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REACTIONS OF ACTIVE OXYGEN AND NITROGEN SPECIES STUDIED BY EPR AND SPIN TRAPPING

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The reactions of hydrogen peroxide (H_2O_2) with nitrite (NO_2^-) and of superoxide ($O_2^{\cdot-}$) with nitric oxide ($NO^{\cdot}$) were studied using EPR and spin trapping techniques. These reactions reportedly have a common peroxynitrite ($OONO^-$) intermediate. It has been suggested that this intermediate when protonated rapidly decomposes producing hydroxyl radicals ($^{\cdot}OH$) and the nitrogen dioxide radical ($NO_2^{\cdot}$). The production of $^{\cdot}OH$ in the reaction between H_2O_2 and NO_2^- was confirmed in spin trapping experiments using the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). H_2O_2 and NO_2^- were mixed at neutral pH and then the pH was decreased to pH 3-3.5 in the presence of DMPO or DMPO and ethanol. In these experiments, the EPR spectrum of the DMPO-OH adduct was obtained in addition to a weak EPR spectrum consisting of a triplet of triplets (a_N = 1.415 mT and a_N^β = 0.35 mT) indicating the addition of a nitrogen centered radical to DMPO. The formation of $^{\cdot}OH$ was confirmed using ethanol as an $^{\cdot}OH$ scavenger. The DMPO-hydroxyethyl adduct was produced from the reaction of $^{\cdot}OH$ with ethanol. However, in experiments using an excess of ethanol, the formation of DMPO-OH was not prevented. This suggests that the DMPO-OH formed in the decomposition of HOONO does not entirely originate from a direct addition of $^{\cdot}OH$ to DMPO.

The reaction of $O_2^{\cdot-}$ with $NO^{\cdot}$ was carried out in deaerated and air-saturated solutions at pH 12.3 where the dismutation of $O_2^{\cdot-}$ is minimal. The pH was then decreased to pH 3-3.5 in the presence of DMPO or DMPO and ethanol. In these experiments, the most prominent EPR spectrum obtained was a triplet of triplets (a_N = 1.415 mT and a_N^β = 0.35 mT) suggesting the addition of a nitrogen centered radical to DMPO. The formation of DMPO-OH was minimal and there was no formation of DMPO-hydroxyethyl adducts in the presence of ethanol. The results suggest that $NO^{\cdot}$ in solution yields additional reactive species which act as nitrating agents in the presence of DMPO.

KEY WORDS: Nitric oxide, superoxide, peroxynitrite, hydroxyl radicals, EPR, spin trapping.

INTRODUCTION

Free radical pathways are thought to play a major role in toxic mechanisms in various organs (gut, lung, heart, kidney, brain, liver), in the toxicity of various xenobiotic agents in these organs and in vascular disorders.[1] In general, the focus of free radical induced pathological conditions revolves around oxygen-related species. These include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). However, in recent years a new important class of biologically generated free radicals has emerged. These are the nitrogen centered radicals nitric oxide ($NO^{\cdot}$) and its by-products (eg. nitrogen dioxide, $NO_2^{\cdot}$). The biology and pharmacology of $NO^{\cdot}$ is the focus of recent reviews.[2-4] $NO^{\cdot}$ is a second messenger and its

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(1) $2H^+ + O_2^{\cdot-} + O_2^{\cdot-} \longrightarrow H_2O_2 + O_2$

(2) $2NO^{\cdot} + O_2 \longrightarrow 2NO_2^{\cdot}$

(3) $H_2O_2 + NO_2^- + H^+ \longrightarrow ONOOH + H_2O$

(4) $O_2^{\cdot-} + NO^{\cdot} + H^+ \longrightarrow ONOOH$

(5) $ONOOH \longrightarrow {}^{\cdot}OH + NO_2^{\cdot}$

(6) ${}^{\cdot}OH + NO_2^{\cdot} \longrightarrow NO_3^- + H^+$

(7) $O_2^{\cdot-} + Fe^{+3} \longrightarrow O_2 + Fe^{+2}$

(8) $Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + {}^{\cdot}OH + OH^-$

(9) $NO_3^- + 3Fe^{+2} + 4H^+ \longrightarrow NO^{\cdot} + 3Fe^{+3} + 2H_2O$

SCHEME 1-9

production is known to occur in several types of cells.[2,4] It is known to be directly involved in the mechanisms controlling vascular tone (endothelial derived relaxing factor, EDRF) and in the cytotoxic mechanisms of macrophages.[5] It is also known that $NO^{\cdot}$ production leads to the formation of nitrous acid. This acid causes transitional mutations on DNA by deaminating adenine residues, which pair with thymine, producing hypoxanthine which pairs with cytosine.[6]

It has been reported that $O_2^{\cdot-}$ inactivates EDRF.[7] This possibly occurs through direct reaction with $NO^{\cdot}$. In a recent study the reaction of $O_2^{\cdot-}$ with $NO^{\cdot}$, following simultaneous generation in solution, was investigated suggesting the production of ${}^{\cdot}OH$.[8] Pulse radiolysis studies have shown that $NO^{\cdot}$ reacts rapidly with $O_2^{\cdot-}$ ($k = 3.7 \times 10^7\ M^{-1}\ s^{-1}$).[9†] Since $NO^{\cdot}$ is a second messenger, $O_2^{\cdot-}$ may play a physiological role in its modulation.

Several important reactions involving active oxygen and nitrogen species indicating their interrelationships are presented in the following equations:

The reactions shown in eqns. (3)–(5) have either reactants or products in common with all the reactions above. Furthermore, the reactions shown in eqns. (3) and (4) are particularly important because they produce the peroxynitrous acid (HOONO). H_2O_2 and NO_2^- are, respectively, the products of $O_2^{\cdot-}$ dismutation and a by-product of $NO^{\cdot}$ decomposition in cells. Equation (3) shows that H_2O_2 and NO_2^- react forming the same intermediate (HOONO) as the reaction between their progenitor species [Eqn. (4)]. The peroxynitrite anion ($OONO^-$) is stable in alkaline solutions, but decomposes upon protonation producing ${}^{\cdot}OH$ and $NO_2^{\cdot}$.[10,11] Peroxynitrite anion is reportedly formed in biological systems and this anion has been suggested as a factor in lipid peroxidation and sulfhydryl oxidation.[11-13] Therefore, the focus of this study is to employ EPR and spin trapping techniques to determine whether the reactions between H_2O_2 and NO_2^- [Eqn. (3)] and between $O_2^{\cdot-}$ and $NO^{\cdot}$ [Eqn. (4)] generate a common intermediate which decomposes forming ${}^{\cdot}OH$ and NO_2^-.

MATERIALS AND METHODS

$NO^{\cdot}$ gas was purchased from Matheson Gas Products, Inc. (Fairfield, NJ). Potassium superoxide was purchased from Sigma (St. Louis, MO). Sodium nitrite and

†Since submission of this article more recent studies have shown that $k_{O_2^{\cdot-} + NO} = 6.7 \times 10^9\ M^{-1}\ s^{-1}$. R.E. Huie, and S. Padmaja, *Free Rad. Res. Communs.*, **18**, 195–199, 1993.

hydrogen peroxide were obtained from Fisher Scientific Co. (Fair lawn, NJ). The concentration of hydrogen peroxide was determined by titration with potassium permanganate.[14] The spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was purchased from Aldrich (Milwaukee, WI) and verified to be free of radical impurities by EPR. The concentration of DMPO was measured spectrophotometrically ($\lambda = 227$ nm; $\epsilon = 8 \times 10^3\,M^{-1}\,cm^{-1}$).[15]

$O_2^{\cdot-}$ was prepared, in a plastic tube sealed with a plastic stopper, as a saturated solution of potassium superoxide in dimethylsulfoxide. Once the excess undissolved potassium superoxide settled, the supernatant solution was continuously purged with nitrogen to avoid water and oxygen contamination of the solution. The concentration of $O_2^{\cdot-}$ was determined by EPR and spin trapping as described previously.[16,17]

To prevent possible trace metal ion contamination $NO^{\cdot}$ solutions were prepared in a plastic tube impermeable to gases and sealed with a rubber septum. $NO^{\cdot}$ gas was bubbled into deaerated N_2-saturated water (N_2 bubbling, 0.5–1 hr.). $NO^{\cdot}$ solutions at high pH were also prepared in the same manner in 0.1 M NaOH solutions. All aqueous solutions were prepared using metal free water obtained from a Sybron/Barnstead NANO pure system.

Between 50 and 100 μl of the $NO^{\cdot}$ in 0.1 M NaOH solutions were added by syringe to 1 ml deaerated (N_2 saturated) sodium hydroxide (0.1 M). The NaOH solution was deareated in a tube with a side arm fitted directly to an EPR quartz flat cell (60 × 10 × 0.25 mm) continuously flushed with nitrogen. Addition of $NO^{\cdot}$ was followed by the immediate addition of an aliquot of the $O_2^{\cdot-}$ solution. The final concentrations of $O_2^{\cdot-}$ added to the reaction mixture ranged between 40–60 μM. After mixing $NO^{\cdot}$ and $O_2^{\cdot-}$ the pH of the solution was pH 12.3. In experiments not requiring N_2 saturation an aliquot of $NO^{\cdot}$ solution was added directly to an air-saturated 0.1 M NaOH solution followed by the addition of $O_2^{\cdot-}$. The advantage of working at high pH is two fold: (1) any $OONO^-$ formed is stable at alkaline pH; and (2) the dismutation of $O_2^{\cdot-}$ at high pH is minimal. A DMPO (0.2 M, final concentration)/HCl mixture was added to the reaction mixture containing $O_2^{\cdot-}$ and $NO^{\cdot}$. The quantity of HCl was sufficient to lower the pH of the reaction mixture to pH 3–3.5. For experiments requiring deaerated conditions the DMPO/HCl mixture was added by syringe and the reaction mixture was allowed to flow into the EPR cell after the cell was sealed. The EPR spectrum was immediately recorded. For the experiments carried out under air-saturated conditions, the DMPO/HCl mixture was added directly to the reaction mixture and this mixture was then transferred to the EPR flat cell followed by immediate recording of the EPR spectrum.

In experiments involving the reaction between H_2O_2 and NO_2^-, sodium nitrite (1×10^{-4} M, final concentration) was added to a H_2O_2 (1×10^{-4} M) solution containing DMPO (0.2 M). The reaction mixture was immediately acidified to pH 3–3.5 with an aliquot of HCl. Ethanol at various final concentrations (0.01 M–1.7 M) was added to the reaction mixture prior to the addition of sodium nitrite and acid to determine $^{\cdot}OH$ scavenging by ethanol.

All EPR spectra were recorded on a Varian E-109 X-band spectrometer at 100 kHz magnetic field modulation. The magnetic field was set at: 338.0 mT; microwave frequency: 9.510 GHz; microwave power: 20 mW; modulation amplitude: 0.1 mT (unless otherwise indicated); time constant: 0.5 s; scan time: 4 min.; scan range: 10.0 mT. Spectral parameters were obtained by computer simulation generating theoretical EPR spectra matching the experimental spectra.

RESULTS AND DISCUSSION

The reaction of H_2O_2 with NO_2^- is known to generate $OONO^-$ which, in an acidic environment, reportedly decomposes forming $^{\cdot}OH$ and $NO_2^{\cdot}$.[12] In order to verify the production of these radical species, solutions of $NaNO_2$ and H_2O_2 were mixed (1×10^{-4} M) in the presence of DMPO (0.2 M). The reaction was carried out at pH 7–8 and pH 3–3.5 after acidifying with HCl. The EPR spectrum in Figure 1A shows that at neutral pH no DMPO spin adducts were obtained. This result is consistent with the slower decomposition of $OONO^-$ at higher pH values. However, when a similar reaction mixture is acidified (pH 3–3.5) with HCl, the EPR spectrum in Figure 1B is obtained. This EPR spectrum consists of the superposition of the EPR spectra corresponding to two DMPO spin adducts. One consists of a 1:2:2:1 quartet which is computer simulated using hyperfine coupling constants, $a_N = a_H^\beta = 1.49$ mT. This EPR spectrum corresponds to the DMPO-OH spin adduct.[18] The second DMPO spin adduct yields an EPR spectrum consisting of a triplet of triplets, indicating the interaction of a secondary nitrogen nucleus with the unpaired nitroxide electron. The computer simulation that matches this EPR spectrum is obtained using hyperfine coupling constants, $a_N = 1.415$ mT and $a_N^\beta = 0.35$ mT. Figure 1C shows the overall computer simulation that matches the experimental EPR spectrum in Figure 1B. Since the EPR spectrum consisting of a triplet of triplets (Figure 1B) indicates the addition of a nitrogen centered radical to DMPO, it is possible that this spin adduct originates from the direct addition of $NO_2^{\cdot}$ or an $NO_2^{\cdot}$ by-product to DMPO. It is important to note that in the EPR spectrum (triplet of triplets) there is no evidence of a β-hydrogen interaction with the unpaired nitroxide electron. This interaction is usually observed in the EPR spectra of most DMPO adducts when the proton at the C (2) DMPO position is present. This observation may suggest the loss of the β-hydrogen from a DMPO adduct after the addition of a nitrogen center, possibly NO_2 or one of its by-products, at the DMPO C(2) position. It is also possible that the β-hydrogen couplings are too small to be observed in the EPR spectrum (triplet of triplets) because of a small interaction between the β-hydrogen nucleus and the unpaired nitroxide electron. Furthermore, the observation of the EPR spectrum corresponding to DMPO-OH (Figure 1B) does not necessarily mean that this spin adduct originates entirely from the production of $^{\cdot}OH$.

Two experiments using ethanol as a $^{\cdot}OH$ scavenger were done in order to verify the production of $^{\cdot}OH$ following decomposition of HOONO. In the first experiment the effect of pH on the spin adduct formation was studied (Figure 2). The second experiment consisted of varying the concentration of ethanol, relative to DMPO, in the reaction mixture at the pH value determined as optimal for spin adduct formation (Figure 3). Figure 2A illustrates the results obtained in a control experiment in which ethanol (1.7 M, final concentration) was added to a solution containing H_2O_2 (1×10^{-4} M) and DMPO (0.2 M) followed by a pH reduction to pH 3.5. The EPR spectrum indicates that no DMPO adducts were formed. A similar result was obtained when an analogous control experiment was done at neutral pH. Reduction of the pH to pH 5 when NO_2^- (1×10^{-4} M, final concentration) is added to the reaction mixture containing H_2O_2, ethanol and DMPO, yields the EPR spectrum in Figure 2B. This EPR spectrum consists of the superposition of the EPR spectra of three DMPO spin adducts. Furthermore, the intensity of the more prominent EPR spectra increases in time consistent with the slower decomposition of HOONO as the pH is slightly decreased below neutrality. Although weak, the EPR

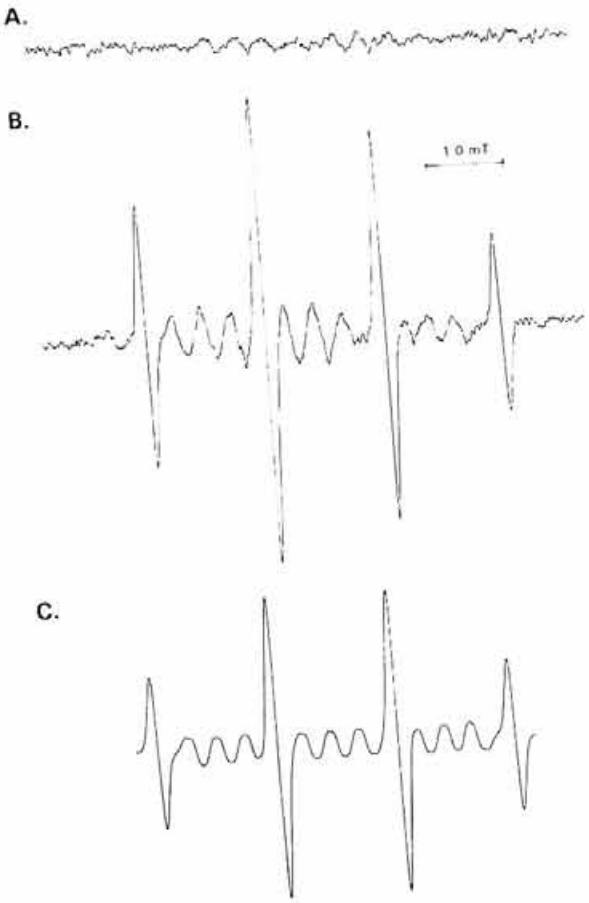


FIGURE 1 DMPO spin adducts obtained following the reaction of H_2O_2 with nitrite. A. pH 7–8. B. pH 3–3.5. C. Computer simulation of the EPR spectrum in B. Receiver gain: 2.5×10^4.

spectrum corresponding to the triplet of triplets observed in Figure 1 is also evident in the low-field and center-field sections of the EPR spectrum in Figure 2B. The more prominent EPR spectra in Figure 2B consist of a 1:2:2:1 quartet which corresponds to the DMPO-OH spin adduct, in addition to, a triplet of doublets. The triplet of doublets and the DMPO-OH EPR spectra are formed more rapidly and to a greater extent when the pH of the reaction mixture is lowered to pH 3.5 (Figure 2C). In this case, the triplet of doublets is computer simulated (Figure 2D) using hyperfine coupling constants, $a_N = 1.58\,mT$ and $a_H^\beta = 2.28\,mT$. These hyperfine coupling constants are consistent with those reported for the DMPO spin adduct formed in the reaction between ethanol and $^{\cdot}OH$.[18] As in Figure 2B, the additional weak EPR signals observed in the low-field and center-field sections of the EPR spectrum in Figure 2C correspond to the triplet of triplets observed in Figure 1. The results obtained in Figures 2B and 2C indicate that the DMPO-OH EPR spectrum persists although sufficient ethanol (1.7 M) is present to scavenge all $^{\cdot}OH$ produced. There

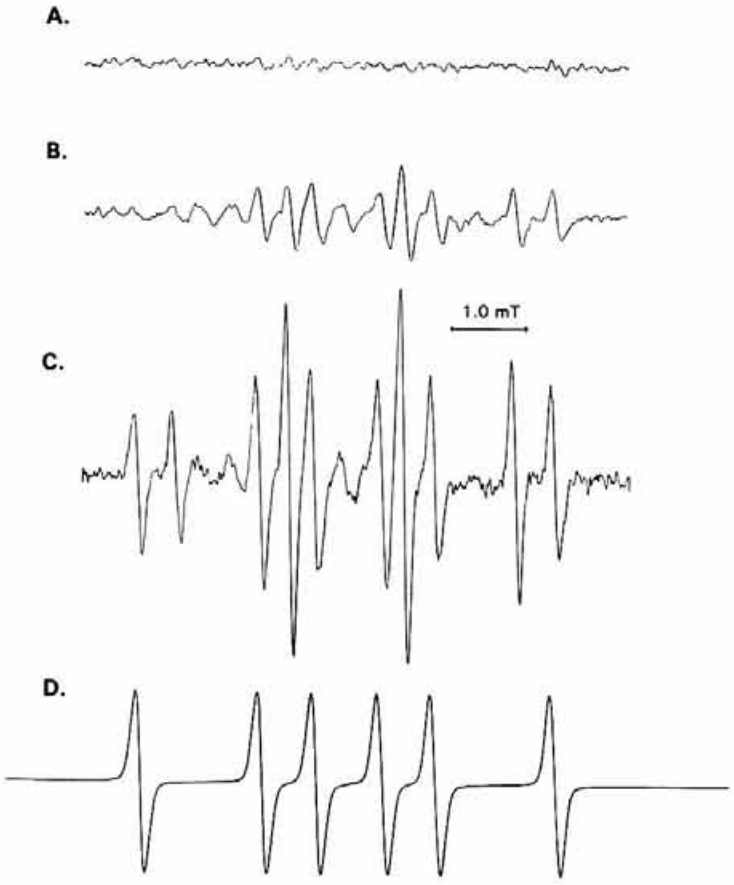


FIGURE 2 DMPO adducts obtained following the reaction of H_2O_2 with nitrite in the presence of ethanol. pH dependance of the decomposition of HOONO. A. Control; B. pH 5; C. pH 3–3.5; D. Simulation of the DMPO-hydroxyethyl adduct. Receiver gain: 5×10^4; modulation amplitude: 0.05 mT.

are two possible explanations for the observed results: (1) ·OH is not produced in the decomposition of HOONO and the DMPO-hydroxyethyl adduct originates by mechanisms not involving reaction of ·OH with ethanol; and (2) a fraction of the DMPO-OH EPR spectrum observed in Figure 1B originates through mechanisms which do not involve direct addition of ·OH to DMPO. Of these alternatives, the latter seems more probable. Beckman *et al.*[11] have shown, during the decomposition of HOONO, the formation of formaldehyde and malondialdehyde from dimethylsulfoxide and deoxyribose, respectively. These products are known to originate in reactions of ·OH with dimethylsulfoxide or deoxyribose.

When reaction mixtures similar to those described for experiments in Figure 2 were prepared with varying concentrations of ethanol (0.01–1.7 M), the DMPO-hydroxyethyl adduct increases as a function of the increase in ethanol concentration (Figure 3). This is consistent with the formation of ·OH in the reaction mixture. The EPR spectra shown in Figure 3A–3D are similar to those described in Figures 1 and 2. Several features must be noted in the EPR spectra shown in Figure 3. First, all

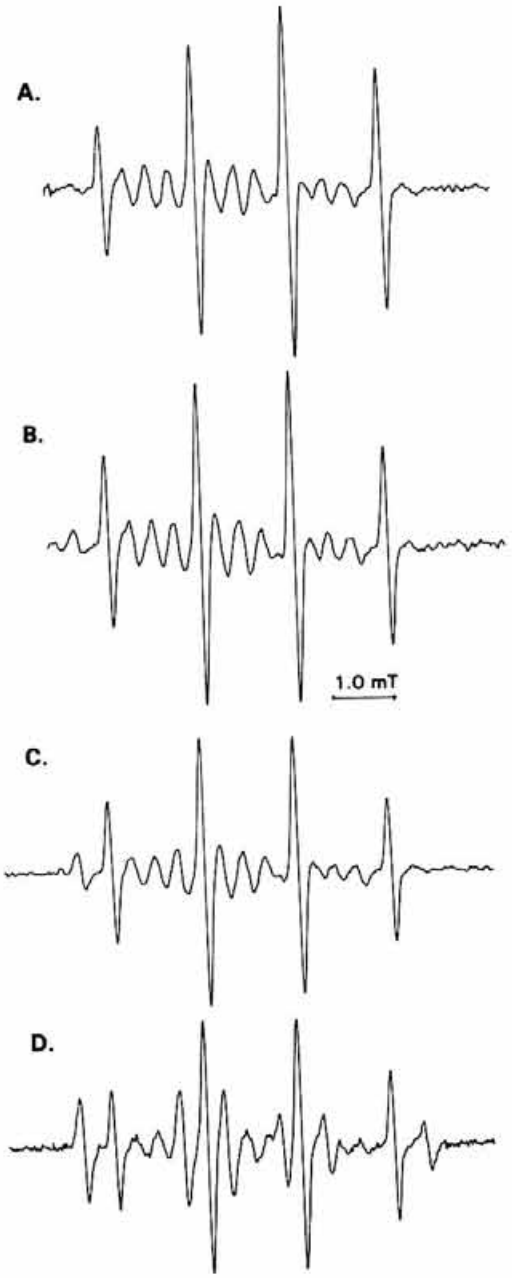


FIGURE 3 DMPO-hydroxyethyl adduct formation as a function of the concentration of ethanol. A. 0.01 M ethanol; B. 0.1 M ethanol; C. 0.8 M ethanol; D 1.7 M ethanol. Receiver gain: 4×10^4 (for A and B) and 2.5×10^4 (for C and D).

DMPO-hydroxyethyl adduct is produced instantly in the reaction mixture as evident by its decay in time during the course of the spectral recording. This observable decay is due to the shorter spin adduct lifetime at low pH. If the DMPO-hydroxyethyl adduct is formed by a mechanism not involving the reaction of $^{\cdot}OH$ with ethanol, the observed decay would not necessarily occur due to the large excess of ethanol compared to H_2O_2 and NO_2^- in the reaction mixtures. At its lowest concentration (0.01 M), ethanol is in a 100-fold excess. Therefore, if the formation of the DMPO-hydroxyethyl adduct does not originate from the $^{\cdot}OH$ reaction with ethanol, it should be expected that the EPR intensity of the DMPO-hydroxyethyl adduct in all spectra in Figure 3 would be similar. On the other hand, the DMPO-OH EPR spectra observed in Figure 3 show a tendency to increase or decay during the spectral recording. This suggests that there are contributions to the DMPO-OH EPR spectra originating from mechanisms not involving the direct addition of $^{\cdot}OH$ to DMPO. A final important feature can be determined from the EPR spectrum in Figure 3D. This spectrum was recorded in an identical fashion as the spectrum in Figure 1B. The reaction mixtures differed only in the presence of excess ethanol (1.7 M, Figure 3D) and in its absence (Figure 1B). In comparing these spectra it is evident that the DMPO-OH EPR signal has decreased by approximately 40% in the presence of ethanol. This observation suggests that approximately 40% of the DMPO-OH observed in the EPR spectrum in Figure 1B originates from the addition of $^{\cdot}OH$ to DMPO. The conclusion drawn from the experiments described in Figures 1–3 strongly suggest that $^{\cdot}OH$ is produced during the decomposition of HOONO. Furthermore, the production of $^{\cdot}OH$ also suggests that the mechanism of HOONO decomposition occurs via the reaction described in Eqn. 5.

It has been suggested that the reaction of $NO^{\cdot}$ with $O_2^{\cdot-}$ forms the $OONO^-$ intermediate.[8,9] Furthermore, this reaction has previously been reported to occur at high pH.[9] This is advantageous due to the minimal dismutation of $O_2^{\cdot-}$ at high pH values. Three basic experiments were done to study the reaction between $NO^{\cdot}$ and $O_2^{\cdot-}$. The reactants were mixed at pH 12.3 and then the pH was immediately lowered to pH 3–3.5. Besides preventing the dismutation of $O_2^{\cdot-}$, mixing the reactants at high pH also prevents any peroxynitrite formed from decomposing prior to addition of DMPO. In the first experiment $NO^{\cdot}$ gas was dissolved in air-saturated and deaerated solutions followed by the addition of DMPO. In the second experiment, $O_2^{\cdot-}$ was added to the deaerated dissolved $NO^{\cdot}$ solutions prior to the addition of DMPO. The third experiment consisted of adding $O_2^{\cdot-}$ to a deaerated reaction mixture containing dissolved $NO^{\cdot}$ and ethanol. The spin trapping results obtained suggest that at pH 12.3 the $NO^{\cdot}/O_2^{\cdot-}$ reaction mixture does not produce a significant quantity of $OONO^-$. Instead, the results suggest that dissolved $NO^{\cdot}$ generates additional reactive species that act as nitrating agents in the presence of DMPO. Figure 4 shows the results obtained when DMPO (0.2 M, final concentration) is added to an air-saturated (Figure 4A) or to a deaerated (Figure 4B) solution of dissolved $NO^{\cdot}$ at pH 12.3 and the pH is immediately lowered (HCl) to pH 3–3.5. The EPR spectra in Figure 4A and 4B are similar and show the formation of identical spin adducts. The presence of oxygen in the solution had no effect on the type of spin adducts formed. $NO^{\cdot}$ is known to react with oxygen producing $NO_2^{\cdot}$. The results in Figure 4 suggest that dissolved $NO^{\cdot}$ and $NO_2^{\cdot}$ yield the same spin adducts in the presence of DMPO or that, during its dissolution, the $NO^{\cdot}$ yields other products which may then interact with DMPO. The latter possibility is not unlikely since various reactions of $NO^{\cdot}$ with bases in alkaline solutions have been previously reported.[19] The EPR spectra in Figures 4A and 4B consist of the super-

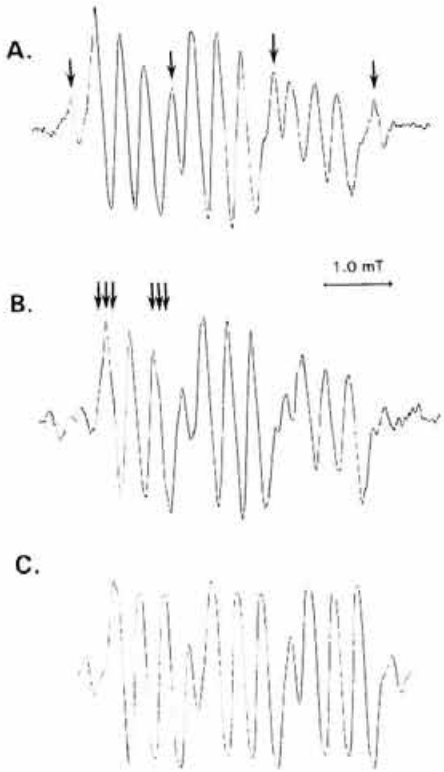


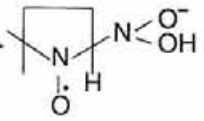
FIGURE 4 DMPO adducts formed in air-saturated and deaerated solutions of $NO^{\cdot}$. $NO^{\cdot}$ solutions were prepared at pH 12.3 and the EPR spectra were recorded at pH 3–3.5 immediately after addition of DMPO. Receiver gain: 5×10^4.

position of the EPR spectra of two DMPO spin adducts. One of the DMPO spin adducts yields an EPR spectrum consisting of a 1:2:2:1 quartet (Figure 4A, arrows) and corresponds to the DMPO-OH spin adduct. The second DMPO spin adduct EPR spectrum consists of a triplet of triplets indicating that the unpaired nitroxide electron is interacting with the nucleus of a secondary nitrogen. This EPR spectrum is computer simulated with identical parameters ($a_N = 1.415$ mT and $a_N^{\beta} = 0.35$ mT) as the triplet of triplets in Figure 1B. Furthermore, similar to the triplet of triplets in Figure 1B, the triplet of triplets in Figure 4A does not show evidence of a β-hydrogen interaction. However, additional triplet splittings are observable in Figure 4A and are enhanced in Figure 4B (arrows). These additional lines possibly correspond to the splitting of each line, in the triplet of triplets, into a 1:2:1 triplet due to the interaction of the two DMPO γ-hydrogens with the unpaired nitroxide electron.

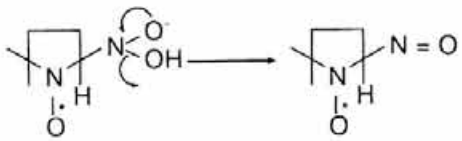
The formation of the observed spin adduct (triplet of triplets) in Figures 4A and 4B can be explained as follows. First, a reactive radical intermediate is formed from the dissolved $NO^{\cdot}$ [Eqn. (10)] followed by the reaction of this intermediate with DMPO to form an unstable adduct [Eqn. (11)].

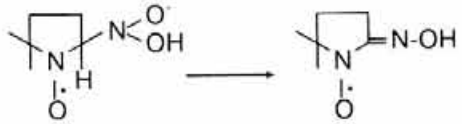
In the presence of acid, this unstable adduct rapidly rearranges into the observed spin adduct [Eqns. (12) and (13)].

(10) $NO^{\cdot} + OH^{-} \longrightarrow HO\dot{N}O^{-}$

(11) $HO\dot{N}O + DMPO \longrightarrow$ 

SCHEME 10-11

(12) 

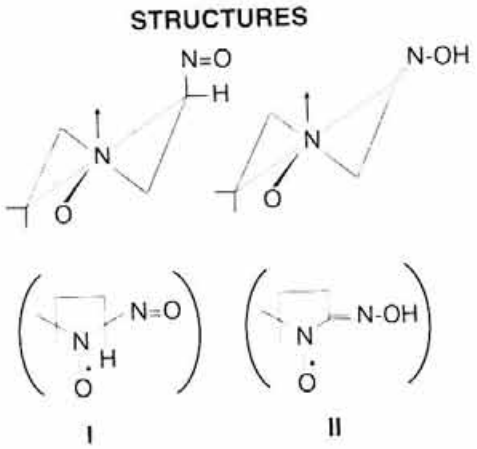
(13) 

SCHEME 12-13

As in the reactions between bases and NO$^{\cdot}$ forming a reactive radical intermediate, BNO,[19] Eqn. (10) involves the reaction of NO$^{\cdot}$ with the hydroxide anion (OH^{-}) yielding a hydrated radical intermediate, possibly $HONO^{-}$. This is possible because in the experiments described in Figures 4A and 4B, OH^{-} is the only base present when dissolving the NO$^{\cdot}$ gas. Therefore, it is possible that in alkaline solutions the dissolved NO$^{\cdot}$ exists as a hydrated radical species in which the unpaired electron is localized on the nitrogen and not delocalized as in NO$^{\cdot}$. It could also be argued that because the unpaired electron is localized in the case of $HO\dot{N}O^{-}$, this species is more reactive than NO$^{\cdot}$ toward DMPO.

The initial DMPO adduct formed [Eqn. (11)] is identical to an unstable intermediate occuring in the reduction of organo nitro compounds.[20,21] Since the analogous organo nitro intermediate rapidly rearranges into a nitroso compound, it would be expected that the initial DMPO adduct would rearrange in the same manner [Eqn. (12)]. It must be noted that in the presence of reducing agents including some metals (eg. Zn, Sn, Fe), the nitroso intermediates formed during the reduction of organo nitro compounds are unstable yielding the corresponding hydroxylamine and subsequently the amine.[20,21] Although possible, the further rearrangement of the nitroso derivative [Eqn. (12)] originating from the initial DMPO adduct in Eqn. (11) appears unlikely because of the absence of metals and common reducing agents in the reaction mixture. Since no β-hydrogen couplings are observed in the EPR spectra in Figures 4A and 4B, it is also conceivable that the initial DMPO adduct [Eqn. (11)] dehydrates and rearranges according to Eqn. (13).

There are two immediate reasons to explain the absence of observable β-hydrogen couplings in the EPR spectra in Figures 4A and 4B. One is that the adduct contains



STRUCTURES I AND II

a β-hydrogen but there is no interaction between the nucleus of this hydrogen and the unpaired nitroxide electron. If this interaction exists it is very small possibly falling within the linewidth of the observed spectra. The other is that the DMPO spin adduct contains no β-hydrogen to interact with the unpaired nitroxide electron.

These alternatives, described as DMPO-NO and DMPO = NOH, are illustrated in Structures I and II which show the more stable chair conformation of DMPO. However, although the EPR spectra (Figure 4) confirm the addition of a nitrogen center to DMPO, the exact nature of the nitrogen substituents cannot be determined from the observed EPR spectra. Structure I contains a β-hydrogen but this hydrogen is in (or close to) an equatorial position. Structure II does not contain a β-hydrogen and, therefore, would allow only the β-nitrogen couplings to be observed by EPR. When the β-hydrogen is close to being in or is in the plane (equatorial position) of the DMPO ring and perpendicular to the nitrogen π-orbital (solid arrow) containing the unpaired nitroxide electron, the interaction of this electron with the nucleus of the β-hydrogen would be at a minimum or would not exist. Therefore, it should be expected that in the conformation shown in Structure I no β-hydrogen couplings would be evident in the spin adduct EPR spectra as is the case in Figures 4A and 4B.

The hyperfine coupling constants, especially the primary nitrogen couplings, should play an important role in distinguishing between the adducts described in Structures I and II. For instance, the electron withdrawing nature of the doubly bonded nitrogen substituent (Structure II) would decrease the interaction of the unpaired nitroxide electron with the nucleus of the nitroxide nitrogen. Furthermore, it could be expected that the electron withdrawing capability of the doubly bonded nitrogen substituent, Structure II, should be similar to the electron withdrawing capability of the doubly bonded oxygen in the acylated DMPO derivative often referred to as DMPOX.[18] Since the reported nitrogen hyperfine coupling constant for the DMPOX is, $a_N = 0.72$ mT, a similar primary nitrogen hyperfine coupling constant could be predicted for a DMPO=NOH adduct in Structure II. This value is approximately half of the normally measured primary nitrogen couplings and, furthermore, is approximately 50% of the primary nitrogen coupling

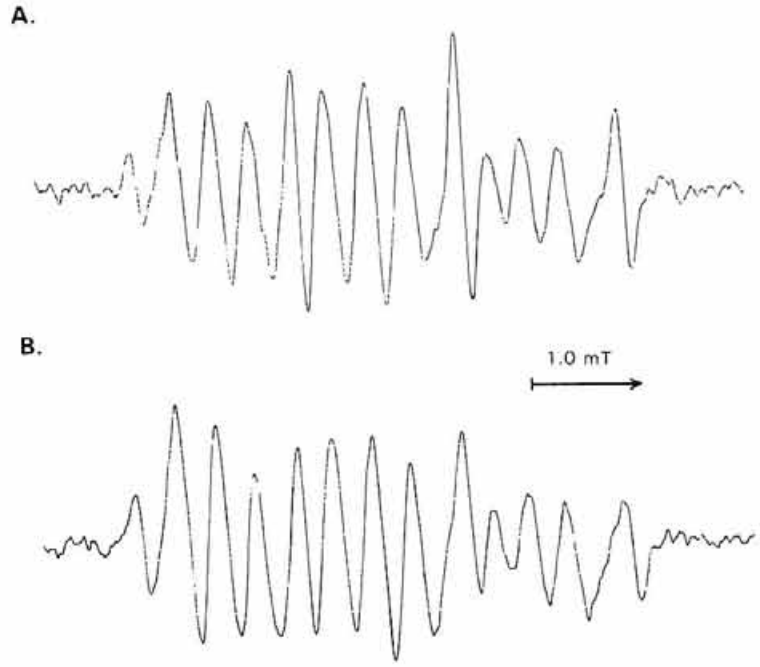


FIGURE 5 Same as Figure 4 with the exception that the experiment was carried out under deaerated conditons in the presence of superoxide. (A) EPR spectrum was recorded immediately after addition of superoxide; (B) Solution was purged (30 min.) after addition of superoxide and prior to addition of DMPO. Receiver gain: 5×10^4.

(a_N = 1.415 mT) measured for the EPR spectra in Figures 4A and 4B. Therefore, the results suggest that the EPR spectra (triplet of triplets) observed in Figures 4A and 4B correspond to the spin adduct conformation in Structure I, possibly DMPO-NO. The spectral parameters obtained for the triplet of triplets observed in Figure 1B are identical to the parameters for the EPR spectra in Figure 4. This suggests that the spin adducts yielding the EPR spectrum consisting of a triplet of triplets in Figure 1B and Figure 4 are similar. The nitrogen centered adduct (triplet of triplets) in Figure 1B could originate from the direct addition of $NO_2^{\cdot}$ to DMPO. It could also originate from the nitration of DMPO in the presence of nitric acid formed in the decomposition of peroxynitrous acid.[22] In either case the initial DMPO adduct formed could rearrange, in a similar fashion as the organo nitro intermediates, to form an identical DMPO adduct to the one observed in the EPR spectra in Figure 4.[20,21]

When $O_2^{\cdot-}$ is added to a deaerated solution at pH 12.3 containing dissolved $NO^{\cdot}$ and the pH is lowered to pH 3–3.5 in the presence of DMPO (0.2 M), the EPR spectrum in Figure 5A is obtained. Similar to the EPR spectrum in Figure 4B, the EPR spectrum in Figure 5A consists of a triplet of triplets (a_N = 1.415 mT and a_N^{β} = 0.35 mT) superimposed on the EPR spectrum corresponding to the DMPO-OH adduct. The triplet of triplets in Figures 4B and 5A are also similar in intensity which may suggest that the reaction between $O_2^{\cdot-}$ and dissolved $NO^{\cdot}$ to yield $OONO^-$ did not significantly occur. However, it must be noted from the experiments

described in Figure 1, an EPR spectrum consisting of a triplet of triplets was formed during the decomposition of peroxynitrous acid. Furthermore, the triplet of triplets in Figures 1, 4 and 5 have identical spectral parameters suggesting that the spin adducts are similar. Therefore, it is also possible that $O_2^{\cdot-}$ reacts with the dissolved NO$^{\cdot}$ at pH 12.3 forming peroxynitrite which, subsequently decomposes when acidified, yielding an EPR triplet of triplets which is responsible for, or contributes to, the overall EPR spectral intensity in Figure 5A. Another feature of interest in Figure 5A is the DMPO-OH EPR spectrum which is substantially larger than the DMPO-OH EPR spectrum in Figure 4B. The difference between the experiments described in Figures 4B and 5A is the addition of $O_2^{\cdot-}$ to the reaction mixture yielding the EPR spectrum Figure 5A. The larger DMPO—OH component in Figure 5A can originate from two sources. First, the experiments in Figures 1–3 have shown that a significant quantity of DMPO—OH is produced during the decomposition of peroxynitrous acid. The second source of DMPO—OH formation in Figure 5A could be the presence of $O_2^{\cdot-}$ in the reaction mixture, since the dismutation of $O_2^{\cdot-}$ at high pH is slow. The addition of $O_2^{\cdot-}$ to DMPO produces DMPO-$O_2^{\cdot-}$ which rapidly decomposes into DMPO-OH. If the DMPO-OH observed in Figure 5A originates from the decomposition of peroxynitrous acid after protonation of peroxynitrite, formed at pH 12.3 in a reaction between $O_2^{\cdot-}$ and dissolved NO$^{\cdot}$, no significant change in the EPR spectrum would be expected when the reaction mixture is allowed to stand for a period of time prior to the addition of DMPO and decreasing the pH of the solution. Peroxynitrite, if formed, is stable at high pH and its concentration will be the same whether its decomposition, in the presence of acid and DMPO, is carried out immediately or after a period of time. On the other hand, the dismutation of $O_2^{\cdot-}$ at high pH will occur even though this dismutation reaction is slow. Therefore, if the DMPO-OH observed in Figure 5A originates from the addition of $O_2^{\cdot-}$ to DMPO, a significant change in the DMPO-OH EPR spectrum is expected when the reaction mixture is allowed to stand for a period of time prior to addition of DMPO and decreasing the pH of the solution. Figure 5B shows the EPR spectrum obtained after a similar reaction mixture to the one described in Figure 5A, is allowed to stand for 30 min. before addition of DMPO (0.2 M) and decreasing the pH to pH 3.5. The solution containing dissolved NO$^{\cdot}$ and $O_2^{\cdot-}$ was continuously purged with nitrogen prior to mixing with DMPO and decreasing its pH. Although the triplet of triplets in Figure 5B is similar in intensity to the one in Figure 5A, the DMPO-OH EPR spectrum in Figure 5B has significantly decreased when compared to the DMPO-OH EPR spectrum in Figure 5A. This result suggests that the DMPO-OH observed in Figure 5 originates mainly from the decomposition of DMPO-$O_2^{\cdot-}$ and not from the decomposition of peroxynitrous acid.

The above observations support the idea that the dissolved NO$^{\cdot}$ at pH 12.3 forms another intermediate, which in this case, would be unreactive toward $O_2^{\cdot-}$. In order to verify this point, an experiment was done in which $O_2^{\cdot-}$ was added to the deaerated solution at pH 12.3 containing dissolved NO$^{\cdot}$ and an excess ethanol (1.7 M). DMPO (0.2 M) was added to the reaction mixture immediately prior to lowering the pH to pH 3–3.5. Similar to the experiments in Figures 2 and 3, ethanol was used as an $^{\cdot}$OH scavenger. The results are shown in Figure 6. Figure 6A is the result obtained in the control experiment done in the absence of $O_2^{\cdot-}$. Figure 6B is the result obtained when $O_2^{\cdot-}$ was added to the reaction mixture. The EPR spectra in Figures 6A and 6B are similar in intensity and consist mainly of the triplet of triplets observed previously. Also present to a minor extent is the EPR spectrum corresponding to the DMPO-OH. The EPR spectrum in Figure 6B shows no evidence of an increase in DMPO-OH

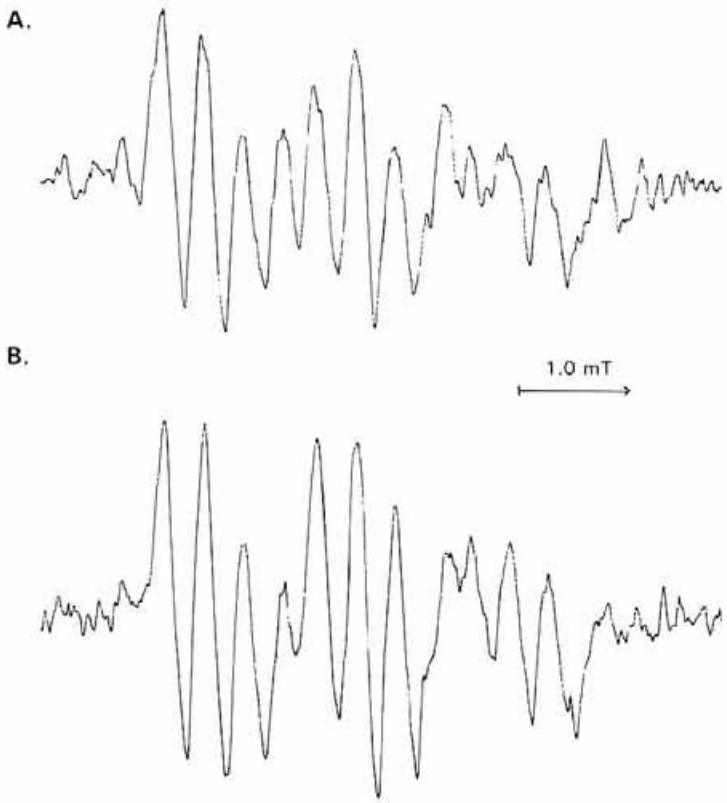


FIGURE 6 Same as Figure 4 with the exception that the experiment was carried out in the presence of ethanol (A) and in the presence of ethanol and superoxide (B). Receiver gain: 1.25×10^5.

spectral intensity nor any indication of the formation of the DMPO-hydroxyethyl adduct. Similar to the experiments described in Figures 1–3, it would be expected that if $OONO^-$ was formed in the experiments described in Figures 5 and 6B, the decomposition of peroxynitrous acid, formed after lowering the pH of the reaction mixture, would yield $^{\cdot}OH$ which in the presence of ethanol leads to the formation of the DMPO-hydroxyethyl adduct. These results suggest that the presence of $O_2^{\cdot-}$ in the reaction mixture had no effect on the spin adducts formed. Instead, the results support the conclusion that in alkaline solutions $NO^{\cdot}$ generates another intermediate [Eqn. (11)] which is apparently unreactive toward $O_2^{\cdot-}$. One observation that should be made regarding the EPR spectra in Figure 6, is that these spectra, although similar in intensity, are approximately 50% lower in intensity than the EPR spectra in Figures 4 and 5. It is possible that some of the dissolved $NO^{\cdot}$ has reacted with the ethanol. The reaction of $NO^{\cdot}$ with ethanol (Traube reaction) in alkaline solutions, yielding methylenediisonitramine, by a mechanism involving a BNO-type radical intermediate has been reported.[19] Although in the EPR spectra in Figure 6 there is no indication of the formation of additional radical species due to the presence of ethanol, this does not necessarily mean that these

intermediates were not formed. DMPO may not be an adequate spin trap for these radical intermediates originating from the reaction between ethanol and dissolved NO$^{\cdot}$, or these may have rearranged into their non-radical products prior to the addition of DMPO to the reaction mixture. However, the results shown in Figure 6 indicate that after the addition of ethanol to the solution containing dissolved NO$^{\cdot}$, there is still sufficient dissolved NO$^{\cdot}$ remaining to react with $O_2^{\cdot-}$. Furthermore, since the reaction mixtures contained an excess ethanol, there is more than sufficient ethanol remaining in the solution in order to scavenge $^{\cdot}$OH produced, in the decomposition of peroxynitrous acid, when the pH of the mixture is lowered to pH 3–3.5.

In conclusion, the experiments described in Figures 4–6 suggest that in alkaline solutions dissolved NO$^{\cdot}$ yields an additional species, possibly a hydrated radical intermediate. This species readily reacts with DMPO but appears to be unreactive toward $O_2^{\cdot-}$. The possibility that such a hydrated radical intermediate exists in equilibrium with dissolved NO$^{\cdot}$ at physiological pH is being investigated.

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