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Kinetics of Spin Trapping Superoxide, Hydroxyl, and Aliphatic Radicals by Cyclic Nitrones

Sara Goldstein,*,[†] Gerald M. Rosen,[‡] Angelo Russo,[§] and Amram Samuni[⊥]

Department of Physical Chemistry, the Hebrew University of Jerusalem, Jerusalem 91904, Israel, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Medical Biotechnology Center, University of Maryland Biotechnology Institute, and Center for Low Frequency EPR Imaging for In Vivo Physiology, the University of Maryland, Baltimore, Maryland 21201, Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892, and Department of Molecular Biology, the Hebrew University of Jerusalem - Hadassah Medical School, Jerusalem 91120, Israel

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Spin trapping coupled with electron paramagnetic resonance (EPR) spectroscopy has surfaced as one of the most specific and reliable methods for identifying free radicals in biological systems. Despite extensive studies focused on the kinetics of radical trapping by cyclic nitrones, the mechanism has not been fully elucidated. Moreover, major controversies still persist even regarding the efficiency and the rate constants of the trapping reaction. The present research used pulse radiolysis for studying the reaction of 5,5-dimethyl-1-pyrroline *N*-oxide **1** (DMPO) and of the ester-containing derivative, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **4**, with $^{\bullet}OH$, $O_2^{\bullet-}$, $CO_2^{\bullet-}$, $^{\bullet}C(OH)(CH_3)_2$, $^{\bullet}CH_2OH$, and $^{\bullet}CH_3$. The results reveal that radical trapping is far more complex then previously realized. Radiation chemical experiments combined with EPR demonstrate that about 30% of $^{\bullet}OH$ add to nitrones **1** and **4** at position 2, yielding the corresponding persistent aminoxyls. The remaining $^{\bullet}OH$ radicals form transient intermediates that rapidly decay bimolecularly. These transient intermediates react with oxygen with rate constants that are significantly lower than those generally reported for alkyl radicals, which suggests that they are not simple carbon-centered radicals generated as a result of H-abstraction from the methyl or methylene groups of the nitrones. It is also shown that the addition of $O_2^{\bullet-}$ and various aliphatic radicals to the nitrones is an equilibrium process. The upper limit for the rate constant of the reaction of nitrone **4** with $O_2^{\bullet-}$ was 3 M^{-1} s^{-1}. The rate constant for the reaction of nitrone **1** with $O_2^{\bullet-}$ was determined to be 170 ± 40 M^{-1} s^{-1}. This value is significantly higher than those previously determined by following the formation of the corresponding aminoxyl by EPR, which indicates that the yield of the aminoxyl is only a small fraction of the reacting $O_2^{\bullet-}$.

Introduction

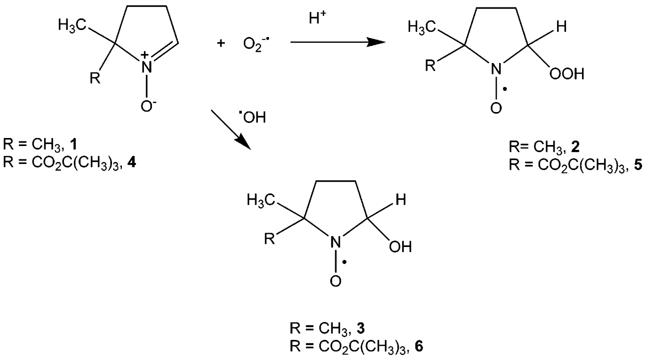
Given the theory that free radicals participate in cell signaling pathways,[1−5] the detection and characterization of these reactive intermediates is critical to understanding the mechanism of these physiological processes. Of the available methods for identifying free radicals in a myriad of biological systems, spin trapping/EPR spectroscopy has surfaced as one of the most specific and reliable techniques.[6] For instance, spin trapping was essential in the discovery that nitric oxide synthase (NOS; EC 1.14.13.39)[7] generates $O_2^{\bullet-}$ [8−16] along with $^{\bullet}NO$ and H_2O_2[17−19] during NOS reduction of O_2.

Since the mid-1970s, 5,5-dimethyl-1-pyrroline *N*-oxide **1** (DMPO) has been the primary spin trap for $O_2^{\bullet-}$ and $^{\bullet}OH$, owing this standing to the ease of synthesis and to the unique EPR spectrum of aminoxyl **2** and aminoxyl **3**, derived from the reaction of nitrone **1** with $O_2^{\bullet-}$ and $^{\bullet}OH$, respectively (Scheme 1).

Nitrone **1** is the most frequently used trap; yet, despite extensive studies focused on the kinetics of radical trapping, the detailed mechanism has not been fully elucidated. Moreover, although many potential artifacts associated with spin trapping have been previously recognized, major controversies still persist even regarding the efficiency and the rate constants of the trapping reaction. Concomitantly, new spin traps have been synthesized in an attempt to answer fundamental questions pertinent to free radicals in biology. For example, the spin trap 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **4**, whose reaction with $O_2^{\bullet-}$ affords aminoxyl **5** yields a product having a more robust EPR spectrum than that of aminoxyl **2**,[20−22] was critical for understanding how (6R)-5,6,7,8-tetrahydro-L-biopterin (H_4B) regulated NOS-generated $O_2^{\bullet-}$ and H_2O_2.[19]

In the present study we have used pulse- and γ-radiolysis and EPR spectroscopy to study the mechanism of the reactions of nitrones **1** and **4** with $^{\bullet}OH$, $O_2^{\bullet-}$, $CO_2^{\bullet-}$, and several aliphatic radicals.

SCHEME 1



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* To whom all correspondence should addressed.

[†] Department of Physical Chemistry, the Hebrew University of Jerusalem.

[‡] Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Medical Biotechnology Center, University of Maryland Biotechnology Institute, and Center for Low-Frequency EPR Imaging for In Vivo Physiology, the University of Maryland.

[§] Radiation Biology Branch, National Cancer Institute, NIH.

[⊥] Department of Molecular Biology, the Hebrew University of Jerusalem - Hadassah Medical School.

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Experimental Section

Chemicals. All chemicals were of analytical grade and were used as received. Solutions were prepared with distilled water, which was further purified using a Milli-Q water purification system. 5,5-Dimethyl-1-pyroline *N*-oxide **1** was purchased from Aldrich. The colored impurity in this commercial product was removed with charcoal followed by filtration. This preparation did not demonstrate any EPR signal before the trapping of the radicals. Moreover, ^{1}H NMR scanning did not detect any contaminant either. The concentration of nitrone **1** was determined using $\epsilon_{228} = 8000$ M^{-1} cm^{-1}. 5-*tert*-Butoxycarbonyl-5-methyl-1-pyroline *N*-oxide **4** was synthesized and purified as previously described[21,22] and showed no spurious EPR spectra. Trace contaminants can lead to side reactions and erroneous conclusions. This is true in the common spin-trapping experiments where relatively high concentrations of nitrones are applied (at the tens and hundreds millimolar range). Moreover, since in the present study the concentration of the nitrones did not exceed 4 mM, potential residual contaminants are not anticipated to affect the reaction kinetics.

Pulse Radiolysis. Pulse radiolysis experiments were carried out with a Varian 7715 linear accelerator delivering 5-MeV electron pulses of 0.2−1.5 μs and 200 mA. All measurements were made at ambient temperature using a 4-cm Spectrosil cell applying three light passes (optical path length 12.1 cm). Each measurement was repeated at least five times. Appropriate cutoff filters were used to eliminate photolysis. Dosimetry was performed with an oxygenated solution containing 5 mM ferrocyanide using $G(\mathrm{Fe(CN)_6}^{3-}) = 2.7 \times 10^{-7}$ M Gy^{-1} and $\epsilon_{420}(\mathrm{Fe(CN)_6}^{3-}) = 1000$ M^{-1} cm^{-1}.

Steady-State γ-Irradiation. γ-Irradiation was carried out at room temperature using either a ^{137}Cs source with a dose rate of 9 Gy min^{-1} or a ^{60}Co source with a dose rate of 37 Gy min^{-1} as determined by Fricke dosimetry.

Electron Paramagnetic Resonance (EPR) Measurements. After irradiation, the samples were immediately frozen in liquid nitrogen to prevent spin adduct decay. The frozen samples were thawed just before recording their EPR spectra. To determine aminoxyl concentration, samples were injected through a narrow Teflon capillary inserted into a quartz tube that is placed within the EPR spectrometer cavity. The EPR spectra were recorded using JEOL JES-RE3X ESR spectrometer operating at X band with center field set at 3290 G, 100 kHz modulation frequency, 1 G modulation amplitude, and 4 mW incident microwave power. The concentration of the nitrone spin adduct was calculated from the EPR signal using a standard solution of 2,2,6,6-tetramethyl-1-piperidinyloxyl and 4-hydroxy-2,2,6,6-tetramethyl-1- piperidinyloxyl.

Results and Discussion

Reaction of Nitrones with $^{\bullet}$OH. Irradiation of aqueous solutions at pH > 3 saturated with N_2O (25 mM) produces ca. 90% $^{\bullet}$OH and 10% H$^{\bullet}$.

$$\mathrm{H_2O} \xrightarrow{\gamma} \mathrm{e^-_{aq}}\ (2.6),\ {}^{\bullet}\mathrm{OH}\ (2.7),\ \mathrm{H^{\bullet}}\ (0.6),\ \mathrm{H_2}\ (0.45),\ \mathrm{H_2O_2}\ (0.7),\ \mathrm{H_3O^+}\ (2.6) \quad (1)$$

$$\mathrm{e_{aq}}^- + \mathrm{N_2O} + \mathrm{H_2O} \rightarrow {}^{\bullet}\mathrm{OH} + \mathrm{N_2} + \mathrm{OH^-} \qquad k_2 = 9.1 \times 10^9\ \mathrm{M^{-1}\,s^{-1}} \quad (2)$$

The numbers in parentheses are *G*-values, which represent the yields of the various species in 10^{-7} M Gy^{-1}, and are about 7% higher in the presence of high solute concentrations.

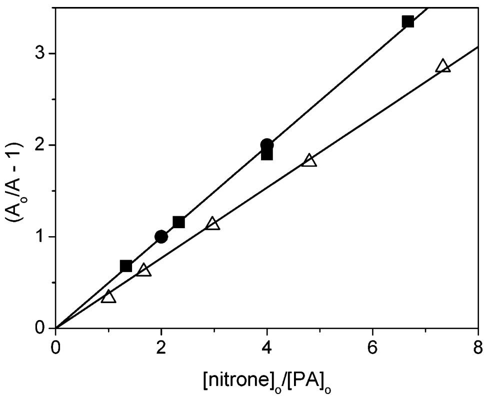

Figure 1. Competition kinetics using phenylalanine as a reference solute. The yield of phenylalanine−OH adduct was measured upon pulse irradiation of N_2O-saturated solution containing nitrone **1** and 100 μM (●) or 300 μM (■) phenylalanine at pH 7.8 or nitrone **4** and 300 μM phenylalanine (△) at pH 7.4. All solutions contained 4 mM PB, and the absorption was measured at 325 nm in the absence (A_o) and presence (A) of various concentrations of the nitrone.

Nitrone **1** or nitrone **4** (≤2 mM) efficiently scavenges $^{\bullet}$OH[23−28] and H$^{\bullet}$ radicals.[26,27]

$$\text{nitrone} + {}^{\bullet}\mathrm{OH} \rightarrow \text{HO-aminoxyl}^{\bullet} + \text{products} \quad (3)$$

$$\text{nitrone} + \mathrm{H}^{\bullet} \rightarrow \text{H-aminoxyl}^{\bullet} + \text{products} \quad (4)$$

The published values for k_3 varied between 1.9×10^9 and 4.3×10^9 M^{-1} s^{-1},[23−28] whereas the reports concerning the yield of aminoxyl **3** are more controversial, namely 33−35%[29,30] in contrast to 94%.[27,31]

We determined the rate constant of reaction 3 using competition kinetics against the reaction of $^{\bullet}$OH with phenylalanine (PA) as a reference solute.

$$ {}^{\bullet}\mathrm{OH} + \mathrm{PA} \rightarrow \mathrm{HO\text{-}PA}^{\bullet} \quad k_5 = 6.5 \times 10^9\ \mathrm{M^{-1}\,s^{-1}}\ ^{32} \quad (5)$$

The yield of HO-PA$^{\bullet}$ was determined by monitoring the absorbance at 325 nm immediately after pulse irradiation of N_2O-saturated solutions containing 100 or 300 μM PA and 4 mM phosphate buffer (PB), pH 7.4−7.8, in the absence (A_o) and in the presence (A) of various nitrone concentrations. When only reactions 3 and 5 take place, eq 6 describes the dependence of the yield of HO−PA$^{\bullet}$ on [nitrone]$_o$ and [PA]$_o$:

$$A = A_o k_5[\mathrm{PA}]_o/(k_3[\text{nitrone}]_o + k_5[\mathrm{PA}]_o) \quad (6)$$

The plot of $(A_o/A - 1)$ vs [nitrone]$_o$/[PA]$_o$ was linear (Figure 1), and from the slope of the lines we calculated $k_3 = (3.2 \pm 0.1) \times 10^9$ and $(2.5 \pm 0.1) \times 10^9$ M^{-1} s^{-1} for nitrone **1** and nitrone **4**, respectively. The value of k_3 determined for nitrone **1** closely agrees with some of the literature values;[23,25,27,33] whereas, k_3 of for nitrone **4** is about half of that previously determined using radiolytically formed $^{\bullet}$OH.[28]

For a direct investigation of the kinetics of $^{\bullet}$OH reaction with nitrones **1** and **4**, we pulse irradiated solutions containing 0.15−2 mM nitrone and 4 mM PB (pH 7.5−7.8), which were saturated either with N_2O or with various mixtures of N_2O and O_2. A rapid first-order formation of a transient with a maximum near 270 nm was observed for both nitrones in the absence and presence of O_2. In the absence of O_2, this transient absorption decayed via a second-order reaction, leaving a residual persistent absorption as demonstrated for nitrone **1** in Figure 2. The observed second-order rate constant and the extent of the residual persistent absorption did not depend on the initial

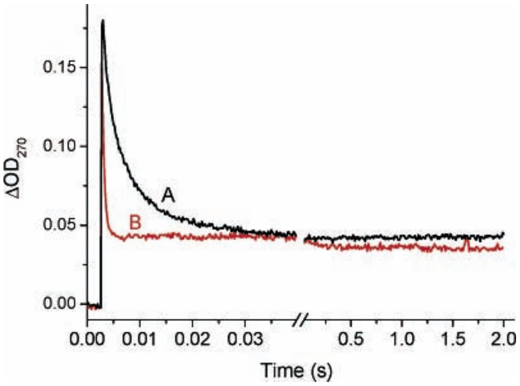

Figure 2. Reaction of $^{\bullet}OH$ with nitrone **1** in the absence and presence of O_2. Typical kinetic traces obtained upon pulse irradiation (13.4 Gy) of solutions containing 300 μM nitrone **1** and 4mM PB at pH 7.5 saturated with N_2O (A) or a mixture of 80% N_2O and 20% O_2 (B). The apparent optical path length was 12.1 cm.

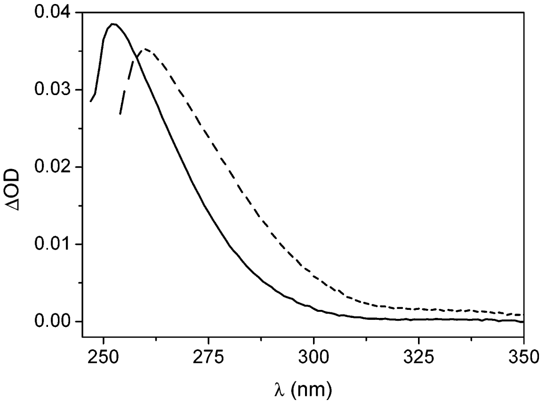

Figure 3. Difference absorption spectra between irradiated and nonirradiated anoxic solutions containing 300 μM nitrone **4** (dashed line) or nitrone **1** (solid line) in 4 mM PB at pH 7.5. The spectra of the irradiated samples were recorded within 1 min after the pulse. The dose was 14.5 Gy (7.7 μM $^{\bullet}OH$), and the optical path length was 4 cm.

concentration of the nitrone between 0.15 and 2 mM. These observations indicate, contrary to the conclusion of Neta et al.,[24] who attributed the absorption with the maximum at 270 nm to the formation of the persistent aminoxyl **3** alone, that the reaction of $^{\bullet}OH$ with the nitrones yields at least two distinct species. The transient absorption reflects the formation of some short-lived nitrone radical (denoted nitrone$^{\bullet}$) via reaction 3, whereas the accumulated aminoxyl **3** (or aminoxyl **6**) is responsible for the residual persistent absorption.

$$\text{nitrone}^{\bullet} + \text{nitrone}^{\bullet} \rightarrow \text{products} \qquad (7)$$

The analysis of the kinetics of the second-order decay yields $k_7/\Delta\epsilon_{270}l = (3.6 \pm 0.3) \times 10^3$ and $(5.6 \pm 0.5) \times 10^3$ s^{-1} for nitrone **1** and nitrone **4**, respectively.

The UV−vis spectrum of the residual persistent absorption of the pulse-irradiated anoxic solution, which was monitored using a diode array spectrophotometer within less than 45 s after the pulse, was stable for at least 15 min. This spectrum was compared with that of the nonirradiated sample, and the difference absorption spectra monitored for nitrones **1** and **4** are shown in Figure 3.

The EPR spectra were recorded following γ-irradiation (4.5−37 Gy) or pulse-irradiation (14.5 Gy/pulse) of 2 mM nitrone in N_2O-saturated solution at pH 7.5. The observed EPR signal exhibited the well-known characteristic signals of aminoxyls **3** and **6**.[24] Considering the total [$^{\bullet}OH$] formed by the radiation and the concentration of the accumulated aminoxyls **3** and **6**, we calculated their yields as 26 ± 6% and 33 ± 5%, respectively. These results suggest that about 30% of $^{\bullet}OH$ add to nitrones **1** or **4** at position 2, yielding the persistent aminoxyls **3** or **6**, respectively. From the knowledge of the yields of nitrone$^{\bullet}$, i.e., ca. 70%, we calculated $\Delta\epsilon_{270}$, which allowed us to evaluate the second-order rate constant for reaction 7 as $k_7 = (7.5 \pm 0.4) \times 10^7$ and $(1.2 \pm 0.1) \times 10^8$ M^{-1} s^{-1} for nitrone **1** and nitrone **4**, respectively. Assuming that the products of reaction 7 have no appreciable absorption at $\lambda > 250$ nm, the residual absorption spectra shown in Figure 3 are attributable to aminoxyls **3** and **6**.

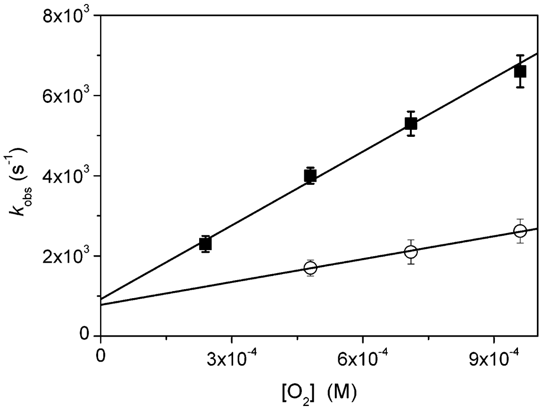

Figure 4. Dependence of the observed pseudo-first-order rate constant of decay of the transient absorption on [O_2]. The solutions containing (■) 300 μM nitrone **1** or (○) nitrone **4** in 4 mM PB at pH 7.5 and saturated with various mixtures of N_2O and O_2 were pulse irradiated applying a dose of 14.5 Gy/pulse (7.7 μM $^{\bullet}OH$).

TABLE 1: Summary of All the Rate Constants Determined in the $^{\bullet}OH$ System

reaction	nitrone **1**	nitrone **4**
k_3, M^{-1} s^{-1}	$(3.3 \pm 0.1) \times 10^9$	$(2.5 \pm 0.1) \times 10^9$
k_7, M^{-1} s^{-1}	$(7.5 \pm 0.4) \times 10^7$	$(1.2 \pm 0.1) \times 10^8$
k_8, M^{-1} s^{-1}	$(6.1 \pm 0.5) \times 10^6$	$(1.9 \pm 0.6) \times 10^6$

In the presence of oxygen the rate of the decay of the transient absorption was enhanced and turned from second- to first-order kinetics as demonstrated for nitrone **1** in Figure 2.

$$\text{nitrone}^{\bullet} + O_2 \rightarrow \text{products} \qquad (8)$$

The observed first-order rate constant increased upon increasing [O_2] (Figure 4), and from the slope of the linear plots we determined $k_8 = (6.1 \pm 0.5) \times 10^6$ and $(1.9 \pm 0.7) \times 10^6$ M^{-1} s^{-1} for nitrone **1** and nitrone **4**, respectively.

All rate constants determined in the present study are summarized in Table 1. The values of k_7 and k_8 are significantly lower than those generally reported for alkyl radicals. These observations suggest that the relatively short-lived nitrone$^{\bullet}$ is not a simple carbon-centered radical. This conclusion does not agree with that of Carmichael et al.[29,30] who assumed that $^{\bullet}OH$ radicals that do not add to the carbon of the N═C bond react with the nitrone by H-abstraction from its methyl or methylene group. Instead, $^{\bullet}OH$ might add to the nitrogen of the N═C bond, and nitrone$^{\bullet}$ might represent a delocalized radical, e.g., an allylic radical, which is generally less reactive than an alkyl radical toward O_2.

Reactions of Nitrones with $CO_2^{\bullet-}$, $^{\bullet}CH_3$, $^{\bullet}CH_2OH$, and $^{\bullet}C(OH)(CH_3)_2$. Employing pulse irradiation of N_2O-saturated solutions (4 mM PB at pH 7.4−7.8) containing 0.1 M formate,

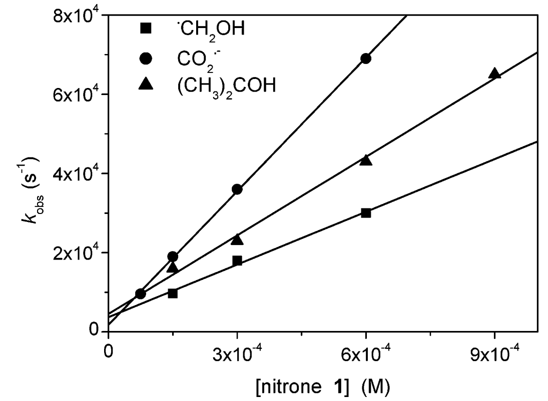

Figure 5. Reaction of nitrone **1** with $R^•$. The observed first-order rate constant of the decay of the absorption formed following pulse irradiation of N_2O-saturated solutions containing 0.1 M formate (●), 0.2 M ethanol (■), or 0.2 M 2-propanol (▲). All solutions contained 4 mM PB at pH 7.5−7.9, the dose was 9−16 Gy, and the decay of the absorption was followed at 260−280 nm.

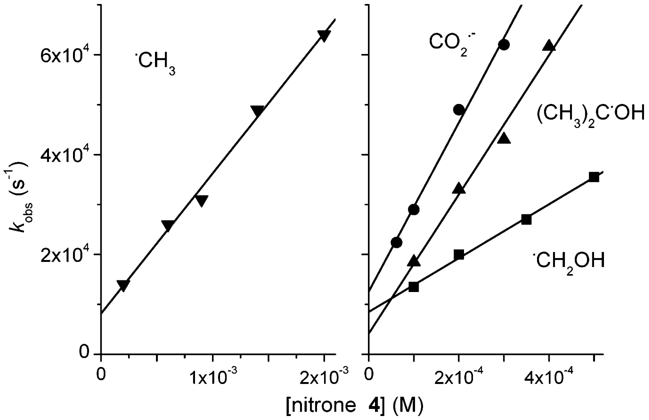

Figure 6. Reaction of nitrone **4** with $R^•$. The observed first-order rate constant of the decay of the absorption formed following pulse irradiation of N_2O-saturated solutions containing 0.1 M formate (●), 0.1 M DMSO (▼), 0.2 M ethanol (■), or 0.1 M 2-propanol (▲). All solutions contained 4 mM PB at pH 7.4−7.5, the dose was 17 Gy, and the decay of the absorption was followed at 290−310 nm.

0.1 M DMSO, and 0.2 M ethanol or 0.1−0.2 M 2-propanol, the reactions of nitrones **1** and **4** with $CO_2^{•-}$, $^•CH_3$, $^•CH_2OH$, or $^•C(OH)(CH_3)_2$ (denoted as $R^•$) were studied. Under such experimental conditions all the primary radicals formed by the radiation were converted into $R^•$, which absorbs at 260−290 nm. In the absence of the nitrones, $R^•$ disappears by a second-order kinetics,[32] whereas in their presence the rate of its decay increased and changed from second- to first-order kinetics. The transient absorption resulting from $R^•$ did not decay to zero but rather left a residual persistent absorption with a maximum near 260 nm. The observed pseudo-first-order rate constant was linearly dependent on $[\text{nitrone}]_o$, demonstrating, particularly in the case of nitrone **4**, relatively high intercepts (Figures 5 and 6). This suggests that the reaction of $R^•$ with the nitrone is an equilibrium process. The knowledge of the radiation dose, i.e., the yield of $R^•$, combined with the determination by EPR of the accumulation of R-aminoxyl$^•$ allowed us to calculate the fractional yield of R-aminoxyl$^•$. Nitrone at 2 mM was used to minimize the competition between the reaction of $R^•$ with the nitrones and the self-decomposition of $R^•$. In the case of nitrone **1** we were unable to study spectrophotometrically its reaction with $^•CH_3$, which absorbs too little at $\lambda > 260$ nm.

The results are summarized in Table 2, and demonstrate that in the case of nitrone **4**, though not nitrone **1**, most or even all $R^•$ add at position 2 of the nitrone, yielding R-aminoxyl$^•$ (reaction 9).

$$\text{nitrone} + R^• \rightleftharpoons \text{R-aminoxyl}^• \quad (9)$$

The reaction of the nitrone with $R^•$ forms also other species (reaction 10). In the case of nitrone **1**, though not nitrone **4**, the contribution of reaction 10 is quite significant.

$$\text{nitrone} + R^• \rightarrow \text{products} \quad (10)$$

Since both reactions 9 and 10 contribute to the loss of $R^•$, the rate constant that characterizes its decay as well as the formation of the persistent R-aminoxyl$^•$ equals ($k_9 + k_{10}$), which were evaluated from the slopes of the lines displayed in Figures 5 and 6. The results are summarized in Table 2 and compared to the values previously reported.

Reaction of Nitrones with Superoxide Radicals ($O_2^{•-}$/$HO_2^•$). The reaction of superoxide radicals with cyclic nitrones has been extensively studied in the past. There are a few cases, e.g., nitrones **1** and **4**, where it was shown that the efficiency of spin trapping of $HO_2^•$ is far greater than that of $O_2^{•-}$, i.e., $k_{11} \gg k_{12}$.[22,23,34]

$$\text{nitrone} + HO_2^• \rightarrow \text{HOO-aminoxyl}^• \quad (11)$$

$$\text{nitrone} + O_2^{•-} + H^+ \rightarrow \text{HOO-aminoxyl}^• \quad (12)$$

Finkelstein et al.[23] have studied the reaction of superoxide radicals with nitrone **1** at pH 5−9 using competition kinetics against superoxide dismutase (SOD), and a reasonable good fit to the experimental data was obtained for $k_{11} = 6.6 \times 10^3\ M^{-1}\ s^{-1}$ and $k_{12} = 10\ M^{-1}\ s^{-1}$. Nevertheless, major disagreements still exist regarding the kinetics of this reaction. Obviously the apparent rate constant, k_{app}, greatly depends on the pH; yet, the values measured at pH 7.2−7.8 vary between 1.2 and 50 $M^{-1}\ s^{-1}$.[23,35−40] In the case of nitrone **4**, k_{app} was determined to be 239 $M^{-1}\ s^{-1}$ at pH 5,[21,22] 0.24−77 $M^{-1}\ s^{-1}$ at pH 7.0−7.4[22,38] and ca. 5 $M^{-1}\ s^{-1}$ at pH 10 (competition kinetics against Cu/Zn−SOD, unpublished results).

Pulse radiolysis enables the generation of superoxide radicals within less than 1 μs, and hence allows direct study of the effect of a tested molecule on their decay. This method has not yet been employed for studying the reaction of nitrones with superoxide radicals, which decay according to the well-established mechanism described by reactions 13−15.

$$HO_2^• + HO_2^• \rightarrow O_2 + H_2O_2 \qquad k_{13} = 8.3 \times 10^5\ M^{-1}\ s^{-1}\ {}^{41} \quad (13)$$

$$O_2^{•-} + HO_2^• + H^+ \rightarrow O_2 + H_2O_2 \qquad k_{14} = 9.7 \times 10^7\ M^{-1}\ s^{-1}\ {}^{41} \quad (14)$$

$$HO_2^• \rightleftharpoons H^+ + O_2^{•-} \quad pK_a = 4.8^{41} \quad (15)$$

Because the dismutation of superoxide at physiological pH is relatively fast, its slow reaction with nitrones can be studied only at pH range where $k_{12}[\text{nitrone}]_o \gg (k_{14}/K_a)\ [H^+][O_2^{•-}]_o$, i.e., alkaline solutions.

In the present study, superoxide radicals were generated by pulse-irradiation of solutions saturated with 50% N_2O (12.5 mM) and 50% O_2 (0.6 mM), which contained 0.5 M 2-propanol, 25 μM DTPA, and 5 mM carbonate at pH 9.6−12.1. Under these

TABLE 2: Summary of All the Rate Constants and Yields

	nitrone **1**		nitrone **4**	
	k, $M^{-1}\,s^{-1}$	yield, %	*k*, $M^{-1}\,s^{-1}$	yield, %
$^{\bullet}OH$	$(3.3 \pm 0.1) \times 10^{9}$ [a]	26 ± 6 [a]	$(2.5 \pm 0.1) \times 10^{9}$ [a]	33 ± 5 [a]
	3.2×10^{9} [23]	94[27]	$(4.5 \pm 0.3) \times 10^{9}$ [28]	
	2.8×10^{9} [27]	35[29]		
	2.6×10^{9} [25]	33[30]		
	1.9×10^{9} [28]			
	4.3×10^{9} [26]			
$^{\bullet}CH_3$	n.d.	63 ± 7 [a]	$(2.8 \pm 0.2) \times 10^{7}$ [a]	n.d.
	1.4×10^{7} [33]			
$^{\bullet}CH_2OH$	$(4.4 \pm 0.4) \times 10^{7}$ [a]	86 ± 10 [a]	$(5.4 \pm 0.2) \times 10^{7}$ [a]	86 ± 7 [a]
	2.2×10^{7} [33]			
$^{\bullet}C(OH)(CH_3)_2$	$(6.6 \pm 0.3) \times 10^{7}$ [a]	73 ± 7 [a]	$(1.4 \pm 0.1) \times 10^{8}$ [a]	96 ± 5 [a]
	6.8×10^{7} [33]			
	1.5×10^{8} [26]			
$CO_2^{\bullet -}$	$(1.15 \pm 0.05) \times 10^{8}$ [a]	55 ± 10 [a]	$(1.8 \pm 0.1) \times 10^{8}$ [a]	95 ± 5 [a]
	6.6×10^{7} [33]			
	7.5×10^{8} [26]			
$O_2^{\bullet -}$	170 ± 40 [a]	n.d.	<3 [a]	n.d.
	50[38]		75 ± 10 [22]	
	10[23]		7[38]	
	2.4[39]		0.53[39]	
	2.0 ± 0.2 [40]			
	1.3[37]			

[a] This study. The yields were determined upon γ-irradiation of N_2O-saturated solutions containing 2 mM nitrone and 4 mM PB (pH 7.4) in the absence and presence of 0.1 formate or 0.1 M DMSO or 0.5 M 2-propanol or 0.5 M methanol. The dose was 4.5−9 Gy.

conditions all the primary free radicals formed by the radiation are converted into $O_2^{\bullet -}$ through reactions 1, 2, 16−19.

$$^{\bullet}OH + (CH_3)_2CHOH \rightarrow {}^{\bullet}C(OH)(CH_3)_2 + H_2O \qquad k_{16} = 1.9 \times 10^{9}\ M^{-1}\,s^{-1}\ [32] \quad (16)$$

$$H^{\bullet} + (CH_3)_2CHOH \rightarrow {}^{\bullet}C(OH)(CH_3)_2 + H_2 \qquad k_{17} = 7.4 \times 10^{7}\ M^{-1}\,s^{-1}\ [32] \quad (17)$$

$$^{\bullet}C(OH)(CH_3)_2 + O_2 \rightarrow (CH_2)_2C(OH)OO^{\bullet} \qquad k_{18} = 4.2 \times 10^{9}\ M^{-1}\,s^{-1}\ [32] \quad (18)$$

$$(CH_2)_2C(OH)OO^{\bullet} + OH^{-} \rightarrow (CH_3)_2C{=}O + O_2^{\bullet -} + H_2O \qquad k_{19} = 5 \times 10^{9}\ M^{-1}\,s^{-1}\ [32] \quad (19)$$

$$H^{\bullet} + O_2 \rightarrow O_2^{\bullet -} + H^{+} \quad k_{20} = 1.2 \times 10^{10}\ M^{-1}\,s^{-1}\ [32] \quad (20)$$

Although the maximum absorption of $O_2^{\bullet -}$ is at 250 nm,[32] the absorption changes associated with its formation and decay were followed at $\lambda = 270-290$ nm due to the relatively high absorption of the nitrones at lower wavelengths. The decay of ca. 4 μM $O_2^{\bullet -}$ at pH 10 obeyed second-order kinetics with $k = (1.9 \pm 0.2) \times 10^{3}\ M^{-1}\ s^{-1}$, and was hardly affected by the presence of 0.5 mM nitrone **4**. Under our experimental conditions $^{\bullet}C(OH)(CH_3)_2$ reacts with O_2 ca. 36-times faster than with nitrone **4** (Table 2), and about 97% of all the radiolytically formed primary radicals are converted into $O_2^{\bullet -}$. Taking into account that 20% acceleration of the rate of $O_2^{\bullet -}$ decay would be detectable, the failure of nitrone **4** at 0.5 mM to affect the decay kinetics of $O_2^{\bullet -}$ implies an upper limit of 3 $M^{-1}\ s^{-1}$ for k_{12}.

In the presence of nitrone **1** at pH 9.6−12.1, the absorption decayed via two sequential processes. Typical kinetic traces at pH 10.6 in the absence and presence of 4 mM nitrone **1** are shown in Figure 7. Under these experimental conditions ca. 90% of all the primary radicals formed by the radiation are converted into $O_2^{\bullet -}$. The contribution of the first process increased with increasing nitrone **1** concentration. This process obeyed first-order kinetics, and k_{obs} increased with increasing [nitrone **1**] as demonstrated in Figure 8. The relatively high intercept seen in Figure 8 suggests that the addition of $O_2^{\bullet -}$ to nitrone **1** is an equilibrium process (eq 21).

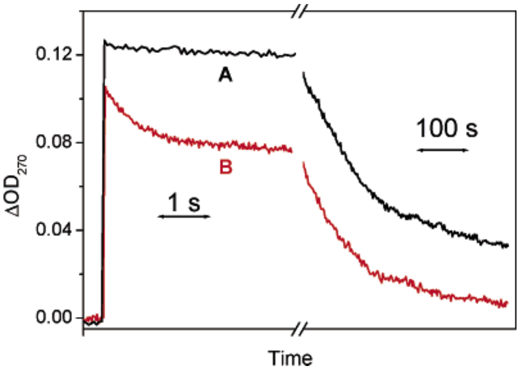

Figure 7. Reaction of $O_2^{\bullet -}$ with nitrone **1**. Typical kinetic traces obtained upon pulse-irradiation of solutions saturated with 50% N_2O and 50% O_2 that contained 0.5 M 2-propanol, 25 μM DTPA, and 5 mM carbonate at pH 10.6 without (trace A) and with 4 mM nitrone **1** (trace B). The dose was 11 Gy, and the apparent optical path length was 12.1 cm.

$$\text{nitrone } \mathbf{1} + O_2^{\bullet -} \rightleftharpoons {}^{-}OO\text{-aminoxyl}^{\bullet} \quad (21)$$

$$^{-}OO\text{-aminoxyl}^{\bullet} + H^{+} \rightleftharpoons HOO\text{-aminoxyl}^{\bullet}\ (\text{aminoxyl } \mathbf{2}) \quad (22)$$

Hence, $k_{obs} = k_{21}[\text{nitrone } \mathbf{1}] + k_{-21}$, and from the slope of the line in Figure 8, we obtained $k_{21} = 170 \pm 40\ M^{-1}\ s^{-1}$.

The pK_a of aminoxyl **2** has not yet been determined but is expected to be close to that of HOOH, namely 11.9. Therefore, drawing the values of k_{obs} obtained at pH > pK_a as a function of [nitrone **1**] should yield a straight line with an intercept equal to k_{-21}. This intercept is expected to decrease at pH close to and below the pK_a of aminoxyl **2**. The effect of the pH on the rate of the faster decay was studied in the presence of 1.8 mM nitrone **1**. We measured $k_{obs} = 1.3 \pm 0.1\ s^{-1}$ at pH 12.1 and 11.1, $0.9 \pm 0.1\ s^{-1}$ at pH 10.1 and $0.6 \pm 0.1\ s^{-1}$ at pH 9.6. These results suggest that the pK_a of aminoxyl **2** is close to 10.

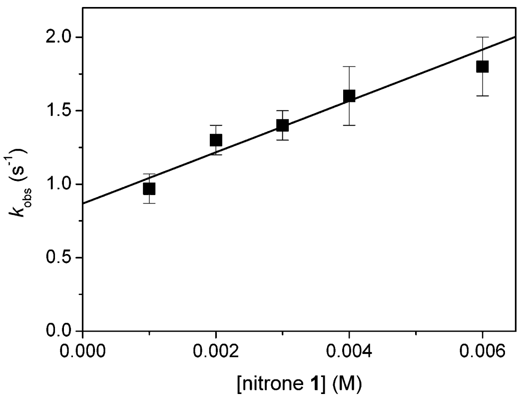

Figure 8. Dependence of k_{obs} of the fast first-order decay of the absorption at 270 nm on [nitrone **1**]$_o$ (trace B, Figure 7). All solutions contained 0.5 M 2-propanol, 25 μM DTPA, and 5 mM carbonate at pH 10.6 and were saturated with N_2O/O_2 (1:1). The dose was 9 Gy, and the optical path length was 12.1 cm.

The half-life of the slower decay decreased upon increasing [nitrone **1**], and at [nitrone **1**] $>$ 1 mM the decay obeyed first-order kinetics. The observed rate constant increased somewhat upon increasing [nitrone **1**] and decreasing the pH, e.g., $k_{obs} = 0.018 \pm 0.002$ s^{-1} and 0.011 ± 0.001 s^{-1} in the presence of 4 mM nitrone **1** at pH 10 and 10.6, respectively, and 0.006 ± 0.001 s^{-1} in the presence of 1.8 mM nitrone **1** at pH 11.1. The analysis of these data is complex because the UV−vis spectra of aminoxyl **2** and its anion and the half-life of the anion are not known. Nevertheless, the measured value in the presence of 4 mM nitrone **1** at pH 10 should approximate the reported values for the half-life of aminoxyl **2** in neutral pH, i.e., $k = 0.014$ s^{-1},[42,43] and even more that previously measured at pH 9, i.e., $k = 0.026$ s^{-1}.[42]

Under our experimental conditions in the absence of radiolytically formed $^{\bullet}OH$ radicals, we never detected the EPR signal characteristic of aminoxyl **3** or **6** following the reaction of superoxide radicals with the nitrones. This was true whether superoxide radicals were produced by pulse- or γ-irradiation. Hence, our results do not support the assumption frequently made that the decomposition of aminoxyl **2** yields aminoxyl **3**.[23,35]

Assuming, as our results suggest, that the pK_a of aminoxyl **2** is close to 10, the measured rate constant for the reaction of $O_2^{\bullet-}$ with nitrone **1** at pH 7.4−7.8 should be close to $k_{21} = 170 \pm 40$ M^{-1} s^{-1}. The latter value is considerably higher than those previously reported, i.e., 1.2 and 50 M^{-1} s^{-1}.[23,35−40] This apparent discrepancy can be explained as follows: As presented above for the reaction of nitrone **1** with $^{\bullet}OH$ or $R^{\bullet}$, only a fraction of the radicals yields the corresponding aminoxyls. The situation is, most likely, the same for $O_2^{\bullet-}$, where only part of the radicals yield aminoxyl **2** and the rest give rise to different species (denoted *X*):

$$\text{nitrone } \mathbf{1} + O_2^{\bullet-} \rightarrow \text{aminoxyl } \mathbf{2} \qquad k_{21a} \qquad (21a)$$

$$\text{nitrone } \mathbf{1} + O_2^{\bullet-} \rightarrow X \qquad k_{21b} \qquad (21b)$$

Hence, rate eqs 23 and 24 are obtained:

$$-d[O_2^{\bullet-}]/dt = (k_{21a} + k_{21b})[\text{nitrone } \mathbf{1}][O_2^{\bullet-}] \qquad (23)$$

$$d[\text{aminoxyl } \mathbf{2}]/dt = k_{21a}[\text{nitrone } \mathbf{1}][O_2^{\bullet-}] \qquad (24)$$

Though the *rate* of the decay of $O_2^{\bullet-}$ differs from that of the formation of aminoxyl **2** or *X*, the half-life of the decay of $O_2^{\bullet-}$ is equal to that of the formation of aminoxyl **2** or *X*, i.e., the *rate constant* of the formation of aminoxyl **2** is the same as that of $O_2^{\bullet-}$ decay. In the present study, we followed the absorption changes associated with $O_2^{\bullet-}$ disappearance and aminoxyl **2** formation and obtained ($k_{21a} + k_{21b}$). However, in previous studies the **differential rate equation** (eq 24) for aminoxyl **2** served for kinetics analysis using the concentration of the nitrone and the **initial rate** of aminoxyl **2** accumulation, which was followed by EPR. Such an analysis does not yield ($k_{21a} + k_{21b}$) but rather k_{21a}. Hence, the yield of aminoxyl **2** does not exceed 30% of $O_2^{\bullet-}$ reacting with nitrone **1**, i.e., 50/170. Such an analysis could be further complicated if $O_2^{\bullet-}$ reacts with the persistent aminoxyl **2** and affect its accumulation. This reaction is also supported by our recent observations where we showed that $HO_2^{\bullet}$ reacts with various aminoxyls much faster than with nitrones.[44] Consequently the analysis of the kinetics of the accumulation of aminoxyls becomes even more complex, which leaves pulse radiolysis solely as a valid tool for determination of accurate rate constants.

Conclusions

The rate constants for the reaction of nitrone **1** with $^{\bullet}OH$, $CO_2^{\bullet-}$, $^{\bullet}C(OH)(CH_3)_2$, and $^{\bullet}CH_2OH$ were determined directly to be $(3.3 \pm 0.1) \times 10^9$, $(1.15 \pm 0.05) \times 10^8$, $(6.6 \pm 0.3) \times 10^7$, and $(4.4 \pm 0.4) \times 10^7$ M^{-1} s^{-1}, respectively. The rate constants for the reaction of nitrone **4** with $^{\bullet}OH$, $CO_2^{\bullet-}$, $^{\bullet}C(OH)(CH_3)_2$, $^{\bullet}CH_2OH$, and $^{\bullet}CH_3$ were determined to be $(2.5 \pm 0.1) \times 10^9$, $(1.8 \pm 0.1) \times 10^8$, $(1.4 \pm 0.1) \times 10^8$, $(5.4 \pm 0.2) \times 10^7$, and $(2.8 \pm 0.2) \times 10^7$ M^{-1} s^{-1}, respectively. About 30% of $^{\bullet}OH$ radicals add to nitrones **1** and **4** at position 2, yielding the corresponding persistent aminoxyls. The remaining $^{\bullet}OH$ radicals form transient intermediates that decay bimolecularly. These transient intermediates react with oxygen with rate constants that are significantly lower than those generally reported for alkyl radicals. This suggests that these transient intermediates are not simple carbon-centered radicals resulting upon H-abstraction from the methyl or methylene groups of the nitrones, but more likely delocalized radicals resulting from the addition of $^{\bullet}OH$ to the nitrogen of the N═C bond that subsequently yields an allylic radical. The failure of nitrone **4** at 0.5 mM to affect the decay kinetics of $O_2^{\bullet-}$ at pH 10 implies an upper limit of 3 M^{-1} s^{-1} for this reaction. The rate constant for the reaction of nitrone **1** with $O_2^{\bullet-}$ has been determined to be 170 ± 40 M^{-1} s^{-1}. This value is significantly higher than those previously determined by following the formation of aminoxyl **2** by EPR, which indicates that the yield of aminoxyl **2** is only a small fraction of $O_2^{\bullet-}$ radicals that react with nitrone **1**.

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