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Modulation of oxidative damage by nitroxide free radicals

ILEANA DRAGUTAN[1] & ROLF J. MEHLHORN[2]

[1]*Institute of Organic Chemistry, Romanian Academy, P.O.B. 35-108, 060023 Bucharest, Romania, and* [2]*Lawrence Berkeley National Laboratory, Berkeley, CA, USA*

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Abstract
Piperidine nitroxides like 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) are persistent free radicals in non-acidic aqueous solutions and organic solvents that may have value as therapeutic agents in medicine. In biological environments, they undergo mostly reduction to stable hydroxylamines but can also undergo oxidation to reactive oxoammonium compounds. Reactions of the oxoammonium derivatives could have adverse consequences including chemical modification of vital macromolecules and deleterious effects on cell signaling. An examination of their reactivity in aqueous solution has shown that oxoammonium compounds can oxidize almost any organic as well as many inorganic molecules found in biological systems. Many of these reactions appear to be one-electron transfers that reduce the oxoammonium to the corresponding nitroxide species, in contrast to a prevalence of two-electron reductions of oxoammonium in organic solvents. Amino acids, alcohols, aldehydes, phospholipids, hydrogen peroxide, other nitroxides, hydroxylamines, phenols and certain transition metal ions and their complexes are among reductants of oxoammonium, causing conversion of this species to the paramagnetic nitroxide. On the other hand, thiols and oxoammonium yield products that cannot be detected by ESR even under conditions that would oxidize hydroxylamines to nitroxides. These products may include hindered secondary amines, sulfoxamides and sulfonamides. Thiol oxidation products other than disulfides cannot be restored to thiols by common enzymatic reduction pathways. Such products may also play a role in cell signaling events related to oxidative stress. Adverse consequences of the reactions of oxoammonium compounds may partially offset the putative beneficial effects of nitroxides in some therapeutic settings.

Keywords: *Oxoammonium, nitroxide, thiol oxidation, oxidative damage*

Abbreviations: *MPS, 3-mercapto-1-propanesulfonic acid; CoM, 2-mercapto-1-ethanesulfonic acid, also known as co-enzyme M; GSH, glutathione; NAC,* N*-acetyl cysteine; DTNB, 3-carboxy-4-nitrophenyl disulfide (Ellman's reagent); MNB, sodium 5-mercapto-2-nitrobenzoate*

Introduction

Nitroxides are organic free radicals that do not dimerize and are inert towards most molecules found in biological environments. They have generated great interest among biologists because of their utility as spin labels and spin probes in biophysical studies and because of their potential as therapeutic agents in medicine. Despite their inherent stability in aqueous solution, nitroxides are susceptible to gradual chemical transformations in biological systems, which include reduction to hydroxylamines, oxidation to oxoammonium species and adduct formation with certain other radicals. Of these, reduction is a benign process and is by far the most common fate of nitroxides in animals [1,2]. A major biological reducing agent is ascorbic acid, which readily reduces six-membered ring piperidine nitroxides, exemplified by the widely studied compound 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPOL). Reduction by ascorbate is particularly rapid for cationic piperidine nitroxides,

Correspondence: R. J. Mehlhorn, Lawrence Berkeley National Laboratory, Berkeley, CA, USA. E-mail: rjmehlhorn@lbl.gov

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e.g. 4-(tetraalkylammonium)-TEMPO at physiological pH, which have been used to study ascorbate metabolism in the human erythrocyte [3]. Other potent reductants of TEMPO-type nitroxides include subcellular organelles like the mitochondrion [4]. Pyrroline- and pyrrolidine-type nitroxides, in which the NO˙ moiety is incorporated within a five-membered ring, are considerably more resistant to reduction by ascorbate [5]. Reactions of nitroxides with strong oxidants or oxidizing radicals can be beneficial but may also be hazardous. Beneficial effects could result if nitroxides acted as free-radical scavengers in reactions whose products would be less damaging than the primary radicals. Adverse reactions are also possible. An example of a potentially hazardous reaction is the oxidation of nitroxides by superoxide radicals [6] producing oxoammonium species that may yield reaction products, e.g. oxoammonium adducts, not expected for superoxide. Another example of a damaging process could be chemical modification of macromolecular free radicals, which may arise if nitroxides form stable adducts with these products instead of being repaired by one-electron transfer.

There has long been interest in the possibility of using nitroxides in medical applications. Potential applications include tumor therapy [7], magnetic resonance imaging [8] and antioxidant-related benefits [9]. Numerous studies document beneficial effects of nitroxide administration to cells or animals (cf Refs. [10,11]). However, pro-oxidant activity of nitroxides has also been reported (cf Ref. [12]). Considerable attention has been devoted to the idea that nitroxide are superoxide dismutase (SOD) mimics (cf Refs. [6,12]), following an early suggestion that SOD activity was responsible for their prevention of oxidative damage in mammalian cells [14]. However, even at the highest concentrations of nitroxides tolerated by animals [15], considerations of rate constants suggest that SOD enzymes are far more effective superoxide scavengers than would be the nitroxides. However, although the rate constant for the reaction of superoxide with nitroxides is significantly lower than that for the reaction of superoxide with superoxide dismutase, there can be no doubt that some conversion of nitroxides to oxoammonium must occur *in vivo*. Other antioxidant-related nitroxide activities may be involved in observed benefits, including the possibility that nitroxides minimize the destructive impact of heme-activated peroxides [16,17].

It seemed plausible to us that the therapeutic potential of nitroxides could be enhanced by understanding and subsequently implementing strategies to minimize adverse effects of nitroxides. This possibility prompted our interest in the reactions of oxoammonium compounds, expected to arise by one-electron oxidation of nitroxides in biologic environments by superoxide or activated heme proteins. Oxoammonium compounds are strong oxidants whose unique chemical properties have been exploited in synthetic organic chemistry [18–20]. We here report on the reactivity of oxoammonium compounds towards common biological targets in aqueous solution.

Methods and procedures

Reagents

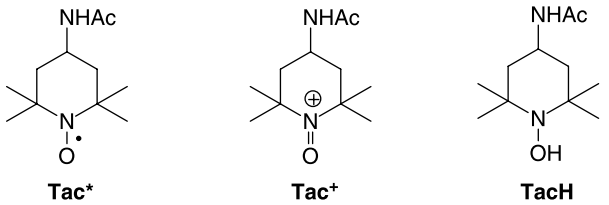
All but bleach were the highest grade available from major commercial vendors, including Sigma and Avanti Lipids. Sodium hypochlorite concentration in fresh household bleach was determined spectrophotometrically in 0.1 M NaOH at 292 nm using an extinction coefficient of 350 $M^{-1}cm^{-1}$ [21].

Probes

The majority of the studies reported here were conducted with 4-acetylamino-2,2,6,6-tetramethylpiperidine-1-oxoammonium tetrafluoroborate, corresponding to 4-acetylamino-2,2,6,6-tetramethylpiperidinyl-1-oxyl [22], generously provided for this study by J. Bobbitt. We refer to this nitroxide as Tac˙, its oxoammonium derivative as Tac^+, and its one-electron reduction product as TacH (see Scheme 1). Stock solutions of the Tac^+ compound (0.1 M) were prepared by dissolving the solid material in either sodium phosphate (0.2 M, pH 2.0) or sodium acetate (0.2 M, pH 4.75) and stored at 4°C. A few studies were conducted with oxoammonium compounds derived from other nitroxides, whose structures differed from that of Tac^+ by having the following substituents at position 4: —H (TEMPO), —OCH_3 (MeTOL), —OH (Tempol), = O (Tempone).

Preparation of MNB

Solid 3-mercaptopropyl-functionalized silica gel (Sigma-Aldrich) was suspended twice in ten volumes of double distilled water and the polymer was pelleted by centrifugation. Treatment of the final supernatant with DTNB (Ellman's reagent) showed that there was no significant release of any disulfide reducing agent from the gel under the conditions subsequently used to prepare MNB. A stock solution of MNB was



Scheme 1. Structure of 4-acetylamino-2,2,6,6-tetramethylpiperidine species.

prepared by intermittent shaking of 1 ml of 10 mM DTNB with 0.1 g of the gel pellet in 0.2 M Chelex-treated sodium phosphate, pH 7.2, for 2 min at 25°C and the gel was separated from the reaction mixture by centrifugation. The product in the supernatant fraction was quantified by its absorbance at 412 nm ($\varepsilon = 1.42 \times 10^4\,M^{-1}\,cm^{-1}$), typically showing that ca 16 mM MNB had been released from the gel.

ESR analyses

ESR measurements were conducted at room temperature with an X-band Varian spectrometer, model E109E. For most experiments equal volumes of oxoammonium salt solution and other compounds were mixed using WIRETROL capillaries and TEFLON-tipped plungers (Drummond Scientific). Reaction mixtures were drawn into 50 µl microcapillaries, which were sealed with Cha-Seal (Chase Instruments) for the ESR analysis. Typical instrument settings were 1.0 G modulation amplitude, 100 G scan range, 4 min scan time, 10 mW microwave power.

Spectrophotometry

Absorption spectra for quantification of Tac species were obtained with a Shimadzu UV-160U spectrophotometer interfaced with an IBM Model 70 computer using the PC160 Plus Personal Spectroscopy Software.

Quantification of Tac species by analyses of UV–visible spectra

Absorbance values at 429, 458, 478, and 511 nm were read for standard solutions of Tac* and Tac^{+} and used in a spreadsheet procedure to calculate amounts of Tac* and Tac^{+} in composite spectra. The use of absorbance measurements at four wavelengths provided a tool for inferring the presence of chemical species having optical spectra different from Tac^{+} and Tac*, while also providing a measure of the effect of instrument noise on the calculated concentrations of the Tac species. The procedure was validated by showing that defined mixtures of Tac species could be accurately quantified.

Rapid mix

Studies carried out on an Olis RSM 1000 spectrometer interfaced with a USA Stopped Flow apparatus.

Oxygen polarography

Polarographic measurements were performed with a Rank Brothers LTD oxygen polarograph Model 10.

Results

Although sensitivity is low, optical spectroscopy allows for the study of oxoammonium reduction to nitroxides, while also providing information about the formation of other optically active products. The hydroxylamine reduction products can be quantified subsequent to treatment of reaction mixtures with oxidants that convert them to nitroxides. Absorption spectra of the oxoammonium and nitroxide are clearly resolved (Figure 1A), while the hydroxylamine has no significant absorption above 280 nm.

Preparation of oxoammonium species from TEMPO nitroxides

A facile synthetic procedure for aqueous solutions, useful for studies of oxoammonium reactions in biologic systems, comprises treatment of nitroxides

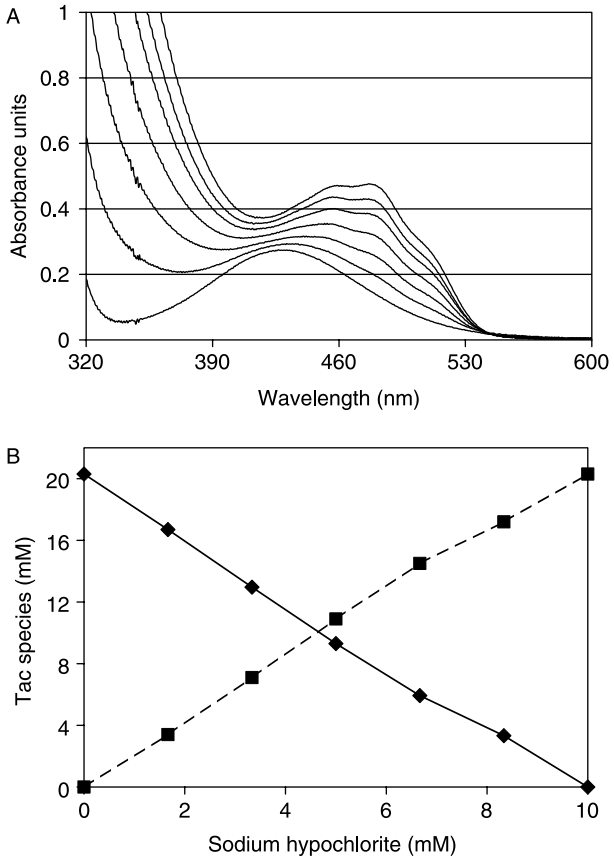


Figure 1. A. The UV–visible spectrum of Tac* (broad absorption peak at 429 nm) progressively changes upon treatment with 1.67 mM increments of sodium hypochlorite in 0.2 M sodium acetate, pH 4.75 until the spectrum of Tac^{+} (peaks at 458 and 478 nm) is seen. After adding six aliquots of the bleach solution, the spectrum was identical to that of freshly dissolved TacBF4 in sodium acetate, did not change with further hypochlorite additions (data not shown) and even with an excess of oxidant (one mole of HOCl added per mole of Tac*), remained stable for at least several hours at room temperature. B. Calculated concentrations, using spreadsheet procedure, of Tac*(◆) and Tac^{+}(■) in composite spectra. About 10 min reaction time was ample to attain equilibrium for all samples.

with ordinary bleach in sodium acetate buffer at pH 4.75 or in phosphate buffer at pH 2. An example of the procedure is illustrated in Figure 1, which shows the conversion of Tac* to Tac^{+} as a function of the hypochlorite concentration in acetate buffer. The reaction stoichiometry is consistent with complete conversion of hypochlorite to chloride and water and is therefore not expected to produce significant residual reaction products that could confound the use of the oxoammonium products for biological studies. Overall progression of the reaction can be monitored quite accurately by combining optical and ESR spectroscopy. Optical spectra can determine the progression of the reaction until about 95% of the starting Tac* has been consumed. By quantifying the remaining Tac* concentration by ESR in response to small additions of hypochlorite complete conversion of Tac* to Tac^{+} can be achieved with no hypochlorite remaining in the reaction mixture. The optical spectrum and aqueous reaction characteristics of the Tac^{+} formed in this manner is indistinguishable from that of freshly dissolved $TacBF_4$ [22] in the sodium acetate buffer system. This simple and rapid synthetic procedure is especially valuable for the preparation of oxoammonium compounds derived from nitroxides that may only be available in small quantities. The sensitivity of the ESR assay of nitroxide loss allows for the preparation of small volumes and low concentrations of oxoammonium compounds. The use of weak acid buffer is convenient for the oxidation of nitroxides that cannot tolerate strongly acidic conditions. Tac^{+} can be prepared as readily in phosphate buffer at pH 2, but the presence of iron impurities in this buffer could affect the results of some studies, particularly if thiols are involved. Stock solutions of Tac^{+} at pH 2 are quite stable—we found virtually no change in the optical absorbance of these solutions after several months of storage at 4°C. Acetate stock solutions are relatively free of iron contamination but are not quite as stable—after five months of refrigerator storage we found that 45% of the oxoammonium had been converted to the nitroxide.

The preceding synthetic procedure was also used to prepare the oxoammonium products of TEMPO and 4-methoxy-TEMPO as well as the chloride and phosphate salt solutions of Tac^{+}. The oxoammonium derivative of TEMPO was also prepared in acidic phosphate buffer by an electrochemical method and was found to have spectral and reactivity properties indistinguishable from that prepared by the hypochlorite treatment.

Stability of oxoammonium species in water

Tac^{+} is moderately stable in water, judging by its persistence in phosphate- or carbonate-buffered solutions (pH 7.1 and 10 respectively, Figure 2).

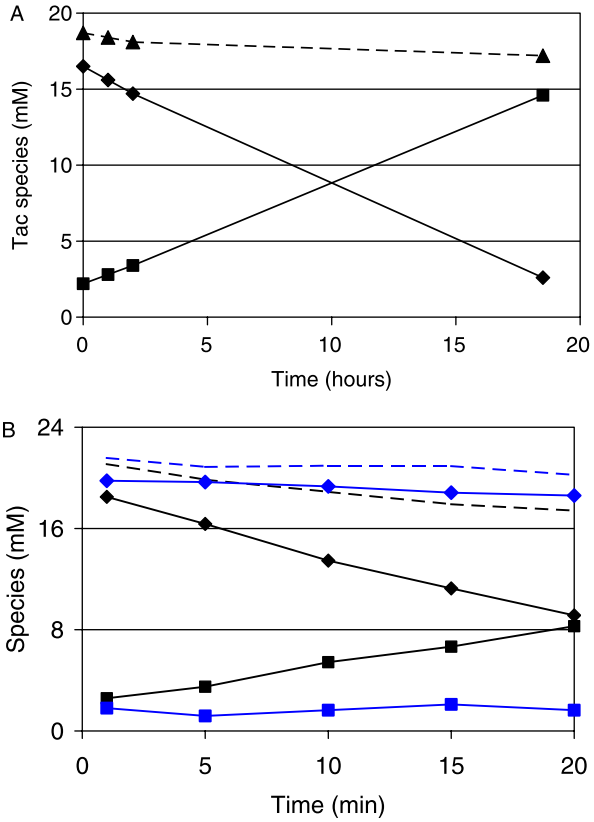


Figure 2. (A) Conversion of oxoammonium to nitroxide at pH 7.1. Symbols: (◆), Tac^{+}; (■), Tac*, (▲) sum of Tac^{+} and Tac*. (B) Conversion of oxoammonium to nitroxide at pH 10.0. Black lines and symbols: MeTOL (results for Tac were indistinguishable from those for MeTOL); blue lines and symbols: TEMPO; squares, oxoammonium; diamonds, nitroxide; and dashed lines, sum of nitroxide and oxoammonium. (See online version for colour).

At alkaline pH Tac^{+} and MeTOL^{+} are only partially converted to Tac* and MeTOL*, respectively (see sum of species plots in Figure 2). It has been suggested that Tac^{+} reacts with OH^{-} to form H_2O_2 and that this product accumulates in the reaction medium [23], but we [16] and others [24] have found that H_2O_2 undergoes rapid oxoammonium-mediated decomposition over a broad pH range. The stability of Tac^{+} is in marked contrast to the stability of Tempone-derived oxoammonium (Tempone^{+}), which has been reported to decompose under a variety of conditions [25,26]. We find that oxoammonium compounds undergo rapid electron exchange as seen in a reaction mixture comprising ^{15}N-Tempol and $TacBF_4$ (observation made at low pH, to minimize decomposition of the oxidized form of Tempol—data not shown). Upon mixing Tac^{+} with Tempone (5 mM each) at neutral pH there is a rapid change of the color of the solution from yellow to blue-green and formation of a complex absorption spectrum comprising that of Tac* (approximately 5 mM) and a new absorption feature at about 650 nm. A similar absorption feature was first reported by Abakumov and Tikhonov [25] who identified this feature as the tertiary nitroso compound

$(CH_3)_2C{=}CH{-}CO{-}CH_2{-}C(CH_3)_2{-}N{=}O$ (absorption peak at 673 nm in benzene) and elucidated the mechanism of its formation. More recently, a study by Goldstein et al. [26] obtained this product by electrochemical oxidation of Tempone in water (absorption peak at 650 nm in water). When higher concentrations of these species are mixed the solutions become quite turbid. Turbidity at higher concentrations is probably also due to the nitroso derivative whose solubility in water is much lower than that of Tempone [25]. We interpret these observations to indicate an electron transfer from Tempone to Tac^+ followed by the decomposition of $Tempone^+$ to form the poorly soluble nitroso product. Mixtures of some other nitroxides with Tac^+ indicate that Tempol and Tempamine also undergo oxidative decomposition. Depending on relative concentrations of Tac^+ and Tempol, reaction products will comprise a mixture of Tempone and the nitroso compound. Spectra for the reaction of Tac^+ with an equal concentration of Tempol (Figure 3) show the nitroso compound absorbance around 650 nm. Assuming an extinction coefficient of $14\,M^{-1}cm^{-1}$ [26] for this compound, we estimate its yield to be about 7 mM in this experiment. The oxidation of Tempamine appears to be more complex—the yield of the nitroso compound is significantly lower than that observed in the oxidation of Tempol.

Accelerated loss of Tac^+ in the presence of many organic compounds

At neutral pH, a diversity of organic and inorganic compounds cause a marked acceleration of the loss of Tac^+ relative to that observed in phosphate-buffered solutions. Some of these compounds can bring about a loss of several equivalents of Tac^+ (Table I).

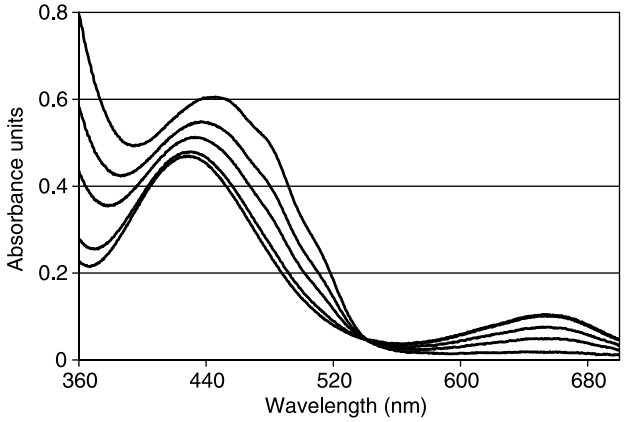


Figure 3. Tempol (20 mM) was mixed with TacBF4 (20 mM), 0.12 M sodium phosphate, pH 7.2. Spectra were recorded at 0.5, 2, 4, 10 and 25 min. Absorbance decreased around 440 nm and increased around 650 nm as function of time.

It has long been known that primary and secondary alcohols in certain solvents can be oxidized to aldehydes and ketones, respectively, in a two-electron transfer reaction with oxoammonium compounds [27,28]. Addition of a small excess of ethanol to oxoammonium in a solvent like acetonitrile bleaches the yellow color of the oxoammonium species within a few minutes, consistent with hydroxylamine formation. In water, even a large excess of ethanol reduces Tac^+ to Tac⋆ rather than the TacH, resulting in a deep yellow solution very different in appearance from the nearly colorless solution seen when this reaction is conducted in acetonitrile even after allowing for solvent-dependent color differences of the nitroxide and oxoammonium. Whereas the oxidation of primary alcohols in inert organic solvents can be controlled to be a two- or four-electron transfer that converts Tac^+ to TacH and alcohols to aldehydes [29,30], in water we could not attain substantial hydroxylamine yields at any alcohol concentration.

The reaction of Tac^+ with Tempol is much faster than is the reaction of Tac^+ with 4-hydroxy-2,2,6,6-tetramethylpiperidine. This suggests that the initial reaction is an electron transfer from nitroxide moiety of Tempol to Tac^+, forming $Tempol^+$, which undergoes intramolecular electron transfer(s) to produce Tempone or its hydroxylamine. The high nitroxide yield suggests little oxidative decomposition of the Tempone that would be formed in this reaction.

Amino acids appear to undergo oxidative deamination—a qualitative test for ammonia in the reaction mixture was positive. Remarkably, with respect to their oxidation behavior with oxammonium, the amino acids in Table I can be divided into two groups exemplified by: (1) alanine and glutamic acid (two-electron reductants of oxammonium); (2) glycine, aspartic acid and proline (2 × two-electron reductants of oxammonium). Several hypotheses can be imagined but not confirmed without isolation and characterization of reaction products. It appears that the α-keto acid derived from the oxidative deamination of alanine (i.e. pyruvic acid) is relatively unreactive with oxoammonium. On the other hand the corresponding oxidation product of aspartate (i.e. oxalylacetic acid) appears to be reactive, causing both reduction and destruction of oxoammonium and, perhaps, nitroxide (compare final nitroxide yield seen with glycine vs. aspartate). As oxalylacetic acid (keto form) mostly exists in solution as its enol isomer (hydroxyfumaric acid), the result is in line with previous reports demonstrating oxidation, in a second step, of easily enolizable ketones to the corresponding diones [31].

The comproportionation reaction of TacH with Tac^+ yields Tac⋆, which is detectable by ESR. We have determined the rate constant of this reaction by ESR to be $1.1 \times 10^2\,M^{-1}\,s^{-1}$ at pH 7.2. This rate constant

Table I. Oxoammonium loss, nitroxide formation and rate constants for some reactions of $TacBF_4$ (initially 20 mM) with organic and inorganic molecules.

Compound	Tac^+ remaining*	Nitroxide (mM)	k ($M^{-1}s^{-1}$)
Sodium acetate (20 mM)	19 mM (after 2 h)	1	
Acetaldehyde (5 mM)	11 mM (after 30 min)	9	0.14
Ethanol (5 mM)	1 mM (after 40 min)	19	0.46
Methanol (5 mM)	3 mM (after 7 min)†	14†	
Glycine (5 mM)	0	20†	40
Alanine (5 mM)	10 mM(after 2 min)†	10†	
Glutamic acid (5 mM)	10 mM (after 2 min)†	10†	
GSSG (2 mM)	10 mM (after 30 min)†	10†	
Lipoic acid (5 mM)	7 mM (after 10 min)†	11†	
Aspartic acid (5 mM)	0	16†	
Proline (5 mM) ‡	5 mM (after 30 min)†	23†	
Taurine (5 mM)	1 mM (after 10 min)	19	
Bisulfite (5 mM)	15 mM (within 30 sec)	0	
H_2O_2 (5 mM)	10mM†	10†	
TacH (10 mM)	10mM†	20†	1.2×10^2
N-acetyl methionine (20 mM)	0 (after 20 min)	14†	0.11
Tempol (20 mM)	0 (after 20 min)†	35†	
Trimethylamine (5 mM)	0 (within 1 min)	20	
2,2,6,6-Tetramethylpiperidine (20 mM)	19.5 mM (after 20 min)	<0.5	
4-Hydroxy-2,2,6,6-tetramethylpiperidine (10 mM)	12 mM (after 20 min)	3	0.09

* All reactions in Chelex 100-treated sodium phosphate buffer, pH 7.2; † Concentration had reached a plateau when spectra were recorded; ‡ Initial concentration of $TacBF_4$ was 25 mM.

is comparable to that reported for the analogous comproportionation reactions of Tempo and 4-hydroxy Tempo species [32]. Since comproportionation reactions are often much faster than oxidation of organic compounds it is relatively easy to estimate rate constants for many of the latter reactions. Some rate constant estimates are listed in Table I.

The reaction of $TacBF_4$ with glycine at pH 7.2 was monitored by the appearance of the Tac* ESR signal, which allowed the use of low concentrations to estimate the rate constant for this reaction. A spreadsheet procedure for numerical integration of the reaction rate equations was used to estimate a rate constant that was of the same order of magnitude as that for the comproportionation reaction. The reaction could be a two-electron reduction process that causes accumulation of the hydroxylamine, followed by a comproportionation of the hydroxylamine with oxoammonium. However, if hydroxylamine formation were the only reaction pathway, we would expect that the yield of Tac* would decrease dramatically with an increase in the glycine concentration, which is not observed at neutral pH. The reaction of $TacBF_4$ with glycine was also conducted at low pH to lower its rate sufficiently to allow for a comparison of a range of glycine concentrations (Figure 4). Only at the highest glycine concentration was there a significant decrease in the sum of Tac^+ and Tac* concentrations, indicating that sequential one-electron transfers to Tac^+ rather than a two-electron transfer followed by comproportionation of Tac^+ with TacH is the dominant reaction mechanism over a broad pH range.

Lipid peroxidation. Although oxoammonium cations would not be expected to partition into the hydrophobic domains of biomembranes, they can initiate lipid peroxidation. The rate of this process appears to be slower than most of the other reactions listed in Table I (Figure 5). The oxygen consumption triggered by Tac^+ is inhibited by Tac*, consistent with the competition of nitroxides and oxygen for carbon-centered lipid radicals, i.e. the reaction of a nitroxide with a carbon-centered lipid radical terminates oxygen-consuming chain reactions. Oxoammonium-mediated oxidation of the carbohydrate moiety of glycolipids has proven useful for the analysis of the lipid bilayer asymmetry characteristics [33]; our data

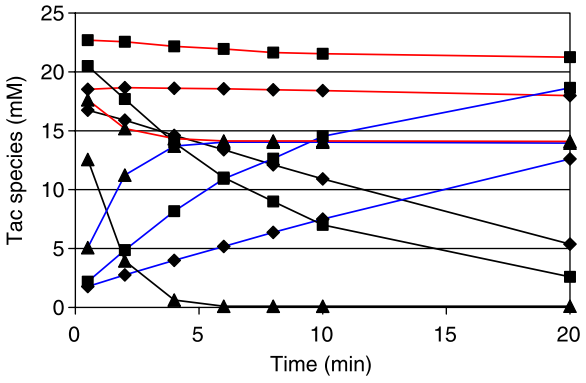


Figure 4. Reaction of TacBF4 with different concentrations of glycine at low pH. (◆) 10 mM glycine, 0.2 M Na acetate, pH 4.7, (■) 50 mM glycine, 0.19 M Na acetate, pH 4.75, (▲) 0.5 M glycine, 60 mM Na acetate, pH 3.7. Black lines Tac^+, blue lines Tac*, red lines sum of Tac^+ and Tac*. (See online version for colour).

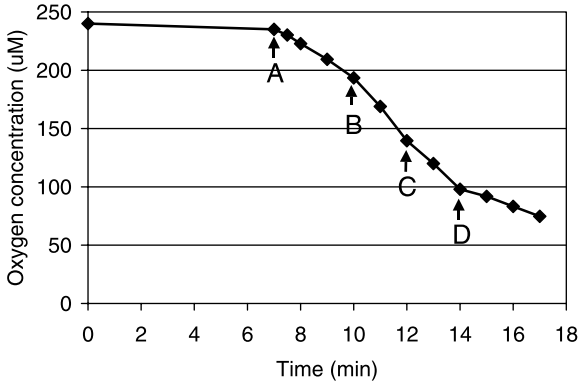


Figure 5. Oxygen consumption in sonicated liposomes (egg phosphatidyl choline, 4 mg/ml, 0.2 M sodium phosphate, pH 7.2). The liposomes were treated with: (A) TacBF4, 100 μM, (B) TacBF4, 100 μM, (C) Tac⋆, 100μM, (D) Tac⋆, 1 mM.

suggest that some oxidation of unsaturated lipids may occur during the application of this assay.

Reaction with phenol. Simple phenols reduce oxoammonium to its hydroxylamine. Rate constants were obtained at low pH, where the reduction of Tac^+ was slow enough to allow us to measure the time dependence of the increase in the ESR signal of Tac⋆. We estimated rate constants of 0.017 and $14\,M^{-1}\,s^{-1}$ at pH 2.0 and 4.75, respectively. The ratio of these values is consistent with an abstraction of an electron from the phenolate anion by Tac^+. Assuming that phenolate-dependent reaction mechanisms extend into the neutral pH range, we infer a rate constant of $4 \times 10^3\,M^{-1}\,s^{-1}$ at pH 7.2 for phenol and, probably other phenolic electron donors, including tyrosine. Optical spectrophotometry of the reduction of Tac^+ by phenol shows a broad absorbance throughout the visible range consistent with the formation of polymeric products known to arise from phenoxyl radicals (data not shown). This observation precludes another conceivable mechanism for the reaction, namely adduct formation of oxoammonium with the phenolate anion, followed by hydrolysis and comproportionation of oxoammonium with hydroxylamine.

Reaction of Tac^+ with sulfur compounds. At neutral pH non-aromatic thiols or cysteine sulfonic acid react rapidly with Tac^+, producing a species that is ESR-silent and cannot be seen by optical spectroscopy above 280 nm. The rapid reaction phase is followed by a slower process that converts Tac^+ to Tac⋆ (examples are shown in Figure 6A and B). Oxygen polarography shows that very little, if any oxygen consumption occurs in the reaction of thiols with Tac^+ in the low millimolar range (data not shown). Therefore, oxygen-consuming free radical reactions do not

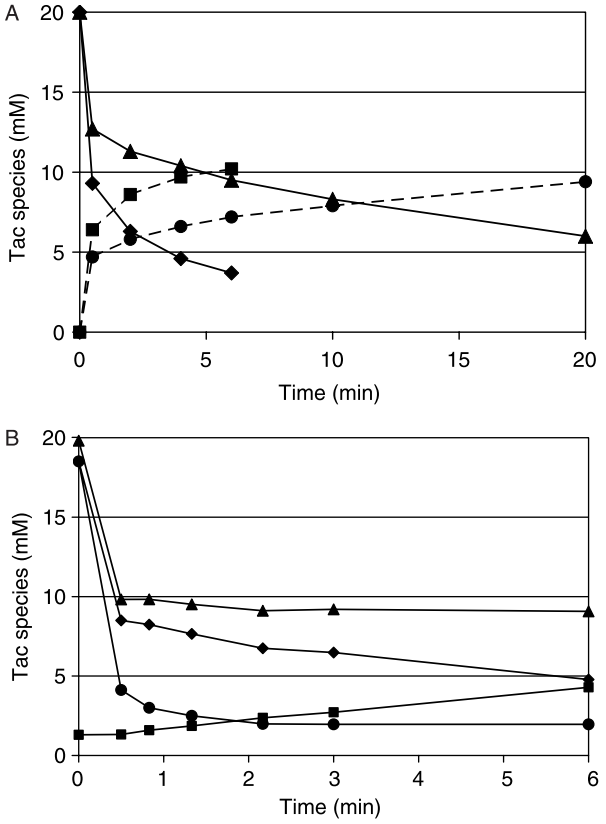


Figure 6. (A) Reaction of TacBF4 (20 mM) with low concentrations of two biologic thiols (2 mM each). Solid lines, Tac^+; dashed lines, Tac⋆. (◆), (■) cysteine; (▲), (•) glutathione. (B) Depletion of oxoammonium by an aliphatic thiol. Equal volumes of MPS (20 mM) and TacBF4 (40 mM) in sodium phosphate buffered solution (0.16 M) were mixed and assayed spectrophotometrically. Symbols refer to: (◆), Tac^+, pH 7.2; (■), Tac⋆, pH 7.2, (▲), sum of Tac^+ and Tac⋆, pH 7.2, (•), Tac^+, pH 2.0. No formation of Tac⋆ was seen at pH 2.

appear to play a significant role in the reaction of oxoammonium with thiols.

The extent of Tac^+ loss can be considerable relative to the thiol concentration, e.g. when the oxoammonium reacts with cysteine (Figure 6A). This is in contrast to the more limited extent of Tac^+ reduction by the non-thiol compounds listed in Table I.

Even at pH 2, the rapid phase is still fast enough to be analyzed without a need to resolve two concurrent processes. We found that at pH 2, bisulfite and cysteine sulfinic acid cause the loss of one Tac^+ per each of these species whereas all thiols that were tested cause the loss of two Tac^+ per thiol (see for example Figure 7). All reactions at pH 2 had gone to completion by the time the samples were scanned, i.e. within 30 s.

Reaction of oxoammonium with MNB. To study the reaction products of a thiol upon reacting with oxoammonium, we examined MNB, whose optical absorbance characteristics at neutral pH allowed us to

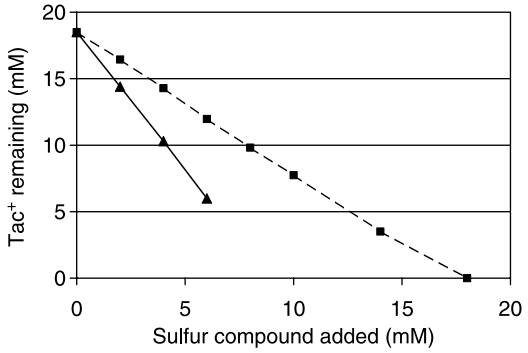


Figure 7. Tac^+ loss in presence of NAC (▲) or cysteine sulfinic acid (■) in acidic solution (0.16 M sodium phosphate, pH 2.0).

quantify this thiol and its disulfide oxidation product by a straightforward analysis of absorption spectra. Unlike the aliphatic thiols examined in this study, almost all of the MNB exists as the thiolate anion in the reaction mixtures. Manual mixing of equal volumes of MNB and $TacBF_4$ solutions by injecting the solutions simultaneously into a magnetically stirred test tube with pipettors, caused a rapid bleaching of the reaction mixture. Treatment of the reaction mixture with 2 mM MPS, a concentration shown to be sufficient to fully reduce any DTNB that could have formed, was used to quantify the concentration of DTNB in terms of the increase in the absorption maximum of MNB. The spectrum of a DTNB solution corresponding to this concentration was subtracted from the spectrum of the reaction mixture to yield the spectrum designated as "other" (Figure 8). The broad absorbance feature extending beyond 530 nm in spectrum c was transient, decaying within several minutes.

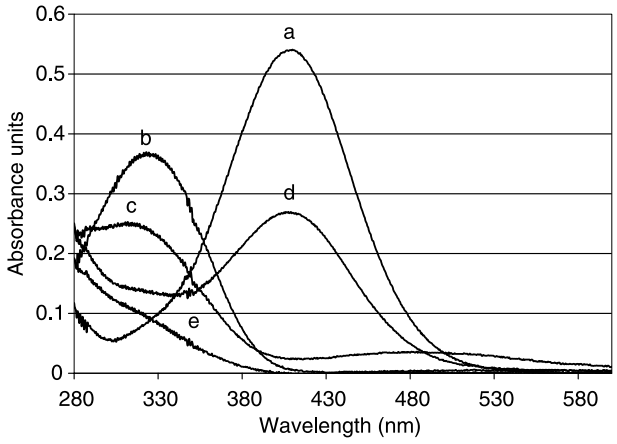


Figure 8. UV–visible spectra of species involved in the reaction of MNB with TacBF4. (a) MNB (40 μM); (b) DTNB (20 μM); (c) reaction product of MNB (40 μM) and TacBF4 (40 μM); (d) after equilibration of the reaction product with 2 mM MPS; and (e) "other" species.

Product yields when equal volumes of MNB and $TacBF_4$ solutions were manually mixed are shown in Table II. The DTNB concentration can be calculated from the difference between columns 4 and 3.

The data show that under all tested conditions the products of the reaction comprised a mixture that included species other than the disulfide. The disulfide yield increased as a function of the ratio of MNB to $TacBF_4$. When this ratio was large, the disulfide was the major thiol oxidation product, whereas other product(s) predominated when this ratio was small. ESR studies of reaction mixtures with high disulfide yields, e.g. treatment of 0.5 mM $TacBF_4$ with 1 mM MNB, showed that the major oxoammonium product was the hydroxylamine (inferred from quantification of Tac⋆ formed after mild oxidation of the reaction mixture, data not shown). There was a correlation between the concentration of other products and the absorbance at 280 nm seen in the spectrum of "other" obtained by the procedure described above, as reflected in the ratio shown in the last column of the table. The chemical species giving rise to this spectral feature is (are) unknown.

The rate of the reaction of MNB with $TacBF_4$ was too fast to be measurable with our stopped-flow instrument, implying a rate constant $> 10^7\ M^{-1}\ s^{-1}$.

Fate of nitroxides during the reaction of oxoammonium with thiols. We attempted to study thiol oxidation products by means of a thiol that had been spin-labeled with a pyrroline nitroxide entity, hoping to exploit the characteristic ESR signature of nitroxide biradicals to resolve thiol oxidation products. These experiments were unsuccessful because we found that the nitroxide moiety of the thiol oxidation products suffered substantial destruction (we use the term destruction to refer to products other than the hydroxylamine). To study this phenomenon experiments were conducted with mixtures of Tac^+, Tac⋆ and a thiol. The ESR signal of Tac⋆ does not change appreciably over the course of several hours in the presence of a thiol like MPS in transition metal-free solutions, e.g. in CHELEX-treated buffer. However, upon treatment of such solutions with oxoammonium a substantial loss of the nitroxide ESR signal is observed. For example, when a mixture of 1 mM Tac⋆ and 0.5 mM CoM was treated with 0.5 mM $TacBF_4$, a rapid decrease in the ESR signal of Tac⋆ was observed (Figure 9). The nitroxide signal could be partially, but not completely, recovered after ferricyanide treatment, suggesting the Tac⋆ had undergone both reduction and destruction by products arising during the reaction of oxoammonium with the thiol. Partially reversible loss of a pyrroline nitroxide ESR signal was also observed when a ^{15}N-labeled nitroxide of this type, whose spectrum is clearly resolved from that of ^{14}N-containing nitroxides, was substituted for Tac⋆ in the preceding reaction mixture.

Table II. Products (in μM) of the reaction of MNB with $TacBF_4$ (μM values).

MNB	$TacBF_4$	MNB remaining	MNB after MPS (2 mM)	Other products	Abs(280)*	Ratio†
80	20	51.5	72	8	0.62	130
80	40	21	59	21	0.15	141
80	60	3.3	50	30	0.22	136
80	80	0	33.5	46	0.32	145
40	80	0	11.6	28	0.22	129
40	160	0	6.9	33	0.23	144

* Calculated by subtracting the absorbance due to MNB at 280 nm; † Other products/Abs(280).

This led us to conclude that the availability of a pyrroline nitroxide spin-labeled thiol would not allow us to quantify thiol oxidation products by ESR methods.

The extensive loss of nitroxides during oxoammonium reactions with thiols was also studied by optical spectroscopy (Figure 10). A rapid initial reaction phase causes the loss of both oxoammonium and nitroxide. Under the chosen reaction conditions a substantial concentration of oxoammonium remains after the initial reaction phase, which would cause any hydroxylamine that was present to undergo virtually immediate oxidation to nitroxide. Therefore, the initial loss of both oxoammonium and nitroxide comprises destruction and not reduction of these species. Subsequently, one observes a loss of oxoammonium with a corresponding increase of nitroxide, such that the sum of the two species remains nearly constant. The conversion of oxoammonium to nitroxide is substantially faster than that observed in buffer (Figure 2A), suggesting that components of the reaction mixture are accelerating this conversion.

Ferricyanide studies of reaction mixtures. Ferricyanide is a mild oxidant (standard reduction potential +0.4 V vs. NHE) relative to the much more strongly oxidizing oxoammonium species (about +0.8 V, 12). The deeply yellow ferricyanide ion is bleached upon one-electron reduction by hydroxylamines and by reactions with thiols, allowing for its use in the detection of these species in some of our reaction mixtures. In an application of this approach, a mixture of $TacBF_4$ and MPS (10 mM each) in 0.17M sodium phosphate buffer, pH 7.2, was allowed to react for ten minutes. An aliquot of this reaction mixture was added to 10 volumes of buffer containing potassium ferricyanide (1 mM). There was no significant bleaching of the ferricyanide, indicating that no hydroxylamine had accumulated and that no thiol remained in the reaction mixture.

Discussion

Important targets of oxoammonium in biological environments are likely to be low molecular weight thiols, pyridine nucleotides [32], phenolic molecules and probably catecholamines. Other reaction targets like lipids could be important if nitroxide oxidation occurred in close proximity to these targets. The relatively sluggish rates of oxoammonium reactions with simple amino acids, amines, alcohols and aldehydes suggest that such reactions would have insignificant biological impacts.

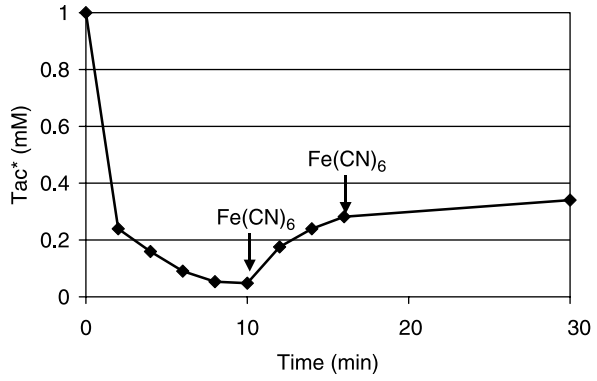


Figure 9. A mixture of Tac* (1 mM) and 2-mercaptoethane sulfonate (0.5 mM) was treated with TacBF4 (0.5 mM) in sodium phosphate buffer, pH 7.2 and the ESR signal of the Tac* was used to quantify its concentration as a function of time. Ferricyanide additions comprised 1 mM each.

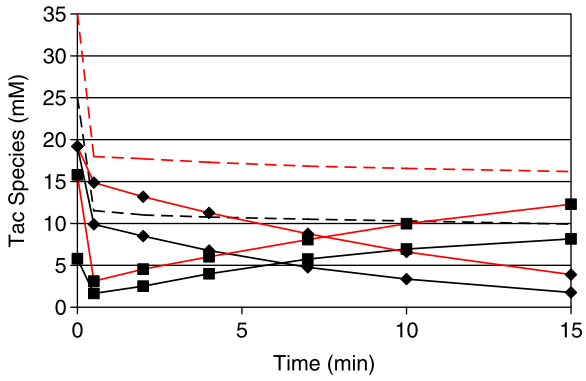


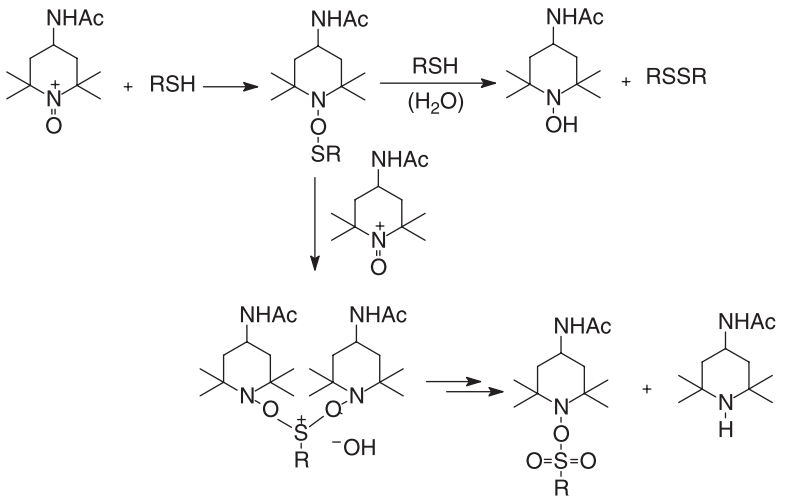
Figure 10. Effect of Tac* on the reaction of TacBF4 with MPS. TacBF4 (20 mM, ◆) was combined with mixtures of Tac* (■) and MPS(10 mM). Concentrations of Tac* were 5 mM (black lines) or 15 mM (red lines). The sum of Tac* and Tac^+ concentrations is shown as dashed lines. (See online version for colour).

Hydroxylamines can arise not only from reduction of nitroxides or oxoammonium species but, in principle at least, by hydrolysis of oxoammonium adducts with other molecular species, e.g. oxoammonium-thiolate adducts ($R_2N—O—SR'$—see upper part of reaction scheme). For the thiolate adduct, a displacement of the hydroxylamine from the adduct by another thiolate anion is also a possibility, particularly at the high thiol concentrations found in most biological environments. Both hydrolysis or displacement (thiolysis) would likely generate the disulfide and some combination of the two could be an important reaction mechanism of oxoammonium with thiols in biology. It is likely that thiolysis would involve the thiolate anion rather than the thiol, suggesting that thiolysis would be more likely to occur in the reaction of oxoammonium with aromatic thiols like MNB than in reactions with aliphatic thiols like MPS. The observation that disulfide (DTNB) formation correlates with hydroxylamine formation (when thiol concentrations significantly exceed oxoammonium concentrations) is consistent with thiolysis, which is expected to yield one hydroxylamine per disulfide (see reaction scheme). However, when thiol concentrations are comparable to or lower than oxoammonium concentrations, the MNB data clearly show that products other than disulfides are formed. The failure to produce nitroxides upon ferricyanide treatment of reaction mixtures containing equal concentrations of MPS and $TacBF_4$ argues against the likelihood that thiolytic or hydrolytic processes (as shown in the reaction scheme) occurred to a significant extent for this aliphatic thiol. Exclusive hydroxylamine formation was claimed to occur in the thiol-dependent reduction of nitroxides in a xanthine/xanthine oxidase system [34], which presumably proceeds via superoxide-generated oxoammonium intermediates. Our efforts to repeat this finding were unsuccessful—only partial reduction of nitroxides in the presence of a variety of thiols could be demonstrated (data not shown). At high oxoammonium concentrations relative to thiol concentrations, we suggest that the oxoammonium-thiolate adduct undergoes further attack by excess oxoammonium to form a second intermediate that possibly could hydrolyze to the piperidine and the hydroxylamine sulfonate. (see reaction Scheme 2).

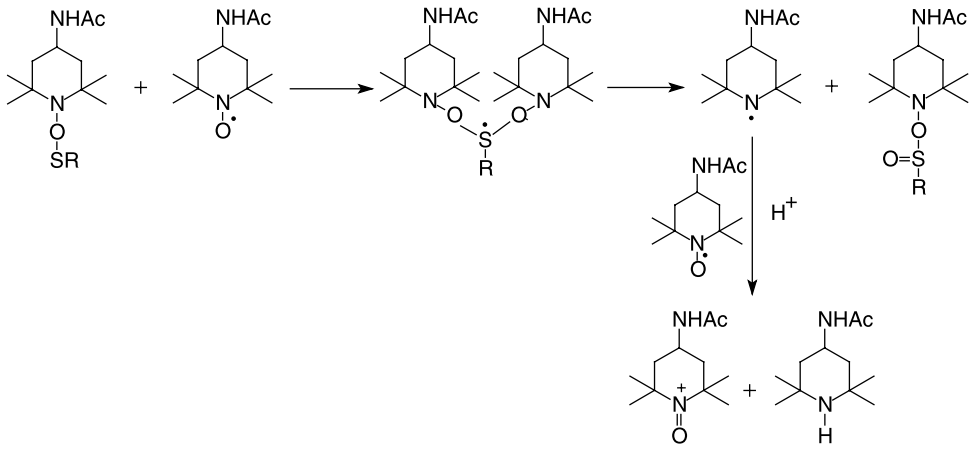
In anhydrous media where hydrolysis is not possible, an analysis of the reaction of oxoammonium with thiols is more straightforward. Disulfide formation was observed when Tempo-derived oxoammonium was treated with 1-dodecanethiol in acetonitrile [35]. Treatment of nitroxides with thiols in organic solvents produces both disulfides and moderately to highly persistent adducts, including sulfenamides, sulfinamides and sulfonamides [36,37]. The amides are derived from the initial sulfur-oxygen bonded adduct, which is presumed to be the adduct initially formed in our studies.

In summary, the reaction products of oxoammonium with thiols can vary substantially, depending on relative concentrations. An excess of thiol correlates with an excess of thiol loss relative to the loss of oxoammonium whereas an excess of oxoammonium correlates with an excess of oxoammonium loss relative to the thiol loss. Moreover, it seems possible that at low concentrations of thiols and oxoammonium, hydrolysis predominates while at higher concentrations of these compounds adduct decomposition would be mediated by either thiols or oxoammonium.

The striking loss of nitroxides during the reaction of oxoammonium with thiols was unexpected, given the persistence of nitroxides in the presence of thiols. A possible mechanism for the nitroxide loss is shown in the following schematic (Scheme 3):



Scheme 2. Proposed reaction mechanism of oxoammonium with thiols.



Scheme 3. Proposed reaction scheme in a mixture of oxoammonium, nitroxide and thiol.

This mechanism indicates a process whereby oxoammonium can be generated from the nitroxides, which would explain the possibility of substantial losses not only of nitroxides, but thiols as well. Importantly, in the presence of appropriate concentrations of nitroxides it is possible that thiol destruction could be much greater than would be expected for a reaction scenario based on oxoammonium and thiols only.

The rate constant for the reaction of superoxide and glutathione has been estimated to be between 30 and 10^3 per mole per second at neutral pH [38,39]. The rate constant for the reaction of superoxide with TEMPO nitroxides is at least two orders of magnitude greater at neutral pH [13]. Superoxide is a weak acid (pKa = 4.7), which oxidizes nitroxides in its protonated state. Therefore nitroxide oxidation by superoxide is favored under acidic conditions. In consideration of the high reactivity of oxoammonium with thiols and the potential of significant formation of products other than disulfides, we suggest that nitroxides can mediate thiol destruction by superoxide and may aggravate superoxide damage *in vivo*. On the other hand, nitroxides may be somewhat effective in protecting against oxidants produced by activated heme proteins. Although capable of inducing a catalatic activity in hemoglobin and myoglobin, this activity is not seen in the presence of physiological concentrations of thiols [16], supporting the conclusion that the reaction rate of oxoammonium with thiols is much greater than is that with H_2O_2. However, the potential protective activity of oxoammonium in heme-catalyzed reactions may be compromised by adverse effects of oxoammonium on thiols. Even in the case of antioxidant enzymes such as catalase and some peroxidases, oxidation states in the enzyme cycle (such as compound I) may exhibit reactions that are deleterious when tested in simple model systems but whose net *in vivo* effects are protective. Oxoammonium reactions are similar to those associated with some enzyme oxidation states and their adverse effects must be weighed against protective effects.

In light of these considerations, we find it difficult to argue for an antioxidant mechanism in the protective effects of nitroxides in animals. However, it is likely that the reduction products of nitroxides (hindered hydroxylamines) can serve a significant protective role against oxidative damage. While they are relatively unreactive towards superoxide, e.g. rate constant of $4 \times 10^2\,M^{-1}s^{-1}$ for the hydroxylamine of Tempol [13], the rate constant of this hydroxylamine with phenoxyl radicals has been estimated to be about $10^7\,M^{-1}s^{-1}$ [40]. Thus the hydroxylamines may "repair" free radical products like tyrosinyl radicals and may be particularly effective in domains of low polarity, where ascorbate would be unavailable. Hydroxylamines partitioning into cell membranes would also be expected to act as powerful free-radical chain terminating agents, possibly acting in concert with vitamin E. A possible caveat to this argument is the rapid oxidation of the hydroxylamine to oxoammonium by hypochlorite. Under some conditions, hydroxylamines could amplify the effects of peroxidase-generated oxidants.

A more plausible mechanism of a therapeutic effect of nitroxides may be that they induce an oxidative shift in the biological environment, which elicits a stress response that would serve a protective function under conditions that have been found to be responsive to nitroxide treatment. Such a stress response may be a direct effect of nitroxides on cell signaling, as has been seen with other free radicals and oxidants [41]. A shift in the redox state of cells could be quite significant at high nitroxide concentrations considering the linkage of nitroxide reduction to ascorbate and thus glutathione metabolism as well as its linkage to the mitochondrial respiratory chain. If redox shifts are responsible for protective effects, optimal nitroxides would be those that undergo rapid reduction by cellular electron donors while also being relatively

resistant to oxidation to the reactive oxoammonium species. While oxoammonium could also trigger a stress response, there would be concurrent damage as these more reactive species attack some of the biological targets identified in our study. Consistent with the potential of oxoammonium-mediated damage, it has been reported that in a breast cancer cell model Tempo (oxidation potential about +0.7 V vs. NHE, 12) is toxic whereas Tempol (oxidation potential about +0.8 V vs. NHE, 12) is not [42].

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