, V. C.; Margerum, D. W. *Inorg. Chem.* **1983**, *22*, 1449–1456. (c) Bandara, B. M. R.; Bernofsky, C. Unpublished results.

conditions, and each chloramine in a given mixture can be quantitatively estimated from its characteristic UV-absorption peak(s). We examined the formation of 1 using varying amounts of chloramines that were controlled by adjusting the reaction conditions.

Studies at pH ≤ 3. EPR spectra of reaction mixtures were recorded for various $[HOCl]_0$ (0.8 to 6.4 mM) with excess $NH_4H_2PO_4$ (40 mM) and DMPO (72 mM) under acidic conditions (pH 3). The signal of 1 (Figure 1A) was discernible over the entire range of $R_{N/Cl}$ = 6.25 to 50. To correlate the concentration of 1 with chloramine concentration, parallel reaction mixtures of $NH_4H_2PO_4$ and HOCl were examined spectrophotometrically. The strong absorption at 206 and 220 nm in the UV spectra indicated that $NHCl_2$ and NCl_3 were present in all the mixtures.[18b,c] Concentrations of $NHCl_2$ and NCl_3 were quantitated from the absorption at 294 and 336 nm[28] and accounted for all the HOCl used in each mixture. The concentration of 1 showed an approximately linear relationship with $[NHCl_2]$, $[NCl_3]$, and $[HOCl]_0$ (Figure 2). However, it was not clear from these data alone whether $NHCl_2$ or NCl_3 was involved in the formation of 1 at pH 3. The lag in reactivity, or induction that is evident in Figure 2 (inset), may be rationalized by a rapid reaction of 1 and DMPO[12] (see below), which precludes the detection of 1 at very low $[HOCl]_0$ and high $[DMPO]_0$ under our experimental conditions.

We next examined radical formation with various $[NH_4H_2PO_4]_0$ (2–40 mM) and constant $[HOCl]_0$ (4 mM) and $[DMPO]_0$ (72 mM) at pH 2.5. The EPR signal of 1 was found over the entire range of $R_{N/Cl}$ = 0.5 to 10. At low $R_{N/Cl}$ values (0.5 and 1), the early scans (0.5–0.6 min) were attributable almost entirely to 1; however, subsequent scans acquired a contribution from DMPOX.

The signal of 1 showed exponential decay for all $[NH_4H_2PO_4]_0$ examined, and its rate of formation was too fast to be measured under our experimental conditions. The initial concentration of 1 was obtained by extrapolating the decay curves to zero time. Attempts were then made to correlate [1] with the concentration of chloramines available for reaction with DMPO. When parallel reaction mixtures of $NH_4H_2PO_4$ and HOCl were examined by UV, the strong absorption at 206 and 220 nm indicated the presence of $NHCl_2$ and NCl_3 for all the $R_{N/Cl}$ values (0.5 to 10). $[NHCl_2]$ and $[NCl_3]$ were estimated from the absorption at 294 and 336 nm,[28] while [HOCl], the HOCl equivalents unaccounted for by chloramines, was calculated from $[HOCl]_0$, $[NHCl_2]$, and $[NCl_3]$.[29] The plot of [HOCl], $[NHCl_2]$, and $[NCl_3]$ vs $[NH_4H_2PO_4]_0$ (Figure 3) shows that [HOCl] decreased and $[NHCl_2]$ increased with $[NH_4H_2PO_4]_0$ as expected, and that at $R_{N/Cl}$ = 10, HOCl was completely consumed by $NH_4H_2PO_4$ to give $NHCl_2$ and NCl_3. The variation of $[NCl_3]$ with $[NH_4H_2PO_4]_0$ was comparatively small, and the concentration of 1 and its half-life (Figure 3 inset) correlated approximately with $[NCl_3]$.

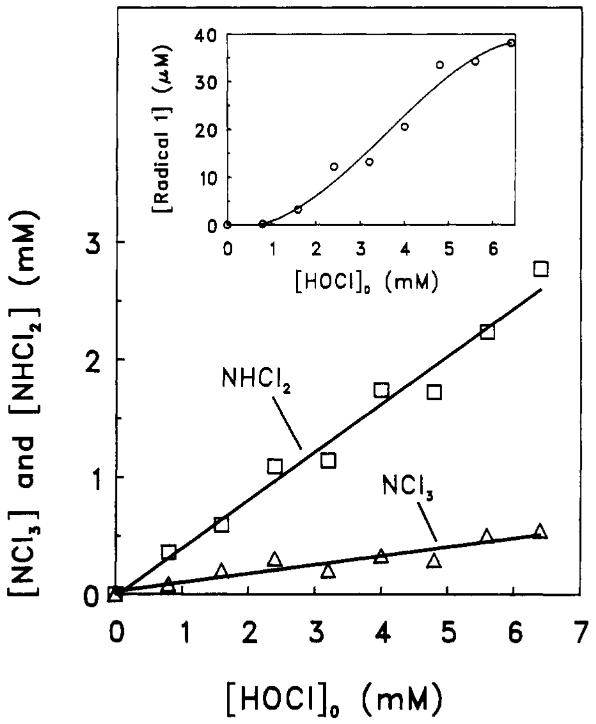

Figure 2. Concentrations of NCl_3 and $NHCl_2$ in solutions that contained various initial concentrations of HOCl (0.8–6.4 mM) and 40 mM $NH_4H_2PO_4$ in 40 mM KH_2PO_4–H_3PO_4 (pH 3). Inset: Concentration of the radical 1 estimated from EPR spectra recorded 0.6 min after the addition of DMPO (72 mM) to the above solutions.

Taken together, the above results suggest that NCl_3 but not $NHCl_2$ participated in the reaction of DMPO to form 1. Assuming that the initial concentration of 1 can represent the rate of radical formation, the correlation of [1] with $[NCl_3]$ indicates that the reaction is first order with respect to NCl_3. Relatively large amounts of HOCl were present in the reaction mixtures when $R_{N/Cl}$ was 0.5 and 1 (Figure 3), and this would explain the appearance of considerable amounts of DMPOX in later scans. The observation that the formation of DMPOX was preceded by that of 1 indicates that the reaction of DMPO and NCl_3 was faster than that of DMPO and HOCl. It is unlikely that DMPOX arose from the hydrolysis of 1 because 1 produced from the reaction of 2-amino-5,5-dimethylpyrroline 1-oxide (6) and HOCl did not yield any DMPOX, even after 24 h.[12,27c]

Effect of Cl^- Ions. The proportions of NCl_3 and $NHCl_2$ in acidic solution can be varied by controlling the Cl^- ion concentration.[27c] To examine this effect, EPR spectra were recorded of reaction mixtures containing varying $[KCl]_0$ (0.2–20 mM) and constant $[NH_4H_2PO_4]_0$ (40 mM), $[HOCl]_0$ (4 mM), and $[DMPO]_0$ (72 mM) at pH 2.8. All the EPR spectra contained the signal of 1 only and showed exponential decay. The initial concentration and the half-life of 1 were obtained from the decay curves. UV spectra of equivalent mixtures of $NH_4H_2PO_4$, KCl, and HOCl were used to estimate the amount of chloramines available for reaction with DMPO. The UV spectra were deconvoluted using a peak-fitting program,[30] and $[NHCl_2]$ and $[NCl_3]$ were calculated from the amplitudes of the component peaks at 220 (NCl_3), 294 ($NHCl_2$), and 336 (NCl_3) nm.[18b,c]

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(28) The concentrations of NCl_3 and $NHCl_2$ were calculated from the following equations:

$$A_{336} = b(\epsilon_{336}^{NCl_3}[NCl_3] + \epsilon_{336}^{NHCl_2}[NHCl_2]) \quad \text{and} \quad A_{294} = b(\epsilon_{294}^{NCl_3}[NCl_3] + \epsilon_{294}^{NHCl_2}[NHCl_2])$$

where A is the absorption, b the path length, and ϵ the extinction coefficient. Subsituting the appropriate ϵ values given by Rizk-Ouaini et al,[26a] the above equations can be reduced to:

$$[NCl_3] = 3.95A_{336} - 0.463A_{294} \quad \text{and} \quad [NHCl_2] = 3.935A_{294} - 2.315A_{336}$$

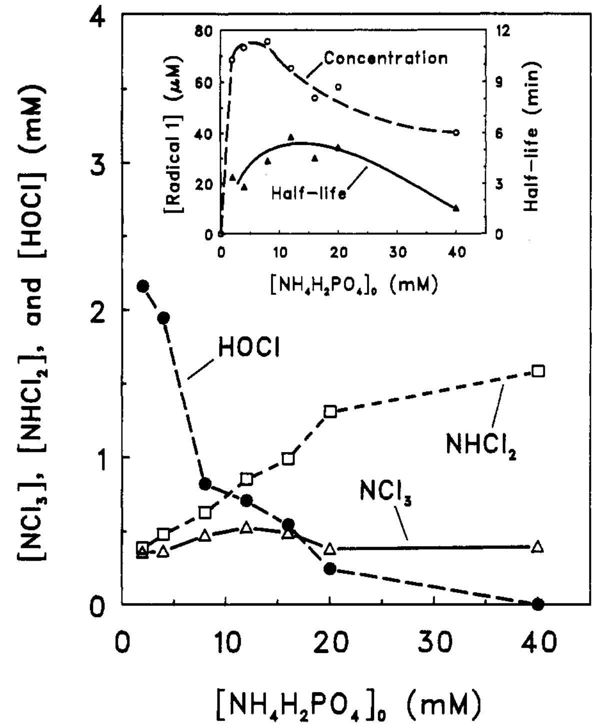

Figure 3. Concentrations of NCl_3, $NHCl_2$, and HOCl in solutions that contained various initial concentrations of $NH_4H_2PO_4$ (2–40 mM) and 4 mM HOCl in 40 mM KH_2PO_4–H_3PO_4 (pH 2.5). Inset: The initial concentration and half-life of 1 estimated from EPR spectra recorded after the addition of DMPO (72 mM) to the above solutions. The concentrations of 1 were obtained by extrapolating the decay curves to zero time.

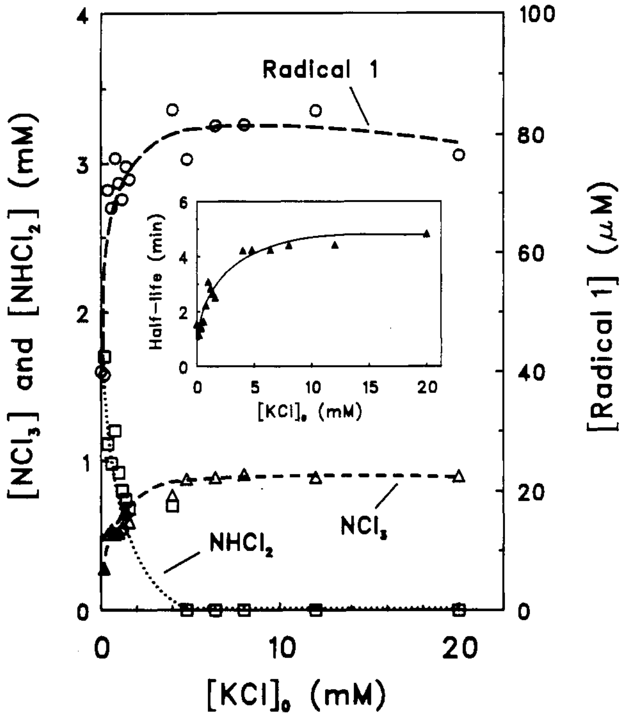

Figure 4. The concentrations of $NHCl_2$, NCl_3, and 1 corresponding to various concentrations of KCl (0.2–20 mM), 40 mM $NH_4H_2PO_4$, 4 mM HOCl, and 72 mM DMPO in 40 mM KH_2PO_4–H_3PO_4 (pH 2.8). The initial concentrations of 1 were obtained by extrapolating the decay curves to zero time. Inset: Half-life of 1 corresponding to various concentrations of KCl in the above reaction mixtures.

A plot of [1], $[NCl_3]$, and $[NHCl_2]$ vs. $[KCl]_0$ is shown in Figure 4. The results indicate that NCl_3 but not $NHCl_2$ was involved in the formation of 1. In addition, [1] appears to correlate with $[NCl_3]$. A similar relationship was inferred from the results presented in Figure 3. The half-life of 1 also increased with $[NCl_3]$ (Figure 4 inset). Although the order of addition of KCl had no effect on [1], it influenced the half-life of 1 which was more stable (about 2-fold) when Cl^- ions were already present in the mixture before the addition of DMPO. It is not clear whether the enhancement in stability of 1 was caused by NCl_3 or Cl^- ions. A Cl^--mediated increase in NCl_3 was responsible for higher [1] when 1 was derived from NH_4Cl rather than from $NH_4H_2PO_4$ under acidic conditions.

Effect of DMPO Concentration. EPR spectra of reaction mixtures at pH 2.8 were also recorded after varying $[DMPO]_0$ over the range of 18 to 359 mM with constant $[HOCl]_0$ (4 mM) and $[NH_4H_2PO_4]_0$ (40 mM). Radical 1 showed exponential decay, and its initial concentration was obtained by extrapolation. The concentration of 1 showed a linear relationship with $[DMPO]_0 < 100$ mM (Figure 5), and, assuming that initial [1] represents the rate of radical formation, one may propose that the reaction is first order with respect to DMPO. Interestingly, the half-life of the radical decreased with increasing $[DMPO]_0$ (Figure 5), which indicates a scavenging of 1 by DMPO.[12] At high $[DMPO]_0$ (180 and 359 mM), the rapid decay of 1 was accompanied by growth of a weak signal of DMPOX indicating that DMPOX could be an end product of the reaction of 1 and DMPO.

At lower concentrations of DMPO (18 and 36 mM), the EPR spectra of early scans contained lines from an additional radical that disappeared rapidly, leaving 1 as the only radical species. Computer subtraction of the EPR lines of 1 from these spectra afforded a 4-line signal ($A_N = A_{H\beta} = 14.87$ G) that was assignable to DMPO-OH (7).[13d,22b] The rate of decay of 7 increased with $[DMPO]_0$ (18 mM: 1×10^{-2} s^{-1}; 36 mM: 3×10^{-2} s^{-1}), and 7 was not detectable at $[DMPO]_0 = 72$ mM under our conditions, indicating that 7 was possibly scavenged by DMPO. Radical 7 was apparently formed by nucleophilic addition of H_2O to DMPO activated by chloramines; we previously demonstrated, using $H_2{}^{17}O$, that DMPO-OH results from the addition of H_2O to DMPO activated by HOCl.[22b]

Studies at pH 4.5–5. From the above experiments, it was inferred that $NHCl_2$ was not involved in the formation of 1. To confirm this, and to examine the fate of $NHCl_2$, we required $NHCl_2$ free of NCl_3. $NHCl_2$ is the least stable of the three chloramines and thus is difficult to isolate. Solutions of $NHCl_2$ decompose rapidly when $NH_4{}^+$ has been removed by ion exchange.[27b] However, $NHCl_2$ is relatively stable at pH 4–5 in the presence of $NH_4{}^+$.[27b,31]

We prepared a solution of $NHCl_2$ (4 mM) free of NH_2Cl and NCl_3 by treating $NH_4H_2PO_4$ or NH_4Cl (12 mM) with

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(29) The equilibrium concentration of HOCl was caculated using the following equation:

$$[HOCl] = [HOCl]_0 - 2[NHCl_2] - 3[NCl_3]$$

(30) The peak-fitting program (PeakFit, Jandel Scientific) was based on a Marquardt-Levenberg non-linear least squares procedure. PeakFit operates in MS-DOS; the LIF file format of the original UV spectra was converted to MS-DOS format by means of software (HPE2080A LIF utilities for the PC) available from Hewlett Packard.

(31) (a) Chapin, R. M. *J. Am. Chem. Soc.* **1929**, *51*, 2112–2117. (b) Fair, G. M.; Morris, J. C.; Chang, S. L. *J. New Engl. Water Works Assoc.* **1947**, *61*, 285–301.

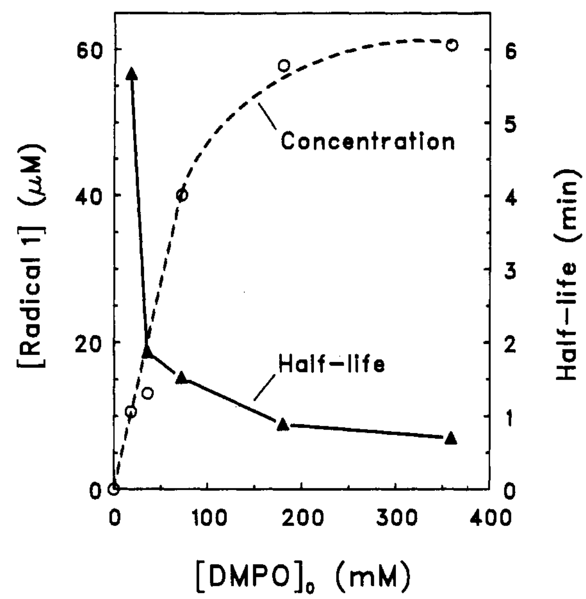

Figure 5. The initial concentration and half-life of 1 corresponding to various concentrations of DMPO (18–359 mM), 40 mM $NH_4H_2PO_4$, 4 mM HOCl, and 40 mM KH_2PO_4–H_3PO_4 (pH 2.8). The concentrations of 1 were obtained by extrapolating the decay curves to zero time.

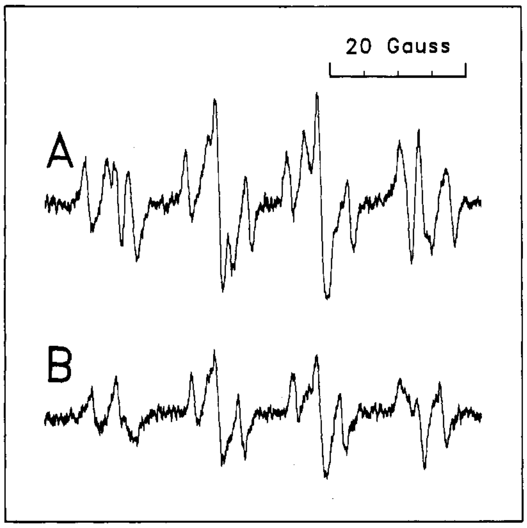

Figure 6. (A) EPR spectrum of a solution of $NHCl_2$ (2.4 mM) and DMPO (72 mM) in 40 mM NaOAc–HOAc buffer (pH 4.7). Recording of the spectrum began 0.7 min after mixing the reagents. The spectrum may represent a chloramine or chloraminium adduct of DMPO (A_N = 14.8 G, $A_H\beta$ = 17.4 G, $A_{14N}\beta$ = 3.2 G, spin = 1) and DMPO-OH (7) (A_N = $A_H\beta$ = 14.95 G). (B) EPR spectrum of a solution of $^{15}NHCl_2$ (2.0 mM) and DMPO (72 mM) in 40 mM NaOAc–HOAc buffer (pH 4.7). Recording of the spectrum was begun 0.6 min after mixing the reagents. The spectrum may represent a chloramine or chloraminium adduct of DMPO (A_N = 14.8 G, $A_H\beta$ = 17.4 G, $A_{15N}\beta$ = 4.0 G, spin = $^1/_2$) and DMPO-OH (7) (A_N = $A_H\beta$ = 14.95 G).

HOCl (10 mM) at pH 4.7. We found that a reaction mixture of $NHCl_2$ (2.4 mM) and excess DMPO (72 mM) did not furnish the EPR signal of 1. Instead, we observed a relatively weak EPR signal (Figure 6A) that appeared to represent a mixture of two radical species. In time, one of these species increased to give the characteristic 4-line spectrum (A_N = A_H = 14.95 G) of DMPO-OH.[13d] Analogous EPR spectra from $^{15}NHCl_2$ and DMPO displayed reduced multiplicity (Figure 6B), indicating that a N atom from dichloramine or ammonium salt was incorporated into the other radical species. The hyperfine splitting constants of this latter radical [A_N = 14.8 G, $A_H\beta$ = 17.4 G, $A_N\beta$ = 3.2 (^{14}N) or 4.0 (^{15}N) G], determined from the signal that remained after subtracting the lines of DMPO-OH from those shown in Figures 6A and 6B, indicated that the radical was a DMPO adduct of a nitrogen species having a tetrahedral β-C atom.[13d] The above $A_N\beta$ values also suggest that the incorporated N may be protonated. For example, the $A_N\beta$ value for the DMPO adduct of $NH_2^•$ is 1.60–1.71 G (2.24 G for ^{15}N), whereas the $A_N\beta$ value for the adduct of $NH_3^{•+}$ is 3.13 G (4.40 G for ^{15}N).[20,32] Alternatively, chlorine atom(s) attached to N could also influence the $A_N\beta$ value. Considering the reactants, the present radical (Figures 6A and 6B) appears to be a DMPO adduct of a chloramine or chloraminium radical of ammonia. The yield of this radical was determined to be 0.2% with respect to $NHCl_2$, an estimate obtained by computer subtraction of DMPO-OH lines from the Figure 6A signal and the use of TEMPOL as an integration standard. The possibility of a DMPO adduct of $NH_3^{•+}$ seems unlikely because the reported hyperfine splitting constants (A_N = 14.0 G, $A_H\beta$ = 18.74 G) of this radical[20] are sufficiently different from those observed above.

Although reaction mixtures of DMPO and $NHCl_2$ did not form 1, the addition of HOCl (2.4 mM, 0.6 μmol) to a mixture of DMPO (72 mM) and $NHCl_2$ (2.4 mM) or $^{15}NHCl_2$ (2.0 mM) furnished a strong EPR signal of 1 (93 μM) or 5 (71 μM), respectively. Three mechanisms may be considered: (a) $NHCl_2$ is converted by HOCl to NCl_3, which then reacts with DMPO to furnish 1; (b) HOCl activates DMPO for reaction of $NHCl_2$ to give 1; and (c) DMPO and $NHCl_2$ form an intermediate(s) that reacts with HOCl to provide 1.

To clarify these possibilities, UV studies were performed with reaction mixtures similar to those used for EPR. Treatment of $NHCl_2$ with HOCl under these conditions did not show absorption peaks corresponding to NCl_3. Moreover, the formation of NCl_3 is unlikely on the basis of kinetic considerations because the reaction of HOCl with $NHCl_2$ to give NCl_3 is 2 × 10^6 times slower than the reaction of HOCl with NH_3 to give NH_2Cl.[21b] Thus, the formation of 1 upon the addition of HOCl above was not dependent on the generation of NCl_3. Only when both DMPO and HOCl were introduced into the reaction mixture was the UV absorption maximum at 294–296 nm (corresponding to $NHCl_2$) reduced. These observations eliminate possibility a but do not distinguish between the HOCl-activation of DMPO (possibility b) and the formation of an intermediate(s) from $NHCl_2$ and DMPO (possibility c).

The detection by EPR of chloramine or chloraminium adducts of DMPO (Figures 6A and 6B) supports possibility c, that is, the involvement of an intermediate(s), and this was further confirmed by the results of an experiment using PbO_2. Brief treatment of a mixture of DMPO and $NHCl_2$ with excess PbO_2 produced a strong EPR signal (not shown) containing the lines of 1 and DMPOX. DMPOX, which decayed rapidly, arose from the oxidation of DMPO by PbO_2.[27c,33] Radical 1 thus arose from oxidation of a DMPO derivative(s) having an *N*-chloro group at the β-carbon. Attempts to isolate these intermediates by TLC, however, were unsuccessful.

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[32] Kirino, Y.; Ohkuma, T.; Kwan, T. *Chem. Pharm. Bull.* **1981**, *29*, 29–34.

[33] Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. *Arch. Biochem. Biophys.* **1980**, *200*, 1–16.

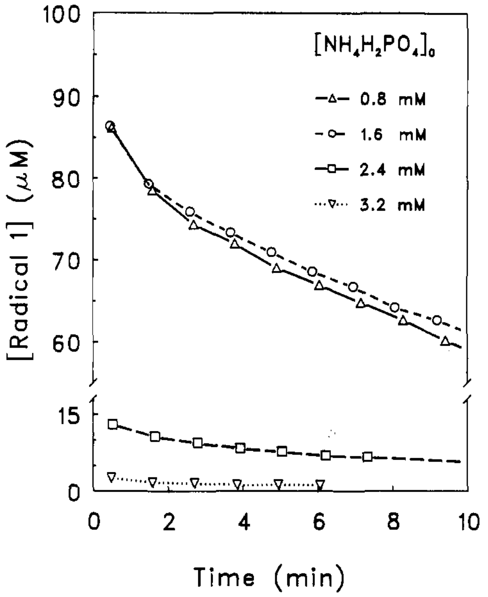

Figure 7. Time course of 1 formed in solutions containing various initial concentrations of $NH_4H_2PO_4$ (0.8–3.2 mM), 4 mM HOCl, 72 mM DMPO, and 40 mM K_2HPO_4–KH_2PO_4 (pH 6.8).

If the rate of formation of the *N*-chloro intermediate is sufficiently slower than the rate of its reaction with HOCl, then only radical **5**, the $[^{15}N]$-isomer of **1**, would be observed when $NHCl_2$ and HOCl are simultaneously introduced into a mixture of $^{15}NHCl_2$ and DMPO. When this experiment was performed, however, a complex EPR spectrum (not shown) comprising the signals of both **1** and **5** was observed. The complex signal was verified by comparing it with (a) a spectrum obtained from a mixture of DMPO, $NHCl_2$, and $^{15}NHCl_2$ with subsequent addition of HOCl, and (b) a spectrum generated by computer summation of the individual signals of **1** and **5**. These results indicate that the rate of formation of the intermediate was very much faster than the rate of its subsequent reaction with HOCl.

Studies at pH 7. EPR and UV studies were performed at pH 7 using varying $[NH_4H_2PO_4]_0$ in reaction mixtures as described above. A strong EPR signal of **1** was observed with low $R_{N/Cl}$ (0.2 and 0.4, see Figure 7) and excess DMPO. At low $R_{N/Cl}$, the EPR signal also contained lines from DMPOX which, however, decayed faster than those of **1**. Interestingly, the concentration of **1** and its rate of decay were almost the same for both the ratios ($R_{N/Cl} = 0.2$ and 0.4, Figure 7). As $R_{N/Cl}$ was increased further, [**1**] decreased considerably (Figure 7). Only a very weak signal of **1** was observed when $R_{N/Cl}$ was 1 or 2 (data not shown), and with $R_{N/Cl} > 2$, the reaction mixture was EPR-silent.

Examination of mixtures of $NH_4H_2PO_4$ and HOCl at pH 7 by UV revealed the following. When $R_{N/Cl}$ was 0.2, the reaction mixtures showed absorptions corresponding to NCl_3 (λ_{max} 220–222 and 336 nm), $NHCl_2$ (λ_{max} 206 and 294), and OCl^- (λ_{max} 292–294 nm).[18,34] As $R_{N/Cl}$ increased from 0.2 to 0.4, OCl^- was converted to $NHCl_2$ while the amount of NCl_3 remained approximately the same. Further increases in $R_{N/Cl}$ resulted in a gradual conversion of NCl_3 and $NHCl_2$ to NH_2Cl (λ_{max} 244 nm).[18a] NCl_3 was almost absent at $R_{N/Cl} = 2$, and only NH_2Cl could be observed at $R_{N/Cl} > 2$. These results indicate that NH_2Cl alone may not produce **1** with excess DMPO. Instead, NCl_3 appears to have formed **1** and to be responsible for the intensity of the EPR signal.

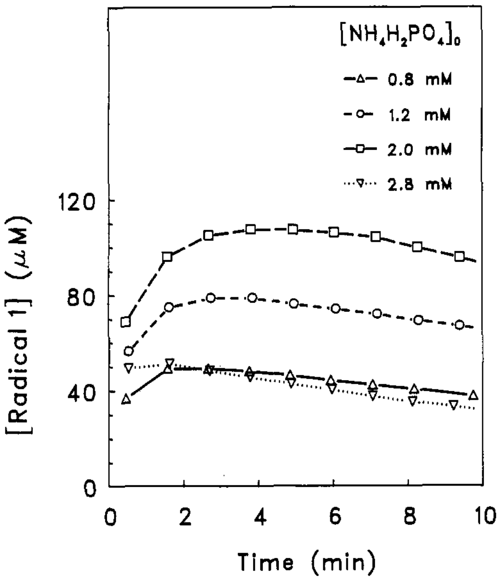

Figure 8. Time course of 1 formed in solutions containing various initial concentrations of $NH_4H_2PO_4$ (0.8–2.8 mM), 4 mM HOCl, 72 mM DMPO, and 40 mM $Na_2B_4O_7$ (pH 9.1).

Studies at pH 9. EPR spectra of reaction mixtures containing $NH_4H_2PO_4$ (0.8–40 mM), HOCl (4 mM), and DMPO (72 mM) were recorded at pH 9. The ratio of the initial concentrations of $NH_4H_2PO_4$ and HOCl appeared to be critical for observing the formation of **1**. For example, the EPR signal of **1** was discernible only for $R_{N/Cl} < 1$ while reaction mixtures were EPR silent for $R_{N/Cl} \geq 1$. The change of [**1**] with time for various $[NH_4H_2PO_4]_0$ is given in Figure 8. In the early scans, the signal was accompanied by lines from DMPOX (not shown), which completely disappeared in later scans. As shown in Figure 8, [**1**] was small for both low (0.8 mM) and high (2.8 mM) values of $[NH_4H_2PO_4]_0$.

UV spectra of the corresponding mixtures of $NH_4H_2PO_4$ and HOCl at pH 9 were also obtained. As $[NH_4H_2PO_4]_0$ increased, the absorption at 292–294 nm (OCl^-) decreased, and the absorption at 244 nm (NH_2Cl) increased (Figure 9). The UV spectra also showed that the higher chloramines ($NHCl_2$ and NCl_3) were virtually absent under these conditions. The isosbestic point at 268–270 nm (Figure 9) indicates that the total active Cl was distributed only between NH_2Cl and OCl^-. The concentration of these two species were calculated from the absorption at 244 and 294 nm and reported values of their extinction coefficients.[35]

The calculated values of $[OCl^-]$ and $[NH_2Cl]$ (from Figure 9), and the maximum [**1**] corresponding to various $[NH_4H_2PO_4]_0$ (from Figure 8) are shown in Figure 10. The signal of **1** was detected only when both NH_2Cl and

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[34] (a) Anbar, M.; Dostrovsky, I. *J. Chem. Soc.*, **1954**, 1105–1108. (b) Morris, J. C. *J. Phys. Chem.* **1966**, *70*, 3798–3805.

[35] The concentrations of NH_2Cl and OCl^- were calculated using the following equations:

$$A_{294} = b(\epsilon_{294}^{NH_2Cl}[NH_2Cl] + \epsilon_{294}^{OCl^-}[OCl^-]) \quad \text{and} \quad A_{244} = b(\epsilon_{244}^{NH_2Cl}[NH_2Cl] + \epsilon_{244}^{OCl^-}[OCl^-])$$

where A is the absorption, b the path length, and ϵ the extinction coefficient. Substituting the appropriate ϵ values given by Rizk-Ouaini et al,[26a] the above equations can be reduced to:

$$[NH_2Cl] = 2.196A_{244} - 0.106A_{294} \quad \text{and} \quad [OCl^-] = 2.736A_{294} - 0.078A_{244}$$

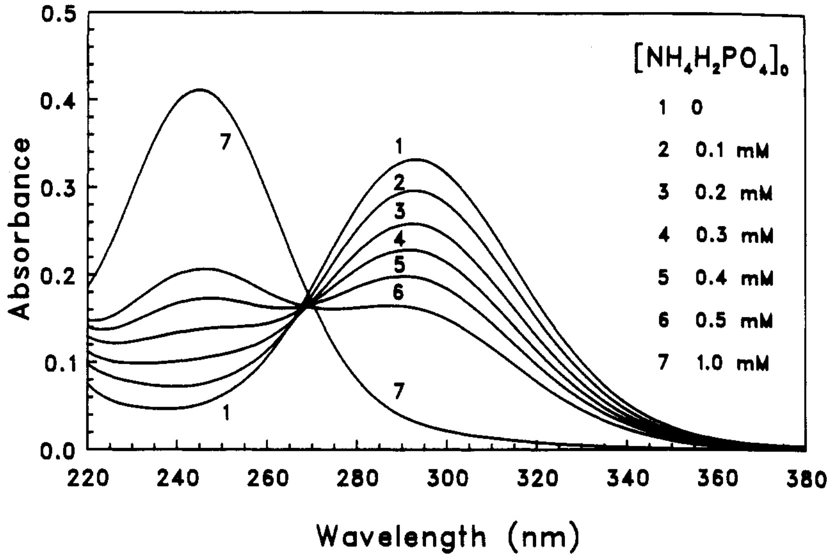

Figure 9. UV spectra of solutions containing various initial concentrations of $NH_4H_2PO_4$ (0–1 mM) and 1 mM HOCl in 40 mM $Na_2B_4O_7$ (pH 9.1). Spectra demonstrate the presence of only OCl^- (λ_{max} 292–294 nm) and NH_2Cl (λ_{max} 244 nm).

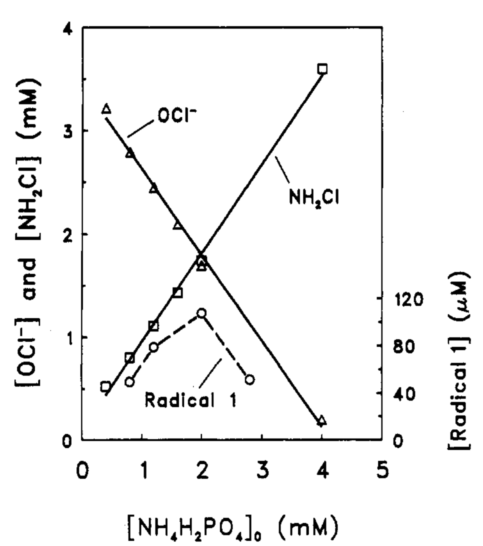

Figure 10. Correlation of the maximum concentration of initial **1** (from Figure 8) with the concentrations of NH_2Cl and OCl^- corresponding to various initial concentrations of $NH_4H_2PO_4$ at pH 9.1.[35] For further details, see legend to Figure 8.

OCl^- were present in the reaction mixture, that is, when $R_{N/Cl} < 1$. The further addition of HOCl (2.4 mM, 0.6 μmol) to an EPR-silent reaction mixture of $NH_4H_2PO_4$ (8 mM), HOCl (4 mM), and DMPO (72 mM) at pH 9 produced a strong signal of **1** (34 μM, $t_{1/2}$ = 7 min) free of DMPOX. Figure 10 also shows that [**1**] correlates with the lower value of [NH_2Cl] and [OCl^-] in the mixture. The results suggest that under alkaline conditions both NH_2Cl and OCl^- are stoichiometrically involved in the formation of **1**. HOCl rather than OCl^- appears to function as the reactive entity,[27a,36] and the HOCl–OCl^- equilibrium may account for the very slow formation of **1** at pH 9 (Figure 8) compared to that at pH 3. The HOCl:OCl^- ratio is pH-dependent: 3:97 at pH 9, 75:25 at pH 7, and 100:0 at pH 3.[37] Alternatively, a pH-dependent conformational equilibrium (or tautomerism) of intermediates or acid catalysis of intermediate reactions may be responsible for the rapid formation of **1** at pH 3.

It is likely that NH_2Cl and HOCl participate in a stepwise manner in the formation of **1** from DMPO because addition of HOCl to an EPR-silent mixture of DMPO and NH_2Cl gave a strong signal of **1**. Involvement of HOCl must be preceded by that of NH_2Cl because HOCl is known to give DMPOX with DMPO[22] and, as shown above, DMPOX did not yield **1** with NH_2Cl. Alternatively, HOCl may activate DMPO for an initial reaction with NH_2Cl. To distinguish between these possibilities, the following experiment was performed. A solution of NH_4Cl and NaOCl containing OCl^- in excess of NH_2Cl was added to an EPR-silent mixture of $^{15}NH_4Cl$, NaOCl, and DMPO at pH 9. If the stepwise process were operative and the rate of formation of the intermediate sufficiently slower than the rate of its subsequent reaction with HOCl, then only radical **5**, the [^{15}N]-isomer of **1**, would be observed. The EPR spectrum of this mixture indeed showed only the presence of **5**. Moreover, when NH_4Cl and $^{15}NH_4Cl$ were interchanged in the above sequence, only the signal of **1** was observed. These results unequivocally demonstrate that NH_2Cl reacts with DMPO to furnish an intermediate diamagnetic species that is converted to **1** by HOCl.

Further, brief treatment of an EPR-silent mixture of DMPO and NH_2Cl with PbO_2 also gave the EPR signal of **1**, indicating that the above intermediate contained an *N*-chloro group at the β-position. Based on these considerations, a good candidate for the intermediate would be the 2-*N*-chloroamino compound **8**. It should be noted that the concentration of **1** obtained from PbO_2 oxidation was approximately 4-fold less than that observed from HOCl treatment of an identical mixture. Thus, the oxidation of **8** to **1** by PbO_2 may not be as effective as that by HOCl, and/or there was another intermediate that reacted with HOCl, but not with PbO_2, to afford **1**. The 2-amino compound **6** would be a possible structure for such an intermediate because this structure is consistent with a reaction product of NH_2Cl and DMPO, and **6** is known to react with HOCl to give **1**.[12] Attempts were

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(36) (a) Weil, I; Morris, J. C. *J. Am. Chem. Soc.* **1949**, *71*, 1664–1671. (b) Margerum, D. W.; Gray, E. T., Jr.; Huffman, R. P. In *Organometals and Organometalloids: Occurrence and Fate in the Environment*; Brinckman, F. E., Bellama, J. M., Eds.; American Chemical Society: Washington, DC, 1978; pp 278–291.

(37) *The Handbook of Chlorination*; White, G. C., Ed.; Van Nostrand Reinhold: New York, 1972; pp 182–227.

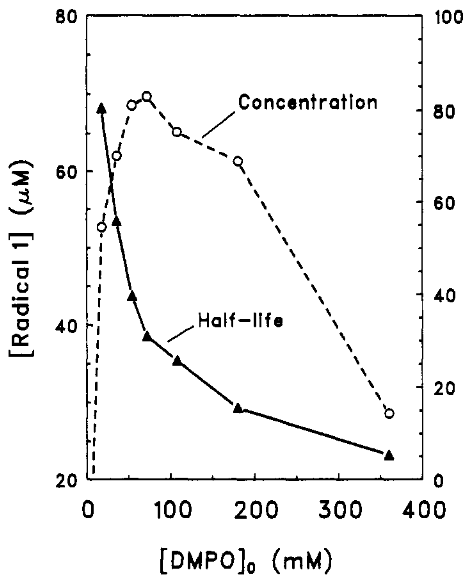

Figure 11. The initial concentration and half-life of 1 in solutions containing 2 mM $NH_4H_2PO_4$, 4 mM HOCl, 18–359 mM DMPO, and 40 mM $Na_2B_4O_7$ (pH 9.1). The concentrations of 1 were obtained by extrapolating the decay curves to zero time.

made to verify the possible intermediacy of 6 by TLC. However, reaction mixtures of NH_2Cl and DMPO did not display a TLC spot corresponding to an authentic sample of 6. Either 6 was not an intermediate or it was present in quantities too small to be detected by TLC.

Effect of DMPO Concentration. To further characterize the formation of 1 at pH 9, EPR spectra were recorded after varying [DMPO]$_0$ (18–359 mM) in reaction mixtures containing $NH_4H_2PO_4$ (2 mM) and HOCl (4 mM). The concentration of 1 was plotted vs time for each [DMPO]$_0$ (data not shown) and was found to increase to a maximum value and then decrease. The pattern was similar to that shown in Figure 8 where $R_{N/Cl} = 0.5$ (i.e., $[NH_4H_2PO_4]_0 = 2$ mM). The maximum [1] obtained from these plots is shown in Figure 11. The maximum [1] first increased and then decreased with [DMPO]$_0$ indicating that DMPO is associated with both the formation and the decay of 1. After reaching its maximum concentration in less than 4 min (see Figure 8), 1 showed exponential decay. The half-life of 1, determined from the decay curves, decreased with increasing [DMPO]$_0$ (Figure 11) as in experiments conducted under acidic conditions (Figure 5). However, 1 survived considerably longer at pH 9, indicating that the reaction of 1 and DMPO is slower under alkaline conditions.

DMPOX was also observed at pH 9 in the early scans, but as usual decayed more rapidly ($t_{1/2} < 2$ min) than 1. Both NH_2Cl and HOCl (or OCl^-) compete for DMPO, and whereas the reaction of NH_2Cl with DMPO would lead to 1, the reaction of HOCl with DMPO would furnish DMPOX. The ratio of [1]:[DMPOX], which represents the ratio of their respective rates of formation, was found to be in the range 2.0 to 2.5 for various [DMPO]$_0$. As mentioned earlier, the addition of HOCl to an EPR-silent mixture of NH_2Cl and excess DMPO gave only 1. The absence of DMPOX in this case suggests that the rate of DMPOX formation was much slower than the reaction of the intermediate 8 with HOCl to give 1. On the basis of these arguments, one may propose that, under alkaline conditions, the rate-controlling step in the formation of 1 is the formation of 8 from DMPO and NH_2Cl. This is also consistent with results from the above experiments with NH_2Cl and $^{15}NH_2Cl$.

UV Irradiation. The ammonia chloramines may participate in reactions via ionic or radical intermediacy.[38] Radical reactions of the chloramines would occur during photochemical activation or in the presence of metal ions such as Fe^{2+}.[38,39] Either of these conditions would enhance the formation of 1 if reaction of the chloramines with DMPO proceeded via free radicals derived from the chloramines. To test this possibility, we examined the reaction at pH 3 and 9 by EPR in the dark and with UV irradiation. Identical EPR spectra were obtained in the absence and presence of ambient fluorescent light, indicating that light has no effect on the formation of 1. Moreover, UV irradiation did not enhance the signal intensity. On the contrary, the signal decayed more rapidly when the reaction mixture was exposed to UV light. Thus, it is unlikely that radical intermediates derived from chloramines were involved in the formation of 1.

Studies in an Aprotic Solvent. Reaction of DMPO and NCl_3 in CCl_4 was examined by UV spectroscopy, TLC, and EPR. The UV absorption band at 344 nm of NCl_3 (in CCl_4)[19,40] immediately disappeared when a solution of DMPO in CCl_4 was added, indicating a rapid reaction of DMPO and NCl_3. Chromatography of the reaction mixture on a silica gel plate using 10% EtOAc in $CHCl_3$ followed by spraying with a solution of KI and starch gave a purple spot at R_f 0.48.[12] This result indicated that 1 was a reaction product. Formation of 1 was further confirmed by EPR analysis. A solution of DMPO and NCl_3 in CCl_4 displayed an EPR spectrum that was identical to that of 1 in $CHCl_3$[12] or CCl_4: $A_N = 7.79$ G, $A_{N\beta} = 3.84$ G, $A_{35Cl} = 0.8$ G, and $A_{37Cl} = 0.7$ G.[41] An authentic sample for 1 in this EPR study was obtained by extracting an aqueous reaction mixture of the 2-amino compound 6 and NaOCl with $CHCl_3$[12] or CCl_4. The results show that DMPO can react with NCl_3 to give 1 even in the aprotic solvent CCl_4.

Reactions with M$_4$PO and PBN. M_4PO (9), a DMPO derivative with two Me groups at the γ-position, and PBN (10), an acyclic nitrone, were treated with hypochlorite-treated NH_4^+ under alkaline (pH 9) and acidic (pH 2.5–3) conditions. However, the analogous *N*-chloroimine radicals 11 and 12 were not observed.

At pH 9, the reaction of M_4PO and HOCl-treated $NH_4H_2PO_4$ ($R_{N/Cl} = 0.5$) produced a relatively stable radical ($t_{1/2} = 15$ min) with a three-line EPR signal and hyperfine splitting constant (A_N) of 6.90 G (Figure 12A). The A_N value of this radical is significantly less than that of known M_4PO-derived radicals (13–16 G) in which the β-carbon is tetrahedral.[13d] The A_N is related approximately linearly to the spin density on the nitroxyl N.[42] In the present case, the low A_N value (6.90 G) indicates that the spin density on the nitroxyl N is diminished, presumably by delocalization over a trigonal functionality at the β-carbon atom.[12] The multiplicity of the signal suggests that the nitroxyl N was not coupled to any atom in the

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(38) Kovacic, P.; Lowery, M. K.; Field, K. W. *Chem. Rev.* **1970**, *70*, 639–665.

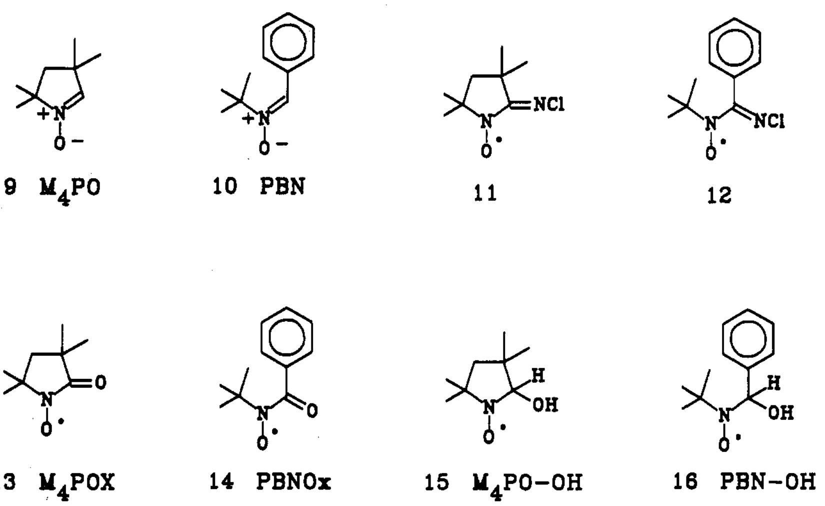
(39) (a) Ogata, Y.; Izawa, Y.; Tomioka, H. *Tetrahedron*, **1967**, *23*, 1509–1514. (b) Neale, R. S. *Synthesis* **1971**, 1–15. (c) Sosnovsky, G.; Rawlinson, D. J. *Adv. Free Radical Chem.* **1972**, *4*, 203–284.

(40) Czech, F. W.; Fuchs, R. J.; Antczak, M. F. *Anal. Chem.* **1961**, *33*, 705–707.

(41) In the EPR spectrum of 1, the fine splitting due to Cl was not so well resolved in CCl_4 and $CHCl_3$ as in H_2O.

(42) (a) Hayat, H.; Silver, B. L. *J. Phys. Chem.* **1973**, *77*, 72–78. (b) Cohen, A. H.; Hoffman, B. M. *J. Phys. Chem.* **1974**, *78*, 1313–1321.

Chart 2



molecule and thus a β-H was absent. The EPR data are consistent with 3,3,5,5-tetramethyl-2-pyrrolidone-1-oxyl (M_4POX, **13**), an oxidation product of M_4PO. The analogous oxidation products of DMPO (DMPOX, **3**) and PBN (benzoyl *tert*-butyl nitroxide, PBNOx, **14**) have A_N values of 7–8 G.[13d]

At pH 3, the EPR signal (Figure 12B) derived from the reaction of M_4PO and HOCl-treated NH_4Cl ($R_{N/Cl} = 2.9$) was assigned to 3,3,5,5-tetramethyl-2-hydroxypyrrolidine-1-oxyl (M_4PO-OH, **15**) by comparison of its hyperfine splitting constants (A_N = 15.33 G, A_H = 16.71 G) with reported values.[43] This radical decayed rapidly ($t_{1/2}$ = 50 s) causing a tapering of the signal with time (Figure 12B). When $R_{N/Cl}$ was changed from 2.9 to 14.3 at constant $[NH_4H_2PO_4]_0$ (40 mM), the initial intensity of the EPR signal, deduced by extrapolating the decay curves to zero time, decreased more than 3-fold (data not shown) while stability of M_4PO-OH was considerably enhanced ($t_{1/2}$ = 2 min). These results suggest that the decay of M_4PO-OH may involve a bimolecular reaction such as a dismutation or dimerization of the radical.[13f,44]

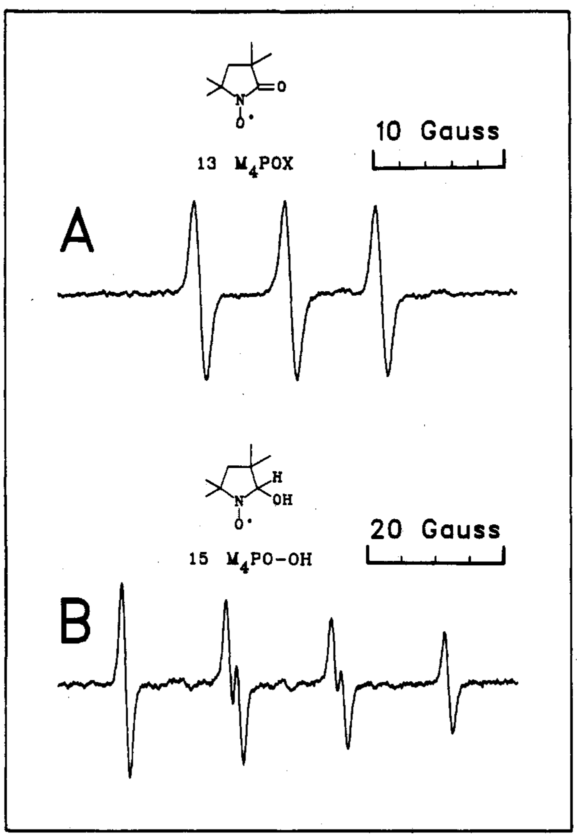


Figure 12. (A) EPR spectrum of M_4POX (**13**) measured in a solution of 2 mM $NH_4H_2PO_4$, 4 mM HOCl, 80 mM M_4PO, and 40 mM $Na_2B_4O_7$ (pH 9). Recording of the spectrum was begun 0.6 min after mixing the reagents: A_N = 6.90 G. (B) EPR spectrum of M_4PO-OH (**15**) measured in a solution of 40 mM NH_4Cl, 14 mM HOCl, 200 mM M_4PO, and 40 mM KH_2PO_4–H_3PO_4 (pH 3). Recording of the spectrum began 0.7 min after mixing the reagents: A_N = 15.33 G, A_H = 16.71 G.

The lack of formation of an *N*-chloroimine radical from M_4PO may be rationalized in terms of steric crowding by the two Me groups at the γ-position which would prevent the approach of a chloramine species (NCl_3, $NHCl_2$, or NH_2Cl) to the β-position. In comparison to DMPO, the lower reactivity of M_4PO toward radicals such as superoxide ($O_2^{\bullet-}$),[45] thiyl ($RS^\bullet$),[46] alkoxyl ($RO^\bullet$),[47] and carbon centered radicals ($R\cdot$)[48] has been similarly ascribed to steric factors. It should be noted that replacement of the two methyl groups at the γ-position by bulkier ethyl groups completely prevented the reaction of $O_2^{\bullet-}$ with the spin trap.[45]

The formation of M_4PO-OH (**15**) from aqueous M_4PO (**9**) and chloramines may follow a mechanism similar to that proposed for the hypochlorite-induced generation of DMPO-OH (**7**) from DMPO.[22b,23] However, in the present case, the chloramine appears to be involved in activating the β-position of M_4PO for nucleophilic addition of H_2O under acidic conditions (Scheme 1). The absence of M_4POX at pH 3 suggests either that M_4POX was not stable at acidic pH or that the chloramine (NCl_3 or $NHCl_2$) was not strong enough to oxidize M_4PO-OH. The latter possibility appears likely because the reaction of M_4PO with HOCl, a stronger oxidant than chloramines,[27a] gave

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[43] (a) Janzen, E. G.; Shetty, R. V.; Kunanec, S. M. *Can. J. Chem.* **1981**, *59*, 756–758. (b) Buettner, G. R.; Britigan, B. E. *Free Radical Biol. Med.* **1990**, *8*, 57–60.

[44] (a) Bullock, A. T.; Gavin, D. L.; Ingram, M. D. *J. Chem. Soc., Chem. Commun.* **1980**, 327–328. (b) Castelhano, A. L.; Griller, D.; Ingold, K. U. *Can. J. Chem.* **1982**, *60*, 1501–1503.

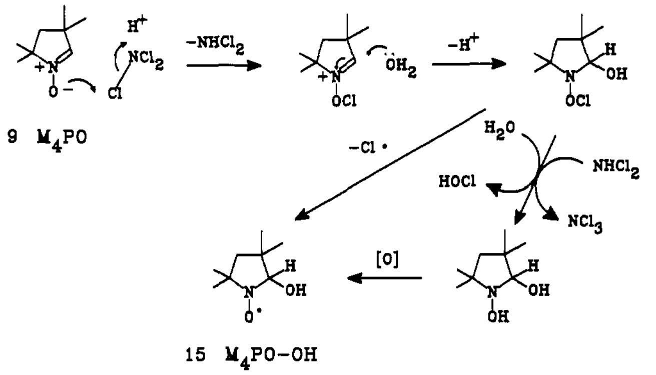
[45] Rosen, G. M.; Turner, M. J., III. *J. Med. Chem.* **1988**, *31*, 428–432.

[46] Davies, M. J.; Forni, L. G.; Shuter, S. L. *Chem.-Biol. Interact.* **1987**, *61*, 177–188.

[47] Haire, D. L.; Janzen, E. G. *Can. J. Chem.* **1982**, *60*, 1514–1522.

[48] Hill, H. A. O.; Thornalley, P. J. *Biochim. Biophys. Acta* **1983**, *762*, 44–51.

Scheme 1. A Possible Mechanism for the Formation of M$_4$PO-OH (15) via Chloramine-Induced Activation of M$_4$PO (9)



M$_4$POX at pH 3, 7, and 9. Moreover, M$_4$POX generated with HOCl was found to be more stable at pH 3 ($t_{1/2}$ = 9 min) than at pH 9 ($t_{1/2}$ = 3 min). As mentioned above, M$_4$POX formed from M$_4$PO and HOCl-treated NH$_4$H$_2$PO$_4$ ($R_{N/Cl}$ = 0.5) at pH 9 had a half-life of 15 min; the increased stability of M$_4$POX in that case may be ascribed to the presence of NH$_2$Cl and/or NH$_4^+$ in the mixture. When $R_{N/Cl}$ = 0.5 at pH 9, both NH$_2$Cl and OCl$^-$ are present in a ratio of 1:1 (see Figure 10), and in accordance with earlier findings on the conversion of DMPO to DMPOX by OCl$^-$/HOCl,[22] only OCl$^-$/HOCl would be expected to react with M$_4$PO to give M$_4$POX.

The reaction mixture of PBN and HOCl-treated NH$_4$H$_2$PO$_4$ ($R_{N/Cl}$ = 3.8) at pH 2.6 gave an EPR signal (Figure 13 inset) that was assigned to PBN-OH (16) on the basis of hyperfine splitting constants (A_N = 15.44 G, A_H = 2.56 G).[13d] However, the reaction of PBN and HOCl under identical conditions furnished a much weaker signal comprised of PBN-OH and PBNOx (14). PBNOx, which was identified from its hyperfine splitting constant (A_N, 8.54 G),[13d] was the minor component and decayed very rapidly (within 2–3 min). At pH 9, PBN and hypochlorite-treated NH$_4$H$_2$PO$_4$ ($R_{N/Cl}$ = 0.4) gave a very weak EPR signal assignable to PBN-OH.

Comparison of the concentration and time-course of PBN-OH formed in the reaction of PBN with chloramines or HOCl at pH 2.6 (Figure 13) provides further evidence for chloramine-induced activation of the nitrone for nucleophilic reaction at the β-position (cf. Scheme 1). A very intense signal of PBN-OH was observed when chloramines (NCl$_3$ and NHCl$_2$) were present in the reaction mixture (Figure 13). However, with PBN and HOCl, the signal intensity of PBN-OH was weak and its rate of formation slow (Figure 13). NCl$_3$ is more hydrophobic than HOCl[49] and would be expected to associate with PBN to a greater extent than HOCl, thus providing enhanced reactivity at the β-position.

Mechanism. Ammonia chloramines formed from NH$_4^+$ and HOCl react with DMPO to provide the *N*-chloro group at the β-position of the chloroimine radical 1. As shown by the above UV-irradiation studies, it is unlikely that this reaction involves radical intermediates of the chloramines. A nucleophilic addition of the N atom of the chloramine to the β-carbon of DMPO seems a likely course of reaction; nucleophilic attack of chloramines has also been considered in the amination of toluene and pyridine.[50] The attachment of the Cl atom of the chloramine to the nitroxide oxygen of DMPO would activate the β-carbon for nucleophilic addition. Similar activation by HOCl has been considered in rationalizing nucleophilic addition of H$_2$O to the β-carbon of DMPO.[22b,23] In the case of chloramine-induced activation of DMPO, the N atom of the chloramine becomes attached to the β-carbon. The initial interaction of the chloramine with DMPO is thus analogous to a 1,3-dipolar addition accompanied by fission of the N-Cl bond. In nitroxides such as M$_4$PO and PBN, where the β-carbon is relatively more crowded, either the approach of a bulky N–Cl entity may not be possible or a requisite restricted conformation for a 1,3-dipolar type of addition may not be achieved, and consequently the analogous chloroimine radicals would not be formed. These nitroxides (M$_4$PO and PBN) afforded radicals corresponding to the nucleophilic addition of H$_2$O (Scheme 1).

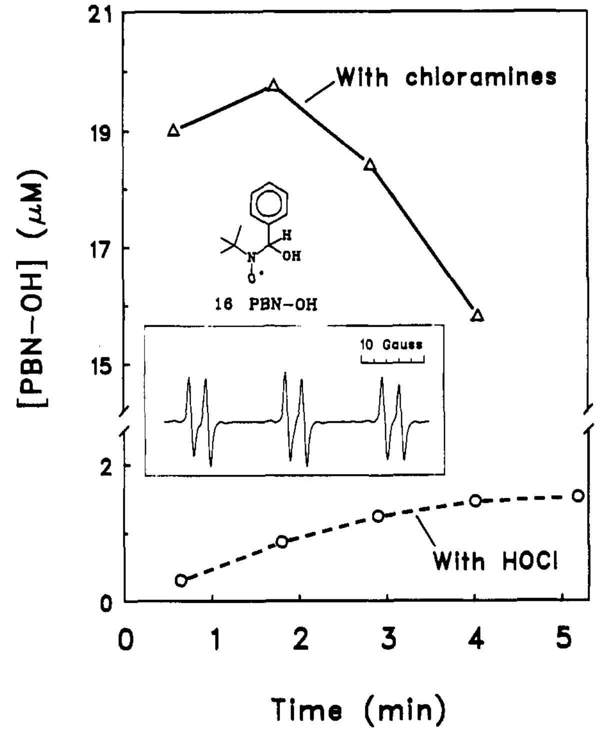


Figure 13. Time course of PBN-OH (16) formed from the reaction of PBN and chloramines and HOCl. Triangles (△) refer to the reaction described below in the inset. Circles (○) refer to the reaction of 4.2 mM HOCl and 30 mM PBN in 40 mM KH$_2$PO$_4$–H$_3$PO$_4$ (pH 2.5). Inset: EPR spectrum of PBN-OH (16) measured in a solution of 16 mM NH$_4$H$_2$PO$_4$, 4.2 mM HOCl, 30 mM PBN, and 40 mM KH$_2$PO$_4$–H$_3$PO$_4$ (pH 2.6). Recording of the spectrum began 0.6 min after mixing the reagents: A_N = 15.44 G, A_H = 2.56 G.

The transfer of Cl from chloramine to the nitroxide oxygen of DMPO does not involve a protonated chloramine.[51] If a protonated chloramine transferred Cl to DMPO, the nucleophile that would be attached to the β-carbon would be H$_2$O or an appropriate N-entity such as NH$_3$ or a chloramine. Being more basic, NH$_3$ would be preferred to a chloramine as a nucleophile. Assuming the nucleophilic attack of H$_2$O, DMPO-OH (7) and/or DMPOX (3)

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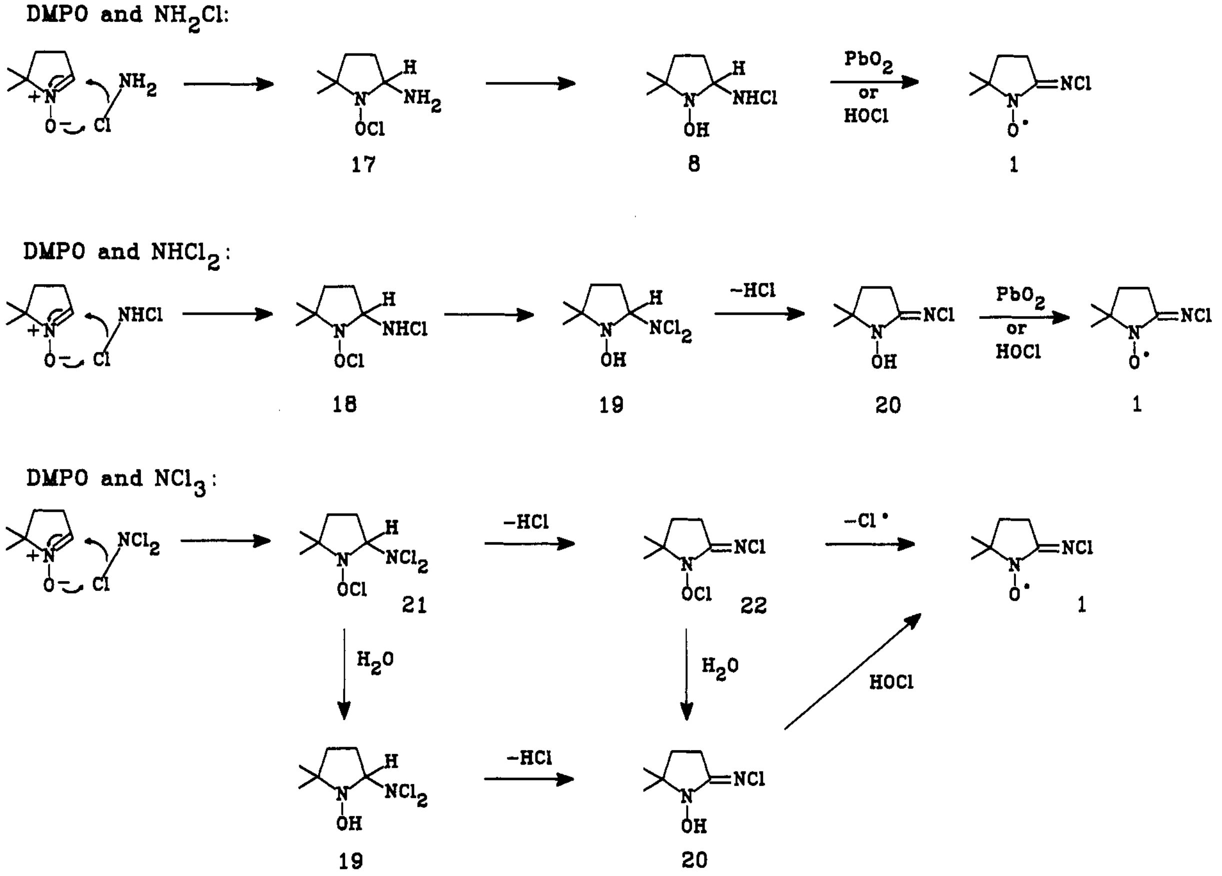
(49) Brunetto, M; Colin, C.; Rosset, R. *Analusis* **1987**, *15*, 393–398.

(50) (a) Kovacic, P.; Goralski, C. T.; Hiller, J. J., Jr.; Leviski, J. A.; Lange, R. M. *J. Am. Chem. Soc.* **1965**, *87*, 1262–1266. (b) Brooks, M. E.; Rudner, B. *J. Am. Chem. Soc.* **1956**, *78*, 2339–2440.

(51) Isaac, R. A.; Morris, J. C. *Environ. Sci. Technol.* **1985**, *19*, 810–814.

Scheme 2. Proposed Mechanisms for the Formation of N-Chloroimino Radical 1 from DMPO (2) and Ammonia Chloramines



rather than 1 would then be expected as the major or perhaps sole radical product(s). However, the studies with ^{15}N-labeled chloramines indicated that N from the activating chloramine rather than N from NH_3 or another chloramine was attached to the β-position. Furthermore, the reaction of DMPO with NCl_3 to give 1 in an aprotic solvent such as CCl_4 suggests that protonation of chloramine or DMPO is not required for the reaction. It should also be noted that DMPO is unlikely to be activated by protonation because its UV spectrum (λ_{max} 228 nm) was found to be unaffected by changes in pH over the range 2 to 11.5.

The addition of NH_2Cl to DMPO would lead to the *N*-chloroxy amino intermediate **17**, which would then undergo an intramolecular transfer of Cl to the amino group to give the *N*-chloro compound **8** (Scheme 2). Evidence for the existence of compound **8** was inferred from PbO_2 treatment of a mixture of DMPO and NH_2Cl as discussed above. As indicated in Scheme 2, the conversion of **8** to **1** involves a 3-electron oxidation, which was accomplished by PbO_2 or HOCl. It is also possible that **8** would undergo dehydrochlorination to give the 2-amino compound **6**. While confirmatory evidence for the intermediacy of **6** is lacking, a comparison of the results from HOCl- and PbO_2-oxidation of mixtures of NH_2Cl and DMPO points to the possibility that *N*-chlorination of **6** by HOCl may also contribute to the yield of **1**.

In an analogous manner, the *N*-chloroxy *N*-chloramino compound **18** formed from $NHCl_2$ and DMPO would sequentially provide **19** and **20**. Oxidation of the latter compound by either PbO_2 or HOCl would give **1** (Scheme 2).

Finally, NCl_3 reacts with DMPO to give **1**. Unlike the case of $NHCl_2$ and NH_2Cl, this reaction does not require additional oxidants because NCl_3, having three active Cl atoms, contains the requisite oxidation equivalents. The *N*-chloroxy intermediate **21**, formed from the addition of NCl_3 to DMPO, would give **1** via the intermediacy of **22** (Scheme 2). Alternatively, **21** might hydrolyze to form HOCl, and **19** would then give **1** via oxidation of **20** as described above.

The final step in the mechanism proposed for the formation of **1** from DMPO and ammonia chloramines involves either the oxidation of *N*-hydroxy compounds **8** and **20** or homolysis of the *N*-chloroxy intermediate **22** (Scheme 2). Generation of *N*-oxyl radicals by oxidation of *N*-hydroxy compounds is known. For example, oxidation of DMPOXH (**4**) by a variety of oxidants[16b,25] including HOCl and ammonia chloramines (see above) yields DMPOX (**3**). Homolysis of an *N*-chloroxy intermediate analogous to that of **22** has also been postulated in the mechanism for the reaction of DMPO and HOCl.[22b,23]

Acknowledgment. The authors thank B. Ivker for English translations.[26,49] This work was supported by grants to C.B. from the Defense Nuclear Agency and National Science Foundation (DMB 90-04557).