Spin Trapping. Kinetics of the Reaction of Superoxide and Hydroxyl Radicals with Nitrones

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Abstract: The pH dependence of the rate constant for superoxide trapping by 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) at 25 °C is examined. The results indicate that either $O_2^{-}\cdot$ or $HO_2\cdot$ can react with DMPO, at rates of 10 and 6.6×10^3 M^{-1} s^{-1}, respectively. Rate constants for superoxide trapping are determined by different methods, and the results are consistent. Mechanistic aspects of the spin-trapping reaction are discussed in light of the kinetic data. The kinetics of hydroxyl radical trapping by several nitrone spin traps and the stability of these adducts are also reported.

Introduction

The use of nitrone spin traps for the detection of superoxide and hydroxyl radical production in aqueous systems is becoming increasingly common, with new studies appearing regularly.[1-6] The proper design and implementation of such experiments requires an understanding of both the reactivity of these radicals with spin traps and the stability of the reaction product. At present, little is known about the nature of the reaction of superoxide with spin traps,[3] and there is little quantitative data on the stability of nitrone spin adducts in aqueous solution.[3,4]

The organic chemistry of superoxide is itself a relatively new area of investigation. Nucleophilic reactions of the superoxide anion in both aprotic solvents[7,8] and aqueous solution[9] have been reported. The reaction of superoxide with alkyl bromides in aprotic solvents has been most extensively studied, and an S_N2 reaction mechanism has been demonstrated.[10] Danen and Warner have reported that superoxide is a powerful nucleophile, having a lower E_a than many other common nucleophiles for reaction with *n*-butyl bromide in Me_2SO.[7] In contrast, there are few examples of superoxide undergoing covalent addition to a double bond,[7,10b,11] as it is presumed to do in spin trapping.[8]

In the original study of Harbour et al.[6a] on superoxide trapping by DMPO, the authors were unable to determine whether $O_2^{-}\cdot$ or $HO_2\cdot$ was the actual reactive species. Indeed, the ability of $O_2^{-}\cdot$ (as opposed to $HO_2\cdot$) to react with spin traps has recently been questioned.[5] The only available evidence that $O_2^{-}\cdot$ can react with DMPO is a study by Harbour and Hair[6b] in which KO_2 in crown ether–Me_2SO reacted with DMPO to yield DMPO–OOH. However, the possibility that $HO_2\cdot$ was the actual reactive species cannot be excluded since the solvents were not dried. Trapping of $O_2^{-}\cdot$ in aprotic solvents also does not necessarily indicate that it can be trapped in aqueous systems, because $O_2^{-}\cdot$ has a lesser reactivity in water than in aprotic solvents.[8]

The $O_2^{-}\cdot/HO_2\cdot$ adduct of DMPO is presumed to be a hydroperoxide, DMPO–OOH. Substantiative evidence for this structural assignment has been reported.[3] Buettner and Oberley have shown that the $O_2^{-}\cdot/HO_2\cdot$ adduct is unstable in aqueous solution and that its rate of decomposition is faster at lower pH.[4] In a recent study, we reported that DMPO–OOH spontaneously decomposes into DMPO–OH and a nonradical species.[3] The use of 2,5,5-trimethyl-1-pyrroline *N*-oxide (TMPO), which forms a more stable adduct, enabled us to measure the rate of reaction of $O_2^{-}\cdot/HO_2\cdot$ with TMPO and DMPO by kinetic competition. At pH 7.8, the rate constants were 7 and 10 M^{-1} s^{-1}, respectively. The question of whether the species reacting with the spin trap was $O_2^{-}\cdot$ or $HO_2\cdot$ was not investigated.

In this study the pH dependence of the rate constant for the reaction between $O_2^{-}\cdot/HO_2\cdot$ and DMPO is examined, in order to determine whether $O_2^{-}\cdot$ or $HO_2\cdot$ is the actual reactive species. The results suggest that either $O_2^{-}\cdot$ or $HO_2\cdot$ can react with DMPO, though the rate of reaction with $HO_2\cdot$ is much greater. A kinetic model is derived which describes the competition between the spin-trapping and dismutation reactions of superoxide. Rate constants for $O_2^{-}\cdot/HO_2\cdot$ trapping are determined by different methods, and the results are in reasonable agreement with each other. Mechanistic aspects of the spin-trapping reaction are discussed in light of the kinetic data. The kinetics of hydroxyl radical trapping by several nitrone spin traps and the stability of these adducts are also reported.

Experimental Section

General. The spin traps 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 2,5,5-trimethyl-1-pyrroline *N*-oxide (TMPO), and 2-carboxy-5,5-dimethyl-1-pyrroline *N*-oxide (CDMPO) were prepared according to the method of Bonnett et al.[12] Both TMPO and DMPO were purified by triple fractional vacuum distillation. DMPO prepared in this manner is a colorless solid, has a melting point of 25 °C and is virtually devoid of spurious EPR signals. The spin trap (α-4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was a gift from Dr. William Yamanashi, Department of Ophthalmology, Duke University. Xanthine oxidase was the generous gift of Dr. Irwin Fridovich, Department of Biochemistry, Duke University. Bovine erythrocyte superoxide dismutase (SOD), Type III cytochrome *c*, diethylenetriaminepentaacetic acid (DETAPAC), and xanthine were purchased from Sigma Chemical Company. Chelex 100 was purchased from Bio-Rad. All other chemicals were of reagent grade. All buffers were passed through a Chelex-100 column according to the method of Poyer and McCay[13] to remove trace metal impurities such as iron. The use of DETAPAC as a chelating agent further minimizes the

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effect of trace metal impurities, since studies have shown that DETAPAC renders Fe^{2+},[14] Cu^{2+}, and Mn^{2+}[15] ineffective as superoxide dismutases. Water was purified with a Continental water purification system and filtered through a 0.2-μm polycarbonate Nuclepore filter. Electron paramagnetic resonance spectra were recorded with a Varian Associates Model E-9 spectrometer. An Orion Research Model 601-A pH meter was used to measure pH values, using pH standards at pH 4 (0.05 M potassium biphthalate, Fisher Scientific) and pH 7 (0.05 M potassium phosphate, Fisher Scientific).

Xanthine–Xanthine Oxidase Superoxide Generating System. The system contained 400 μM xanthine, 1 mM DETAPAC, 50 mM phosphate buffer, pH 8.1, and xanthine oxidase such that the rate of superoxide formation was 9.75 μM/min at 25 °C. Superoxide formation was measured optically by the reduction of cytochrome *c* at 550 nm, using an extinction coefficient of 20 mM^{-1} cm^{-1}.[16] The concentration of TMPO used ranged from 0.016 to 0.16 M. The reaction was initiated by addition of xanthine oxidase. By monitoring the conversion of xanthine to uric acid at 290 nm, it was found that TMPO did not inhibit xanthine oxidase under these conditions. The formation of the TMPO–superoxide adduct was monitored by EPR as the low-field peak and was observed to increase linearly with time for several minutes. The production of the TMPO–superoxide adduct was completely inhibited by superoxide dismutase, but not by catalase, as expected. No radical could be trapped if any component of the system were missing, i.e., xanthine, xanthine oxidase, and TMPO were all necessary, and no signal was produced in the absence of any of these components.

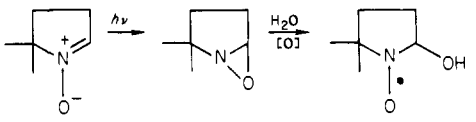
Use of Light–Riboflavin System in Spin-Trapping Studies. The types and intensities of spin adducts observed in a light–riboflavin–electron donor–DMPO system were dependent on the electron donor used and concentration of spin trap used. In general, three spin adducts could be observed, namely, DMPO–OOH, DMPO–OH, and a third species. The nature of the third species varied with the electron donor used. For example when DETAPAC was the electron donor, a species with A_N = 15.8 G and A_H = 22.0 G was present; whereas when cysteine was used as an electron donor, a spectrum with A_N = 15.3 G and A_H = 21.1 G was observed. This suggests that the third species was due to trapping of an electron donor derived radical. Consistent with our observations, it has been suggested that EDTA radicals can result from the oxidation of EDTA by light–riboflavin.[17]

When DETAPAC was used as an electron donor and at DMPO concentrations under 0.1 M, the observed spectra consisted mainly of DMPO–OOH and to a lesser extent DMPO–OH. At DMPO concentrations over 0.3 M, the DETAPAC radical adduct predominated, and the production of DMPO–OOH was inhibited.

When DMPO was incubated with light–riboflavin in the absence of an electron donor, spin-trapped adducts were still observed. The spectrum consisted mainly of DMPO–OH and to a much lesser extent DMPO–OOH. Superoxide dismutase completely prevented DMPO–OOH formation but did not affect DMPO–OH production, indicating that most of the DMPO–OH was not due to decomposition of DMPO–OOH.[3] Similarly, it was found that ethanol could not prevent DMPO–OH formation, indicating that DMPO–OH production was not due to hydroxyl radical trapping. (For comparison, the effect of ethanol on hydroxyl radical trapping by DMPO is shown in Figure 3.)

Although DMPO does not have a visible absorbance at either the fluorescence excitation or emission wavelengths of riboflavin, it could partially quench riboflavin fluorescence, indicating that direct energy transfer from riboflavin to DMPO was occurring. DMPO could also stimulate oxygen uptake by light–riboflavin, as determined polarigraphically. Unlike the stimulation of oxygen uptake caused by DETAPAC, the oxygen uptake caused by DMPO was of much lesser magnitude and exhibited a lag phase.

The behavior of the light–riboflavin–DMPO system may be explained by oxazirane formation, which has been previously reported to occur during light irradiation of DMPO,[18] as shown below. Ring opening of



the oxazirane would yield a hydroxylamine; oxidation of the hydroxylamine (either directly by oxygen or indirectly via the riboflavin) would produce DMPO–OH and $O_2^{-}\cdot$. The lag phase in oxygen uptake can also be explained by the intermediary formation of the hydroxylamine. An alternate explanation would be the direct oxidation of DMPO by excited flavin, although we consider this less likely, as this would not account for the lag in oxygen uptake.[19]

Therefore the behavior of this system is complex. The validity of using this system in the following kinetic study of $O_2^{-}\cdot$ trapping is indicated by the specific inhibition of DMPO–OOH production by superoxide dismutase and by the good agreement among rate constants obtained in this and other $O_2^{-}\cdot$ generating systems (Table I).

Table I. Apparent Rate Constants for Superoxide Trapping[a]

spin trap	rate constant, M^{-1} s^{-1}	conditions
TMPO	7	pH 7.8, xanthine-xanthine oxidase, competition with SOD[3]
TMPO	1.44	pH 8.1, xanthine-xanthine oxidase, competition with spontaneous dismutation of superoxide
DMPO	10	pH 7.8, xanthine-xanthine oxidase, competition with TMPO[3]
DMPO	15.7	pH 8.0, light–riboflavin–DETAPAC, competition with SOD

[a] Rates were determined by kinetic competition.

A light–riboflavin–DETAPAC superoxide-generating system, similar to that described by Buettner and Oberley,[4] was used for the kinetic experiments. A buffer containing 0.1 M potassium phosphate, 0.02 M DETAPAC, and 4.2 × 10^{-5} M riboflavin was used at pH values ranging from 5 to 9. The EPR cell was irradiated directly in the cavity, using a slide projector as a light source. No change in pH occurred with illumination. Superoxide dismutase was used as a competitive inhibitor of superoxide trapping. The choice of SOD as a suitable competitive inhibitor was based on the following reasons: (1) SOD has negligible visible absorbance at the concentrations used and thus does not interfere optically with the production of superoxide by this system. (2) Its rate constant for reaction with superoxide has been directly demonstrated to be independent of pH over the range 5.0 to 9.5.[20] (3) Due to the catalytic nature of its reaction, the concentration of active SOD remains constant during the experiment. (4) It has a high degree of substrate specificity for superoxide.[21]

The SOD solution used was standardized against a solution of cytochrome *c* at pH 7.8 according to the method of McCord and Fridovich.[16] A rate constant for the reaction of superoxide with cytochrome *c* of 6 × 10^5 M^{-1} s^{-1} at pH 7.8 was assumed.[22] It was also determined that no photoinactivation of SOD and that no inhibition of SOD by DETAPAC occurred in the light–riboflavin–DETAPAC system.

For the kinetic experiments, a DMPO concentration of 0.18 M was found to be optimal. The concentration of DMPO was fixed while the SOD concentration was varied. Although the DMPO–OOH adduct is relatively unstable,[3,4] the production of DMPO–OOH was linear with time for the first 10 to 20 s, depending on pH. This initial rate of DMPO–OOH formation was used in the calculations. SOD could completely inhibit DMPO–OOH formation, indicating that DMPO–OOH formation was due to superoxide trapping. SOD had no effect on the stability of preformed DMPO–OOH. Boiled SOD had no effect on the formation of DMPO–OOH at any pH value tested.

Studies on OH· Trapping. For the H_2O_2 photolysis experiments, a buffer containing 50 μM chelexed potassium phosphate (pH 7.4), 1 mM DETAPAC, 0.09 M DMPO, and 0.6% H_2O_2 was used. The EPR cell was irradiated in the cavity with an Ultraviolet Products Model SCT-1 UV light source. The formation of the DMPO–OH adduct was monitored as the low-field EPR peak for a period of 1 min. Under these conditions, the production of DMPO–OH was linear with time.

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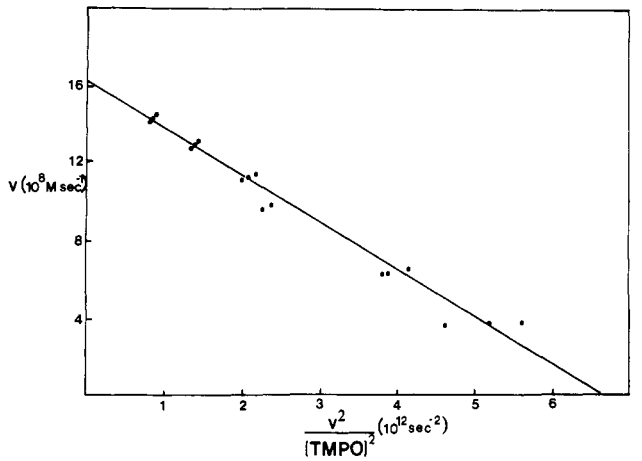

Figure 1. Effect of varying TMPO concentration on superoxide trapping. Superoxide was generated at a rate of 1.63×10^{-7} M/s by a pH 8.1 xanthine-xanthine oxidase system containing TMPO. The rate of EPR adduct formation (v) was dependent on the TMPO concentration. According to these data and eq 7, the apparent rate constant for superoxide trapping by TMPO is 1.44 M^{-1} s^{-1}.

The Fenton reaction system contained 50 mM chelexed potassium phosphate buffer (pH 7.4), 0.6% H_2O_2, 0.09 M DMPO, and 2×10^{-4} M $FeSO_4$.

No solvent effect on the height of the low-field peak of DMPO–OH was seen with the low ethanol concentrations used.

Results

Derivation of Rate Expression for Spin Trapping of Superoxide in the Xanthine–Xanthine Oxidase System. Two different kinetic derivations were developed to determine rate constants for superoxide trapping. The first derivation examines the effect of varying the spin-trap concentration on the rate of the spin-trapping reaction. The xanthine-xanthine oxidase system was used to generate superoxide for these experiments, because the rate of superoxide production by this system was found to be unaffected by the spin-trap concentration. In contrast, systems which produce superoxide by the reaction of hydroxyl radical with either hydrogen peroxide or formate will be affected by the spin-trap concentration, because the spin trap reacts much faster with hydroxyl radical than it reacts with superoxide. A disadvantage to the xanthine-xanthine oxidase system was that it was useful only over a limited pH range.

Therefore, for the pH-dependence studies, a light–riboflavin–DETAPAC superoxide-generating system was used. Superoxide generation by this system was found to be affected by spin-trap concentration, as described in the Experimental Section. Thus a different kinetic treatment was used, where the level of spin trap was fixed at a concentration high enough to trap essentially all of the superoxide being generated. The rate constant for superoxide trapping was then determined by the relative ability of superoxide dismutase to inhibit spin trapping.

Consider an aqueous superoxide-generating system containing the spin trap TMPO. Superoxide produced can decay either by spontaneous dismutation or by reaction with TMPO. As the spin-trap concentration is increased, the amount of superoxide trapped increases, while the amount decaying by spontaneous dismutation decreases. If both the rate of spin trapping and the rate of dismutation are known, the rate constant for TMPO spin trapping can be determined, as shown by the following derivation (eq 1–3). Superoxide is being produced at a constant rate, s, by the superoxide generating system.

$$HO_2\cdot \overset{K_a}{\rightleftharpoons} O_2^{-}\cdot + H^+ \quad (1)$$

$$H^+ + O_2^{-}\cdot + HO_2\cdot \rightarrow O_2 + H_2O_2 \quad (2)$$

$$H^+ + O_2^{-}\cdot + TMPO \rightarrow TMPO\text{–}OOH \quad (3)$$

Let k_d and k_t be pH-dependent apparent rate constants for superoxide dismutation and spin trapping, respectively.[23] Since a large excess of TMPO is used, the TMPO concentration is assumed to remain constant during the course of the experiment. Use of the steady-state assumption[24] that $d[O_2^{-}\cdot]/dt = 0$ leads to eq 4.

$$k_d[O_2^{-}\cdot]^2 + k_t[TMPO][O_2^{-}\cdot] - s = 0 \quad (4)$$

Solving the quadratic expression for $[O_2^{-}\cdot]$ gives eq 5.

$$[O_2^{-}\cdot] = ((k_t^2[TMPO]^2 + 4k_d s)^{1/2} - k_t[TMPO])/2k_d \quad (5)$$

Let the rate of spin trap adduct formation equal V (see eq 6).

$$V = k_t[TMPO][O_2^{-}\cdot] \quad (6)$$

Substituting for $O_2^{-}\cdot$, one obtains eq 7.

$$V = (k_t[TMPO])((k_t^2[TMPO]^2 + 4k_d s)^{1/2} - k_t[TMPO])/2k_d \quad (7)$$

Squaring both sides and rearranging gives eq 8.

$$V = s - (k_d V^2/k_t^2[TMPO]^2) \quad (8)$$

Thus according to eq 8 a plot of V vs. $V^2/[TMPO]^2$ should be linear with a slope of $-k_d/k_t^2$ and an intercept of s. Equation 8 can alternatively be expressed in a form which does not include apparent rate constants. Let $[TMPO] = T$ at $V = s/2$ (see eq 9).

$$V = s - (2T^2/s)(V^2/[TMPO]^2) \quad (9)$$

In a pH 8.1 xanthine–xanthine oxidase superoxide generating system, the rate of the TMPO–superoxide adduct formation was dependent on TMPO concentration, and the results were consistent with 8, as shown by Figure 1. In this figure, the extrapolated value of V at infinite TMPO concentration was set equal to the actual rate of $O_2^{-}\cdot$ formation, as measured by cytochrome c reduction. The straight line in this figure was determined by linear regression; the correlation coefficient was 0.986. The numerical value of the slope was -2.46×10^3 M·s. The value of T, the concentration of TMPO required to trap half of the superoxide, was 0.044 M.

Since the slope is equal to $-k_d/k_t^2$, k_t may be determined if k_d at pH 8.1 is known. By use of a value of k_d equal to 5.1×10^4 M^{-1} s^{-1}, calculated from the expression derived by Behar et al.[25] in their studies on the dismutation of superoxide, k_t becomes 1.44 M^{-1} s^{-1}.

The low rate constant for superoxide trapping means that in practice high spin-trap concentrations must be used to trap superoxide before it can decompose via spontaneous dismutation. This consideration was applied to the other kinetic experiments described in this paper.

pH Dependence of Superoxide Spin Trapping. The pH dependence on the rate constant for DMPO trapping of $O_2\cdot/HO_2\cdot$ was investigated to determine whether $O_2^{-}\cdot$ or $HO_2\cdot$ is the actual

(23) These apparent rate constants include the contributions of both $O_2^{-}\cdot$ and $HO_2\cdot$ to the rate equations. For example the rate of TMPO–OOH formation is equal to the rate of $O_2^{-}\cdot$ trapping plus the rate of $HO_2\cdot$ trapping, i.e., $(d[TMPO\text{–}OOH]/dt) = k_{O_2^-}[TMPO][O_2^{-}\cdot] + k_{HO_2}[TMPO][HO_2\cdot]$, but $[HO_2\cdot] = [O_2^{-}\cdot][H^+]/K_a$, thus $(d[TMPO\text{–}OOH]/dt) = (k_{O_2^-} + k_{HO_2}[H^+]/K_a)[TMPO][O_2^{-}\cdot]$ or $(d[TMPO\text{–}OOH]/dt) = k_t[TMPO][O_2^{-}\cdot]$. A similar derivation can be used to show that at any given pH, the rate of dismutation can be expressed as $k_d[O_2^{-}\cdot]^2$, where k_d is a function of pH. The use of apparent rate constants enables us to express both spin trapping and dismutation as a function of a common term, $O_2^{-}\cdot$, and thus to solve the combined rate equation explicitly.

(24) The validity of using a steady-state assumption for the $O_2^{-}\cdot$ concentration in a xanthine-xanthine oxidase system is supported by earlier work of McCord and Fridovich on cytochrome c reduction by xanthine oxidase. The data they obtained was consistent with a steady-state assumption for the $O_2^{-}\cdot$ concentration. See: McCord, J. M.; Fridovich, I. "Superoxide and Superoxide Dismutases", Michelson, A. M.; McCord, J. M.; Fridovich, I., Ed.; Academic Press: New York, 1977; pp 1–10, and McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1968**, *243*, 5753–5760.

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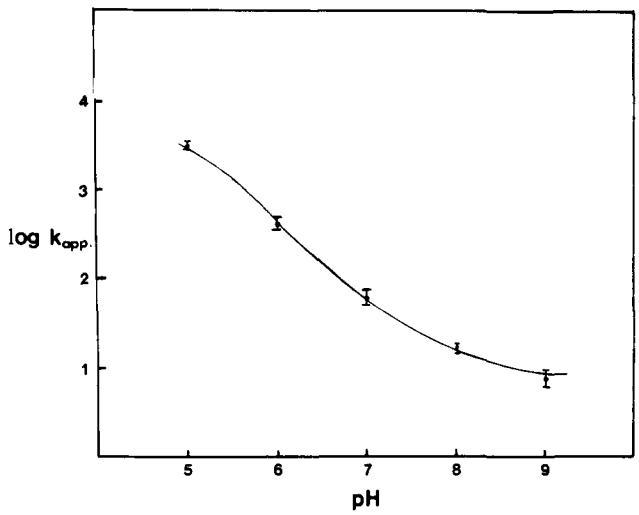

Figure 2. Effect of pH on the apparent rate constant for $O_2^{-}\cdot/HO_2\cdot$ trapping by DMPO. Superoxide was generated by a light–riboflavin–DETAPAC system. Rate constants were determined by the relative ability of superoxide dismutase to inhibit DMPO spin trapping. The solid line is a plot of eq 16 using values of 6.6×10^3 M^{-1} s^{-1}, 10 M^{-1} s^{-1}, and 4.88 for $k_{HO_2\cdot}$, $k_{O_2^{-}\cdot}$, and pK_a of superoxide, respectively. The circles are the experimentally determined rate constants.

reactive species. The light–riboflavin–DETAPAC system was used as a source of superoxide. The method of kinetic competition was used to determine the rate constant, with superoxide dismutase as the competitive inhibitor.[26] The reaction of superoxide with superoxide dismutase has been shown to be first order with respect to superoxide.[20] Thus, the following model (eq 10–14) describes two competing reactions which are both first order with respect to superoxide. The rate constant k_{app}, shown below, is a pH-dependent apparent rate constant which includes the contribution of both $O_2^{-}\cdot$ and $HO_2^{-}\cdot$ trapping. The concentration of DMPO used, 0.18 M, was high enough to trap essentially all of the superoxide in the absence of SOD.

$$H^+ + O_2^{-}\cdot + \text{DMPO} \xrightarrow{k_{app}} \text{DMPO–OOH} \qquad (10)$$

$$H^+ + O_2^{-}\cdot + \text{SOD} \xrightarrow{k_{SOD}} {}^1/_2O_2 + {}^1/_2H_2O_2 \qquad (11)$$

$$\frac{-d[O_2^{-}\cdot]}{dt} = k_{app}[\text{DMPO}][O_2^{-}\cdot] + k_{SOD}[\text{SOD}][O_2^{-}\cdot] \qquad (12)$$

$$\frac{d[\text{DMPO–OOH}]}{dt} = k_{app}[\text{DMPO}][O_2^{-}\cdot] \qquad (13)$$

Dividing eq 12 by eq 13, one obtains eq 14.

$$\frac{-d[O_2^{-}\cdot]/dt}{d[\text{DMPO–OOH}]/dt} = 1 + \frac{k_{SOD}[\text{SOD}]}{k_{app}[\text{DMPO}]} \qquad (14)$$

At a saturating level of DMPO and in the absence of SOD, the rate of spin trapping is equal to the rate of superoxide generation, $d[O_2^{-}\cdot]/dt$. Thus, let V and v represent the rate of spin trapping in the absence and presence of SOD, respectively (see eq 15).

$$V/v = 1 + (k_{SOD}[\text{SOD}]/k_{app}[\text{DMPO}]) \qquad (15)$$

The data were consistent with eq 15. The round circles in Figure 2 represent the experimentally determined apparent rate constants for reaction of DMPO with $O_2^{-}\cdot/HO_2\cdot$. The rate decreased sharply with increasing pH from pH 5 to pH 7, whereas the rates at pH 8 and 9 were similar. The variation in apparent rate constant with pH can be explained by assuming that DMPO

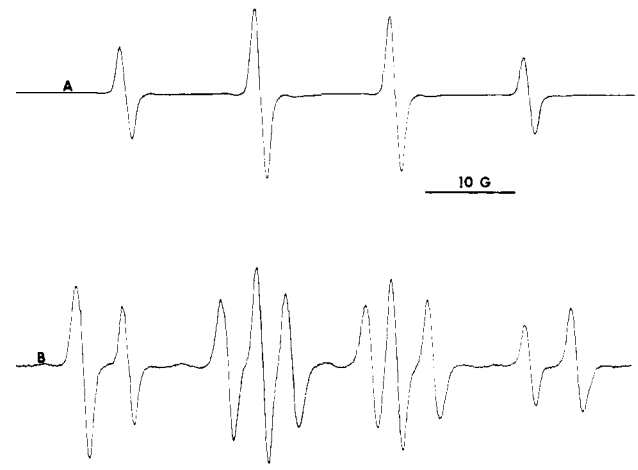

Figure 3. Effect of ethanol on hydroxyl radical trapping by DMPO. (A) ESR spectra produced by UV photolysis of a 0.6% H_2O_2 pH 7.4 phosphate buffer containing 0.09 M DMPO. The spectrum is of DMPO–OH, $A_N = A_H = 14.9$ G. (B) Conditions were the same as those for A, except that 0.33 M ethanol was present and the instrument gain setting was higher. Spectra are a combination of the hydroxyl and α-hydroxyethyl radical adducts. For the α-hydroxyethyl adduct, $A_N = 15.8$ and $A_H = 22.8$ G.

can react with $HO_2\cdot$ and $O_2^{-}\cdot$ at different rates.

Let $x = K_a/[H^+] = 10^{(pH-pK_a)}$, where K_a is the acid dissociation constant of the perhydroxyl radical. The apparent rate constant due to reaction of $HO_2\cdot$ with DMPO as a function of pH is then $k_{HO_2\cdot}/(1 + X)$; the apparent rate constant for reaction of $O_2^{-}\cdot$ with DMPO as a function of pH is $(X)k_{O_2^{-}\cdot}/(1 + X)$. The total apparent rate constant which includes the contributions of both $O_2^{-}\cdot$ and $HO_2\cdot$ is shown in eq 16.

$$k_{app} = (k_{HO_2\cdot} + (X)k_{O_2^{-}\cdot})/(1 + X) \qquad (16)$$

This equation provides a reasonable fit to the experimental data, when values of 6600 M^{-1} s^{-1}, 10 M^{-1} s^{-1}, and 4.88[25] are used for the values of $k_{HO_2\cdot}$, $k_{O_2^{-}\cdot}$, and pK_a, respectively. Comparison between experimental and theoretical points is shown by Figure 2. According to this kinetic model, the reaction between DMPO and $HO_2\cdot$ predominates below pH 7.7, whereas the reaction between DMPO and $O_2^{-}\cdot$ predominates above this value.

The relative pH independence of the spin-trapping reaction above pH 7.7 was further investigated in a kinetic competition experiment using SOD, the xanthine-xanthine oxidase superoxide generating system, and TMPO as the spin trap. The conditions used were identical with those previously described,[3] except that 50 mM glycyl-glycine was used as a buffer instead of phosphate. The data were analyzed by using eq 15. At pH 7.8 and 9.3, apparent rate constants for the reaction of $O_2^{-}\cdot/HO_2\cdot$ with TMPO were 3.2 and 2.7 M^{-1} s^{-1}, respectively, suggesting that the reaction of TMPO with the anionic form, $O_2^{-}\cdot$, also predominates above pH 7.8.[27]

A summary of rate constants for $O_2^{-}\cdot/HO_2\cdot$ trapping is given in Table I. The discrepancy between the values of 7 M^{-1} s^{-1} and 1.4 M^{-1} s^{-1} for reaction of TMPO with $O_2^{-}\cdot$ is probably due to the uncertainty associated with the assumed values of the rate constants.[28]

Kinetic Studies of ·OH Trapping. Two different ·OH generating systems were used, UV photolysis of H_2O_2[6a] and the Fenton reaction.[29] These systems have been used previously in studies on hydroxyl radical spin trapping.[5,6a] A sample experiment in the UV photolysis system is shown in Figures 3 and 4. Ethanol was used as the competitive inhibitor because it has little UV ab-

(26) A similar analysis has been used in other kinetic competition experiments involving superoxide. See: Asada, K.; Takahashi, M.; Nagate, M.; *Agric. Biol. Chem.* **1974**, *38*, 471–473, and Sawoda, Y.; Yamazaki, I.; *Biochim. Biophys. Acta* **1973**, *327*, 257–265.

(27) The reason for the discrepancy between the value of 3.2 M^{-1} s^{-1} at pH 7.8 measured by glycl-glycine and the value of 7 M^{-1} s^{-1} previously determined in phosphate buffer[3] is unknown, although these values were reproducible.

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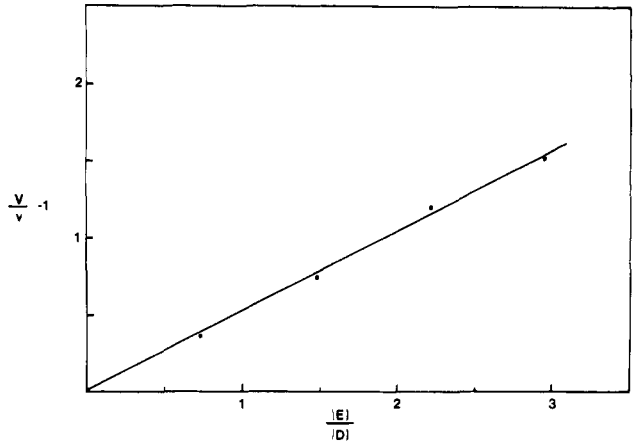

Figure 4. Inhibition of hydroxyl radical trapping by ethanol. Hydroxyl radical was produced by UV photolysis of a 0.6% H_2O_2 pH 7.4 phosphate buffer containing 0.09 M DMPO. The data were plotted according to the equation $V/v - 1 = k_E[E]/k_D[D]$, where V and v are the rates of hydroxyl radical trapping in the absence and presence of ethanol, k_E and k_D are second-order rate constants for the reaction of ·OH with ethanol and DMPO, respectively, and [E] and [D] refer to the ethanol and DMPO concentrations. Values shown are the mean of three determinations. The correlation coefficient was 0.999.

Table II. Rates of Reaction of Various Nitrones with Hydroxyl Radical at 25 °C[a]

rate constant, 10^9 M^{-1} s^{-1}	·OH generating system	ratio	assumed rate constant, 10^9 M^{-1} s^{-1}
$k_{DMPO} = 3.4$	photolysis	$k_{DMPO}/k_{ETOH} = 1.91$	$k_{ETOH} = 1.8$
$k_{DMPO} = 2.1$	Fenton	$k_{DMPO}/k_{ETOH} = 1.12$	$k_{ETOH} = 1.8$
$k_{TMPO} = 3.8$	Fenton	$k_{TMPO}/k_{DMPO} = 1.13$	$k_{DMPO} = 3.4$
$k_{4\text{-POBN}} = 1.9$	Fenton	$k_{4\text{-POBN}}/k_{DMPO} = 0.55$	$k_{DMPO} = 3.4$
$k_{CDMPO} = 1.55$ to 2.68	Fenton	$k_{CDMPO}/k_{DMPO} = 0.45$ to 0.78	$k_{DMPO} = 3.4$

sorbance and thus does not interfere optically with the production of ·OH. The α-hydroxyethyl radicals, formed by reaction of ethanol with ·OH, reacted with DMPO to yield a spectrum with A_N = 15.8 G and A_H = 22.8 G, which was completely distinct from the DMPO–OH spectrum (Figure 3). The ethanol inhibition of DMPO–OH production was calculated by an analysis similar to eq 15, as shown in Figure 4. The ratio of $k_{DMPO}/k_{Ethanol}$ thus determined was 1.91. Assuming a rate constant of 1.8×10^9 M^{-1} s^{-1} for reaction of ·OH with ethanol,[30] k_{DMPO} is 3.4×10^9 M^{-1} s^{-1}. The results of similar experiments are summarized in Table II. As shown in this table, the rate constants for reaction of ·OH with various nitrone spin traps are all very high, with TMPO being most reactive and 4-POBN being least reactive.

A discrepancy between rate constants for reaction of DMPO with ·OH in the photolysis and Fenton reaction systems was noted. Ethanol appeared to be more effective at inhibiting the formation of DMPO–OH in the Fenton system than in the photolysis system. Since the α-hydroxyethyl radical, formed by reaction of ·OH with ethanol, is a strong oxidizing agent, the enhanced ability of ethanol to inhibit the formation of DMPO–OH in the Fenton system can be due to the oxidation of Fe^{2+} by this radical. Oxidation of Fe^{2+} by alkyl radicals produced in the Fenton reaction is known to occur, and these reactions can be quite rapid.[29] Therefore the value obtained in the photolysis system was considered to be more accurate and was used in calculating the other rate constants shown in Table II. The apparent rate constant for reaction of ·OH with carboxy-DMPO was dependent upon the concentration of this spin trap and was higher at lower carboxy-DMPO concentrations. This may reflect dimer formation by this spin trap.

The stability of the hydroxyl radical adducts varied with each nitrone. The decay kinetics could be measured by measuring the decrease in peak height after termination of illumination. The decay of 4-POBN–OH produced by photolysis of 0.6% H_2O_2 in pH 7.4 phosphate buffer was pure first order and had a half-life of only 23 s. The decay of DMPO–OH produced under these conditions, however, was much slower and had both first- and second-order components. The second-order component predominated at higher DMPO–OH concentrations, whereas the first-order components predominated at lower DMPO–OH concentrations. The half-life of the first-order component was 2.6 h. In general, it was found that cyclic nitrones such as DMPO and TMPO have relatively stable hydroxyl radical adducts, whereas aryl nitrones such as PBN and 4-POBN have less stable hydroxyl radical adducts and have a more pronounced first-order decay component.

The stability of the DMPO–OH adduct was also a function of H_2O_2 concentration. At higher H_2O_2 concentration, the first-order component becomes more prominent and is faster. For example, at 30% H_2O_2 the half-life of the DMPO–OH adduct was only 40 s. This suggests that the first-order component of DMPO–OH decay may be due to direct oxidation of the nitroxide group by hydrogen peroxide. The second-order decay component seen at lower hydrogen peroxide concentrations is probably due to disproportionation.

Discussion

The data presented here indicate that aqueous HO_2· and O_2^-· react with DMPO at rate constants of 6.6×10^3 and 10 M^{-1} s^{-1}, respectively. These rate constants are much lower than published reaction rates of other oxygen-centered radicals with DMPO.[31] This is consistent with the relatively low reactivity of O_2^-· and HO_2^-· compared to other oxygen-centered free-radical species. The rate constants we obtain for hydroxyl radical trapping are likewise consistent with the high reactivity of hydroxyl radical, whose reaction rates with most organic compounds fall within the range of 10^8–10^{10} M^{-1} s^{-1}.[32]

The pH dependence on the apparent rate constant for superoxide trapping is an important consideration in aqueous systems. Although the apparent rate constant for superoxide trapping increases at lower pH, the spontaneous dismutation of superoxide also increases.[8,25] At low pH, any advantages due to the higher rate of trapping are therefore offset by the increased rate of dismutation. At higher pH, the spontaneous dismutation rate decreases linearly with pH, whereas the spin-trapping reaction becomes less sensitive to pH, as shown in Figure 2. Thus, the ability of DMPO spin trapping to compete with the spontaneous dismutation of superoxide is actually enhanced at higher pH. Our data can therefore explain the results of Harbour et al.,[6a] who observed a similar pH dependence but did not consider the competition between the dismutation and spin-trapping reactions of superoxide as an explanation for the pH effect.

The rate constants for superoxide trapping are low compared to the rate constants of other methods used to detect O_2^-·.[33] For example, the rate constants for reaction of superoxide with cytochrome *c* and tetranitromethane at pH 7.8 are about 6×10^5 and 2×10^9 M^{-1} s^{-1}, respectively. Spin trapping differs from these other methods in that it involves the covalent reaction of superoxide with the spin trap, as opposed to an electron-transfer-mediated reduction of a dye molecule by superoxide.

Although the actual mechanism of the reaction between O_2^-· and DMPO cannot be determined on the basis of the information presented, certain possibilities can be ruled out. In some instances,

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electron-transfer reactions of $O_2^{-}\cdot$ mimic nucleophilic attack, as has been shown to occur in the reaction of $O_2^{-}\cdot$ with nitro substituted aromatic halides.[10a,34] In these instances, $O_2^{-}\cdot$ reduces the compound to form a one-electron reduced intermediate, which then reacts with molecular oxygen. This type of mechanism would require the production of DMPO-e^- as a free intermediate. However, DMPO-e^- is known to rapidly protonate to form the hydrogen atom adduct, DMPO-H.[2] DMPO-H formation was not observed in the current study, thus ruling out this reaction mechanism as a possibility.

The spin-trapping (i.e., radical addition) reaction is not the only way in which $O_2^{-}\cdot$ can react with nitrones; an ionic mechanism is also possible. Therefore a possible explanation for the lower rate of $O_2^{-}\cdot$ addition to DMPO, as compared to $HO_2\cdot$, is that $O_2^{-}\cdot$ reacts primarily by an ionic mechanism. To the best of our knowledge, this is the first study to report kinetic data on the addition of superoxide to a double bond, and there is little information in the literature with which to compare our observations.

The extent to which an ionic mechanism participates in adduct formation is dependent upon the nucleophilicity of $O_2^{-}\cdot$ in aqueous solution and the susceptibility of DMPO to nucleophilic attack. Danen and Warner have reported rates of nucleophilic displacement of $O_2^{-}\cdot$ with alkyl bromides in DMSO; the rates they reported ranged from $<1\ M^{-1}\ s^{-1}$ to $6.7 \times 10^2\ M^{-1}\ s^{-1}$ at 25 °C.[7] Although one group has reported that $O_2^{-}\cdot$ can act as a nucleophile in an aqueous system,[9] Fee and Valentine have argued that the nucleophilicity of $O_2^{-}\cdot$ would be expected to be greatly decreased in water by analogy to the effect of solvation on the nucleophilicity of fluoride ion.[8] DMPO is known to undergo nucleophilic additions in both aqueous[35] and nonaqueous systems.[12] Thus further study is required in order to determine if $O_2^{-}\cdot$ reacts with DMPO by means of an ionic or radical mechanism, or a combination of both reaction pathways.

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Oxidation of Hydroquinone Silyl Ethers to Quinones

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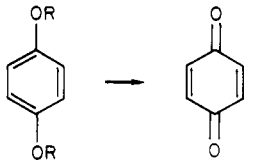
Abstract: The electrochemical oxidation of trialkylsiloxybenzene derivatives was investigated using platinum and graphite anodes in either acetonitrile or methylene chloride solvent. Preparative oxidation of the bis(trimethylsilyl) ethers of hydroquinone, chlorohydroquinone, 2,5-di-*tert*-butylhydroquinone, and 9,10-dihydroxyanthracene gave the corresponding quinones in 80-90% yield. Oxidation of 1,4-bis(trimethylsiloxy)-2-methoxybenzene in acetonitrile gave 3,6-dihydroxy-2,7-dimethoxydibenzofuran in 65% yield. Oxidation of 1-methoxy-4-(trimethylsiloxy)benzene in methanolic acetonitrile produced 1,1-dimethoxy-2,5-cyclohexadien-4-one in 99% yield. The cyclic voltammograms of the trimethylsilyl, triethylsilyl, and *tert*-butyldimethylsilyl ethers of 2,5-di-*tert*-butylhydroquinone were recorded using acetonitrile and methylene chloride solvents at 25 and -60 °C. These compounds were found to react by initial one-electron oxidation to generate a cation radical which decomposes by silicon-oxygen bond cleavage to eventually form quinone. The relative rates for decomposition of the trimethylsilyl, triethylsilyl, and *tert*-butyldimethylsilyl ether cation radicals at -60 °C were 10:5:1. *tert*-Butyldimethylsilyl chloride and *tert*-butyldimethylsilyl triflate were found to be stable toward cathodic reduction in dry methylene chloride and acetonitrile at potentials as negative as -2.1 vs. Ag/0.1 M $AgNO_3$. Cyclic voltammetry indicated that reduction of 2,5-di-*tert*-butylbenzoquinone, anthraquinone, and naphthoquinone in the presence of *tert*-butyldimethylsilyl chloride resulted in reductive silylation.

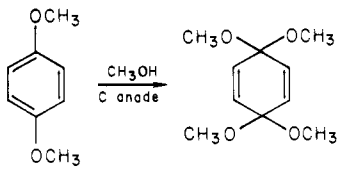
The quinone/hydroquinone couple is the classic organic redox system and, because this interconversion is so facile, quinones and hydroquinones are widely used both naturally and in man-made products.[2] We felt that a reaction which cleanly converted a nonphenolic compound into a quinone would find utility in a variety of applications. We have, therefore, considered masking hydroquinones as diethers and releasing them by oxidation, e.g., eq 1. The importance of this reaction has already been recognized



(1)

1a, R = CH_3
b, R = $(CH_3)_3Si$

and the oxidative conversion of 1,4-dimethoxy aromatics to quinones has been accomplished in yields of 50-90% using AgO in acidic dioxane.[3] The present study employed anodic oxidation and there is a report that oxidation of **1a** on a PbO_2 anode in sulfuric acid produced quinone in 49% yield.[4] When nonaqueous electrochemical solvents are used, quinone formation is not observed and instead dimerization or nucleophilic attack at a ring position dominates.[5] A reaction of this type[6,7] which has provoked some synthetic interest is eq 2.



(2)

A special case in which a quinone was produced anodically from an ether is the oxidation of the dimethyl ether of durohydroquinone

(1) Taken in part from the Ph.D. Thesis of Ray F. Stewart, University of Minnesota.

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