Kinetics and Mechanism of Peroxyl Radical Reactions with Nitroxides

Sara Goldstein*,† and Amram Samuni‡

Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, and Department of Molecular Biology, The Hebrew University of Jerusalem, Hadassah Medical School, Jerusalem 91120, Israel

Received: August 29, 2006; In Final Form: November 21, 2006

Cyclic nitroxides (>NO•) are stable radicals of diverse size, charge, lipophilicity, and cell permeability, which provide protection against oxidative stress via various mechanisms including SOD-mimic activity, oxidation of reduced transition metals and detoxification of oxygen- and nitrogen-centered radicals. However, there is no agreement regarding the reaction of nitroxides with peroxyl radicals, and many controversies in the literature exist. The question of whether nitroxides can protect by scavenging peroxyl radicals is important because peroxyl radicals are formed in biological systems. To further elucidate the mechanism(s) underlying the antioxidative effects of nitroxides, we studied by pulse radiolysis the reaction kinetics of piperidine, pyrrolidine, and oxazolidine nitroxides with several alkyl peroxyl radicals. It is demonstrated that nitroxides mainly reduce alkyl peroxyl radicals forming the respective oxoammonium cations (>N^+═O). The most efficient scavenger of peroxyl radicals is 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TPO), which has the lowest oxidation potential among the nitroxides tested in the present study. The rate constants of peroxyl reduction are in the order $CH_2(OH)OO^\bullet$ > $CH_3OO^\bullet$ > *t*-$BuOO^\bullet$, which correlate with the oxidation potential of these peroxyl radicals. The rate constants for TPO vary between 2.8×10^7 and 1.0×10^8 M^{-1} s^{-1} and for 3-carbamoylproxyl (3-CP) between 8.1×10^5 and 9.0×10^6 M^{-1} s^{-1}. The efficacy of protection of nitroxides against inactivation of glucose oxidase caused by peroxyl radicals was studied. The results demonstrate a clear correlation between the kinetic features of the nitroxides and their ability to inhibit biological damage inflicted by peroxyl radicals.

Introduction

The ever-increasing knowledge of the involvement of radicals in diverse pathological processes has expanded the search for more efficient antioxidants that will diminish radical-induced damage. The most prevalent dogma attributes biological injury mostly to •OH radicals that are highly reactive. Accumulating evidence implicates other secondary radicals, such as peroxyl radicals, which are less reactive, migrate longer distances and are much more selective. Cyclic nitroxides (>NO•) are cell-permeable stable radicals of diverse size, charge, and lipophilicity, which effectively protect cells, tissues, organs, and whole animals from radicals-induced damage.[1−6] Their protective effects apparently derive from their ability to scavenge radicals,[7−13] catalyze superoxide dismutation,[14−16] facilitate catalase-like activity of heme-proteins,[17] detoxify hypervalent metals, and oxidize reduced transition metals ions.[17,18] Nitroxides react readily with •OH radicals.[7,10] However, efficient scavenging of •OH radicals within the cell is practically impossible as a result of their rapid reaction with cellular constituents. Instead, the protective activity of nitroxides may result from reactions with secondary intermediates such as peroxyl radicals, which are less reactive than •OH radicals but potentially more toxic because they are much more selective. It has been shown that nitroxides inhibit radical-mediated lipid peroxidation in fatty acid micelles,[19,20] liposomal membranes,[21] low-density lipoproteins[22] and microsome.[4,23,24] These processes require O_2 and involve formation of peroxyl intermediates. The information concerning the reaction of nitroxides with peroxyl radicals is scarce. Barton et al.[25] reported that 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TPO) reacts with *tert*-butylperoxyl radicals to catalyze the formation of dioxygen, and found a rather stable peroxide product, which they assigned to a trioxide species. Offer and Samuni[26] showed that TPO, 4-OH-TPO and 4-NH_2-TPO protected by reacting with and detoxifying the peroxyl radical against DNA damage and enzyme inactivation. However, Brede et al.[9] determined by pulse radiolysis an upper limit of 1×10^5 M^{-1} s^{-1} for the reaction of several alkylperoxyl radicals with TPO and 4-OH-TPO. This upper limit is considerably lower than the rate constants 1.2×10^8 or 2.8×10^7 M^{-1} s^{-1}, previously determined for the reaction of $HOO^\bullet$ with TPO or 4-OH-TPO, respectively.[16,27] To date, the mechanism(s) underlying the protective effects of nitroxides have not been sufficiently elucidated, and the reaction products derived from their reactions have not been yet identified. In fact, it is still debated whether nitroxides detoxify peroxyl radicals at all. To gain better understanding of the antioxidative activity of nitroxides, we studied by pulse radiolysis the reactions of different piperidine, pyrrolidine, and oxazolidine nitroxides with several alkyl peroxyl radicals, and we correlated the kinetics features with their effect on inactivation of glucose oxidase induced by peroxyl radicals.

Experimental

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

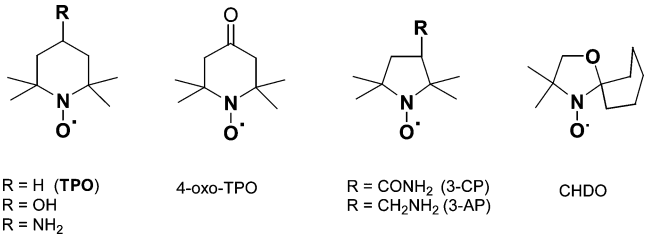
* Corresponding author. Telephone: 972-2-6586478. Fax: 972-2-6586925. E-mail: sarag@vms.huji.ac.il.

† Department of Physical Chemistry, The Hebrew University of Jerusalem.

‡ Department of Molecular Biology, The Hebrew University of Jerusalem, Hadassah Medical School.

10.1021/jp0655975 CCC: $37.00
Published on Web 01/19/2007

SCHEME 1



system. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{2-}$) and glucose oxidase (GOx) from *Aspergillus niger* (EC 1.1.3.4) were purchased from Sigma. The nitroxides TPO, 4-OH-TPO, 4-amino-TPO, 3-carbamoylproxyl (3-CP), 3-aminomethylproxyl (3-AP) and 2-cyclohexane-5,5-dimethyl-3-oxazolidine-1-oxyl (CHDO) were purchased from Aldrich, and 4-oxo-TPO was obtained from Alexis Biochemicals. Scheme 1 displays the structures of the nitroxide derivatives studied.

The hydroxylamines derived from TPO, 4-OH-TPO, and 3-CP were prepared by catalytic reduction using H_2 bubbled over Pt powder or by bubbling HCl gas through ethanolic solution of the nitroxide followed by drying. Solutions of hydroxylamines were freshly prepared before each experiment in order to minimize their oxidation to the corresponding nitroxides. Stock solutions of CHDO and 3-AP were prepared in CH_3CN, which reacts relatively slow with all the radicals formed by radiolysis. The concentration of formaldehyde was determined using the Nash reagent (2 M ammonium acetate, 0.05 M acetic acid, and 0.02 M acetylacetone).[28] The reagent was mixed with an equal volume of the tested solution, and after incubation for about an hour at 38 °C, the absorption at 412 nm was read. Hydroperoxides were analyzed by a molybdate-activated iodide assay.[29] The hydroperoxide concentration was determined by the addition of 1 mL of 0.15 M biphthalate buffer (pH 4), 1 mL of 0.3 M NaI, and 50 μL of 2.5 mM ammonium molybdate to 1 mL of irradiated solution. In view of the relatively slow oxidation of iodide by the organic hydroperoxides,[30] the buildup of I_3^- was followed at 350 nm until a plateau was reached. The concentration of the hydroperoxide was calculated using $\epsilon(I_3^-)_{350} = 25\,000\ M^{-1}\ cm^{-1}$. The organic hydroperoxide concentration was determined after the addition of 50 μL of 1.5×10^4 unit/mL of catalase to 1 mL of the irradiated sample.

GOx Activity. The activity of GOx was determined by measuring the initial rate of the formation of H_2O_2 at 240 nm ($\epsilon = 39.4\ M^{-1}\ cm^{-1}$), which was formed when the enzyme was added to O_2-saturated solution containing 0.1 M D-glucose and 20 mM phosphate buffer (PB) at pH 5.1 and 35 °C.

Apparatus. Pulse radiolysis experiments were carried out with a Varian 7715 linear accelerator with 5-MeV electron pulses of 0.2−1.5 μs and 200 mA. All measurements were made at ambient temperature in a 4 cm spectrosil cell using three light passes (optical path length 12.1 cm). Dosimetry was done using aerated solutions containing 10 mM KSCN by following the formation of $(SCN)_2^{\bullet-}$ at 475 nm ($G\epsilon_{475} = 2.3 \times 10^{-4}$ M $Gy^{-1}\ cm^{-1}$) or solutions saturated with 80% N_2O and 20% O_2 containing 10 mM KSCN and 100 μM $ABTS^{2-}$ by following the formation of $ABTS^{\bullet-}$ at 416 nm ($G\epsilon_{416} = 2.1 \times 10^{-4}$ M $Gy^{-1}\ cm^{-1}$).

Steady-state γ-irradiation experiments were carried out at room-temperature using a ^{137}Cs source. The dose rate was determined by the Fricke dosimeter to be 8.1 Gy min^{-1}.

Results

Production of Alkyl Peroxyl Radicals (ROO•). ROO• was generated upon irradiation of aqueous solutions saturated with $N_2O/O_2 = 4{:}1$ (20−12.5 mM N_2O and 0.24−0.6 mM O_2, respectively), which contained the organic compound RH (CH_3OH, $(CH_3)_3COH$ (*t*-BuOH), or dimethyl sulfoxide (DMSO)) and 1−4 mM PB at pH >3. Under these conditions ca. 90% of all the radicals formed by the irradiation are converted into ROO•, and ca. 10% form superoxide (eqs 1−6):

$$H_2O \xrightarrow{\gamma} e^-_{aq}(2.7),\ {}^{\bullet}OH\ (2.8),\ H^{\bullet}\ (0.6),\ H_2\ (0.45),\ H_2O_2\ (0.7),\ H_3O^+\ (2.6) \quad (1)$$

Reaction 1 represents the radiation-induced formation of the primary radicals and molecular species. The numbers in parentheses are the *G* values at low scavenger concentrations, which represent the concentrations of the species (in 10^{-7} M Gy^{-1}), and are somewhat higher in the presence of high solute concentrations.

$$e^-_{aq} + N_2O + H_2O \rightarrow {}^{\bullet}OH + N_2 + OH^- \quad k_2 = 9.1 \times 10^9\ M^{-1}\ s^{-1}\ [31] \quad (2)$$

$$^{\bullet}OH + RH \rightarrow R^{\bullet} + H_2O \quad k_3 \geq 6 \times 10^8\ M^{-1}\ s^{-1}\ [31] \quad (3)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad k_4 \geq 1.6 \times 10^9\ M^{-1}\ s^{-1}\ [31] \quad (4)$$

$$H^{\bullet} + O_2 \rightarrow HOO^{\bullet} \quad k_5 = 1.2 \times 10^{10}\ M^{-1}\ s^{-1}\ [31] \quad (5)$$

$$HOO^{\bullet} \rightleftharpoons H^+ + O_2^{\bullet-} \quad pK_a = 4.8\ [32] \quad (6)$$

Peroxyl radicals decay either bimolecularly or undergo a number of unimolecular processes.[33] Alkyl peroxyl radicals, e.g., $CH_3OO^{\bullet}$ and *t*-$BuOO^{\bullet}$, react bimolecularly, forming tetroxide

TABLE 1: Summary of Rate Constants for Reactions 4 and 7−9

	$^{\bullet}CH_2C(CH_3)_2OH$	$^{\bullet}CH_3$	$^{\bullet}CH_2OH$
k_4	$1.6 \times 10^9\ M^{-1}\ s^{-1}$ [31]	$4. \times 10^9\ M^{-1}\ s^{-1}$ [31]	$4.5 \times 10^9\ M^{-1}\ s^{-1}$ [31]
k_7	$4.0 \times 10^8\ M^{-1}\ s^{-1}$ [34]	$4.0 \times 10^8\ M^{-1}\ s^{-1}$ [50]	$(2-11) \times 10^8\ M^{-1}\ s^{-1}$ [31]
			$4.1 \times 10^8\ M^{-1}\ s^{-1}$ [a]
k_8			$10 + 2 \times 10^6[HPO_4^{2-}]\ s^{-1}$ [31]
k_9 (TPO)	$1.5 \times 10^8\ M^{-1}\ s^{-1}$ [7]	$2.4 \times 10^9\ M^{-1}\ s^{-1}$	$4.4 \times 10^8\ M^{-1}\ s^{-1}$ [7]
			$9.9 \times 10^8\ M^{-1}\ s^{-1}$ [a]
k_9 (4-oxo-TPO)	$2.8 \times 10^8\ M^{-1}\ s^{-1}$ [7]	$7.6 \times 10^8\ M^{-1}\ s^{-1}$ [a]	$7.2 \times 10^8\ M^{-1}\ s^{-1}$ [7]
k_9 (4-OH-TPO)	NA	$8.8 \times 10^8\ M^{-1}\ s^{-1}$ [a]	$8.7 \times 10^8\ M^{-1}\ s^{-1}$ [a]
k_9 (4-amino-TPO)	NA	$6.7 \times 10^8\ M^{-1}\ s^{-1}$ [a]	NA
k_9 (3-CP)	$1.8 \times 10^8\ M^{-1}\ s^{-1}$ [37]	$7.5 \times 10^8\ M^{-1}\ s^{-1}$ [37]	$3.5 \times 10^8\ M^{-1}\ s^{-1}$ [37]
			$4.4 \times 10^8\ M^{-1}\ s^{-1}$ [a]
k_9 (3-AP)		$4.7 \times 10^8\ M^{-1}\ s^{-1}$ [a]	NA

[a] This study. [b] NA: not available.

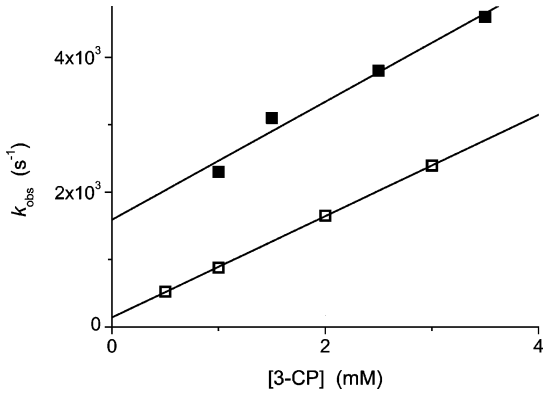


Figure 1. Reaction of *t*-BuOO• with 3-CP. Dependence of kobs of the decay of *t*-BuOO• on [3-CP] in the absence (solid symbols) and the presence (empty symbols) of 100 μM $ABTS^{2-}$. Solutions were saturated with 80% N_2O and 20% O_2 and contained 0.5 M *tert*-butanol and 4 mM PB at pH 7.0. The difference between the intercepts reflects the use of 1.3 and 0.12 Gy/pulse in the absence and presence of $ABTS^{2-}$, respectively.

intermediates, which decompose to give different products including ca. 27% superoxide.[33,34]

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOOOR \qquad (7)$$

When there is a hydroxyl functional group in the α-position to the peroxyl radical, e.g., $CH_2(OH)OO^{\bullet}$, it undergoes also a base-catalyzed elimination of $HOO^{\bullet}$ (reaction 8).[35,36]

$$CH_2(OH)OO^{\bullet} \rightarrow CH_2O + H^+ + O_2^{\bullet -} \qquad (8)$$

$CH_2(OH)OO^{\bullet}$ radicals were produced by pulse irradiation of N_2O/O_2 saturated solution containing 0.5 M CH_3OH and only 1 mM PB at pH 5−6 to slow down the self-decomposition of the peroxyl radicals. Indeed, the decay of $CH_2(OH)OO^{\bullet}$ followed at 290 nm ($\epsilon_{290} = 500\ M^{-1}\ cm^{-1}$) obeyed second-order kinetics ($k_7 = (4.1 \pm 1.1) \times 10^8\ M^{-1}\ s^{-1}$) indicating that under such conditions reaction 7 competes efficiently with reaction 8.

Kinetics of the Reaction of ROO• with Nitroxides. Nitroxides readily react with carbon-centered radicals forming relatively stable adducts (reaction 9).[8,37]

$$>NO^{\bullet} + R^{\bullet} \rightarrow >NO-R \qquad (9)$$

Therefore, the yields of $ROO^{\bullet}$ depend on the ratio $k_4[O_2]/k_9$[nitroxide], and the contribution of reaction 10 depends on the yield of $ROO^{\bullet}$ and to a lesser extent also on the ratio k_{10}[nitroxide]/($2k_7$[$ROO^{\bullet}$] + k_8) (Table 1).

$$ROO^{\bullet} + >NO^{\bullet} \rightarrow \text{products} \qquad (10)$$

The rate constant of reaction 10 was determined either by following directly the absorption decay of $ROO^{\bullet}$ at 270−300 nm or indirectly using $ABTS^{2-}$ as a detector molecule. $ABTS^{2-}$ reacts relatively slowly with nitroxides, $CH_3OO^{\bullet}$,[31] *t*-BuOO• and $CH_2(OH)OO^{\bullet}$ (present results), but is readily oxidized by the product of reaction 10 to $ABTS^{\bullet -}$, which absorbs highly in the visible region ($\epsilon_{416} = 36\,000\ M^{-1}\ cm^{-1}$; $\epsilon_{660} = 12\,000\ M^{-1}\ cm^{-1}$). The use of $ABTS^{2-}$ was particularly useful in cases where the absorption of the nitroxide in the UV region interferes with the direct determination, and when low concentrations of peroxyl radicals were essential to minimize their bimolecular self-decomposition (reaction 7).

The same k_{10} values were obtained either directly or indirectly using $ABTS^{2-}$ as a marker. Typical results are shown in Figure 1 for the reaction of 3-CP with *t*-BuOO•, resulting in $k_{10} = (8.1 \pm 0.6) \times 10^5\ M^{-1}\ s^{-1}$. The intercept in Figure 1 reflects the contribution of the self-decomposition of *t*-BuOO•, and is about 10-times higher in the direct experiment following the decay of *t*-BuOO• because its yield was about 10-times higher than that in the indirect experiment following the formation of $ABTS^{\bullet -}$. Figures 2 and 3 show the results for the reactions of the nitroxides in the presence of $ABTS^{2-}$ with $CH_3OO^{\bullet}$ and $CH_2(OH)OO^{\bullet}$, respectively.

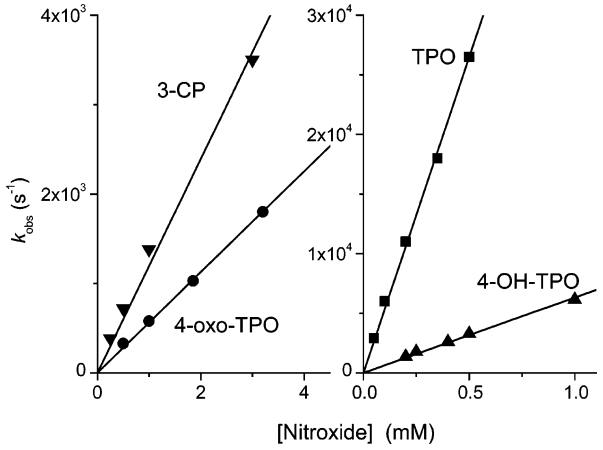


Figure 2. Reaction of $CH_3OO^{\bullet}$ with nitroxides. Dependence of k_{obs} of the decay of $CH_3OO^{\bullet}$ on [nitroxide] upon pulse-irradiation of solutions saturated with 80% N_2O and 20% O_2 and containing 0.2 M DMSO, 100 μM $ABTS^{2-}$, and 4 mM phosphate buffer (pH 7.0). The dose was 0.12 Gy/pulse.

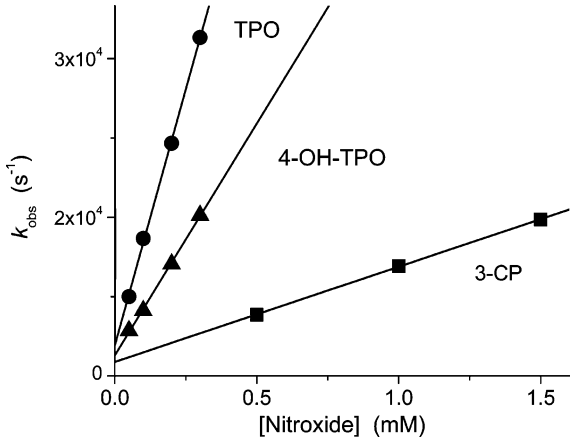


Figure 3. Reaction of nitroxides with $CH_2(OH)OO^{\bullet}$. Dependence of k_{obs} of the decay of $CH_2(OH)OO^{\bullet}$ on [nitroxide] upon pulse-irradiation (0.12 Gy/pulse) of solutions saturated with 80% N_2O and 20% O_2 and contained 0.5 M methanol, 100 μM $ABTS^{2-}$, and 1 mM PB at pH 5.0 (3-CP) or 6.0 (TPO, 4-OH-TPO).

The rate constants of the reactions of $CH_3OO^{\bullet}$ and *t*-BuOO• with the pyrrolidine nitroxides were hardly affected by increasing [PB] from 4×10^{-3} to 0.4 M at pH 7.0, but they increased in the case of the piperidine nitroxides (Figures 4 and 5). The apparent bimolecular rate constants (k_{app}) in Figures 4 and 5 were obtained from the dependence of k_{obs} on [nitroxide] at constant [PB] and pH. A pronounced downward curvature of the plot of k_{app} dependence on [PB] was observed only in the case of 4-amino-TPO (Figures 4 and 5). For all piperidine nitroxides, k_{app} decreased ca. 5-folds upon increasing the pH from 6.9 to 8.0 at constant [PB] indicating that the reaction is catalyzed by $H_2PO_4^-$. The reaction could not be studied in acidic solutions because of too rapid oxidation of the nitroxides by $HOO^{\bullet}$ ($pK_a = 4.8$) (Table 2).[16,27] In the case of $CH_2(OH)OO^{\bullet}$,

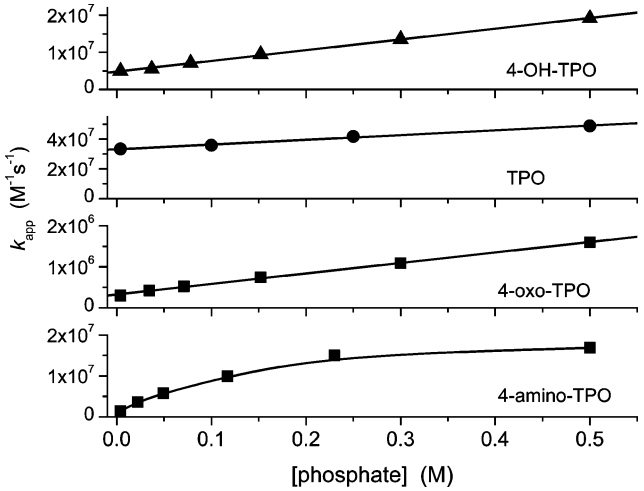


Figure 4. Effect of phosphate concentration on the rate of the reaction of *t*-BuOO$^{\bullet}$ with piperidine nitroxides. The formation of ABTS$^{\bullet-}$ upon pulse irradiation (0.14 Gy/pulse) of N_2O/O_2 saturated solutions containing 0.5 M *tert*-butanol, 100 μM $ABTS^{2-}$ and PB at pH 6.9 was followed. k_{app} was obtained from the dependence of k_{obs} on [nitroxide] at constant [PB].

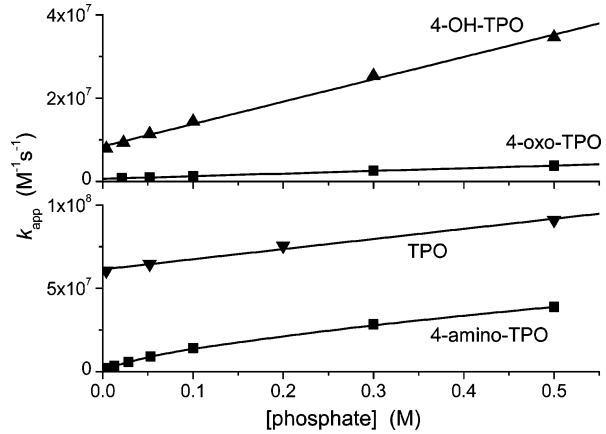


Figure 5. Effect of phosphate concentration on the rate constant of $CH_3OO^{\bullet}$ reaction with piperidine nitroxides. The formation of ABTS$^{\bullet-}$ upon pulse irradiation (0.14 Gy/pulse) of N_2O/O_2 saturated solutions containing 0.2 DMSO, 100 μM $ABTS^{2-}$, and PB at pH 6.9 was followed.

the concentration of PB could not be increased because the self-decomposition of $CH_2(OH)OO^{\bullet}$ is catalyzed by PB.

The k_{10} values derived from the dependence of k_{obs} on [nitroxide] are summarized in Table 2. In the case of the piperidine nitroxides, the extrapolated values to zero [PB] are included in Table 2.

Product Analysis. The optical absorption of the nitroxides following γ-irradiation was studied under conditions where the contribution of the reaction of the nitroxide with the alkyl radical (reaction 9) was negligible, i.e., solutions saturated with 50% N_2O and 50% O_2 containing 50−200 μM nitroxide. In the presence of 20 mM DMSO (4 mM PB, pH 7.0), the absorption peak of the nitroxide decreased with increasing the dose and $-\Delta[\text{nitroxide}]/[CH_3OO^{\bullet}]_T \approx 1$. In the presence of 0.5 M *t*-BuOH (4 mM PB, pH 7.0), no effect on the spectrum of 3-CP was observed whereas TPO^+ and the products of the self-decomposition of 4-oxo-TPO^+ were identified spectrophotometrically.[10,12] In the presence of 0.5 M CH_3OH (1 mM PB, pH 5.7), no effect on the spectrum of 3-CP was observed while TPO was consumed $-\Delta[TPO]/[CH_2(OH)OO^{\bullet}]_T \approx 1$, i.e., $G(\text{-TPO}) \approx 5.5$. In the latter systems we determined the yields of CH_2O and hydroperoxides. Under all experimental conditions these yields increased linearly with the dose. In the absence of the nitroxides we determined $G(CH_2O) = 5.6 \pm 0.2$ and $G(H_2O_2) = 3.8 \pm 0.1$. These results are in agreement with reaction 8, dismutation of $O_2^{\bullet-}$ into O_2 and H_2O_2 and the radiolytically produced $O_2^{\bullet-}$ ($G = 0.6$) and H_2O_2 ($G = 0.72$). In the presence of 150 μM TPO we found that $G(\text{-TPO}) \approx G(H_2O_2 + HOCH_2OOH) \approx 0.5G(CH_2O) \approx 5.7 \pm 0.3$, and in the presence of 150 μM 3-CP $G(CH_2O) \approx G(H_2O_2 + HOCH_2OOH) = 5.6 \pm 0.3$. The yields of the hydroperoxides decreased to zero after the addition of catalase indicating that under our experimental conditions $HOCH_2OOH$ decomposes into CH_2O and H_2O_2.[38]

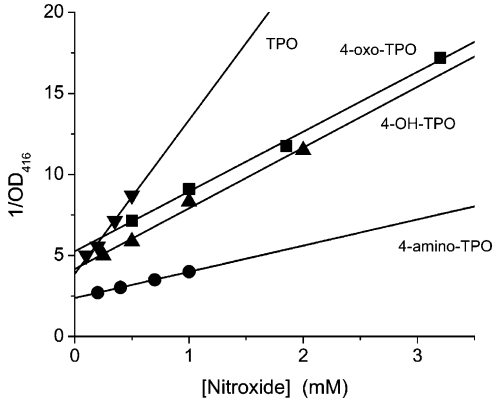


Figure 6. Yields of ABTS$^{\bullet-}$ formed in the reaction of $CH_3OO^{\bullet}$ with TPO, 4-oxo-TPO, 4-amino-TPO, or 4-OH-TPO. The reciprocal yield of ABTS$^{\bullet-}$ vs [nitroxide] measured upon pulse-irradiation of solutions saturated with 80% N_2O and 20% O_2 and contained 0.2 M DMSO and 100 μM $ABTS^{2-}$. In the case of TPO, 4-oxo-TPO and 4-OH-TPO, [PB] = 4 mM and the dose was 0.11 or 0.12 Gy/pulse. In the case of 4-amino-TPO, [PB] = 0.2 M and the dose 1.7 Gy/pulse.

The yield of ABTS$^{\bullet-}$ formed as a result of reaction 10 was determined under the conditions where the contribution of nitroxide reaction with R$^{\bullet}$ (reactions 9) and the self-recombination of the ROO$^{\bullet}$ (reaction 7) are negligible, i.e., when varying [nitroxide] had no effect on the yield. Alternatively, we determined the effect of [nitroxide] on the yield of ABTS$^{\bullet-}$ at constant radiation dose, i.e., from the intercept of the line obtained by plotting 1/[ABTS$^{\bullet-}$] vs [nitroxide] (e.g., Figure 6). The results are summarized in Table 3. In some of the cases, e.g., relatively low k_{10}, even the use of relatively high [nitroxide]

TABLE 2: Summary of the Rate Constants (M^{-1} s^{-1}) for the Uncatalyzed Reaction of ROO$^{\bullet}$ with Nitroxides

	t-BuOO$^{\bullet}$	$CH_3OO^{\bullet}$	$CH_2(OH)OO^{\bullet}$	$HOO^{\bullet}$ [a]
3-CP	$(8.1 \pm 0.6) \times 10^5$	$(1.1 \pm 0.1) \times 10^6$	$(9.0 \pm 0.1) \times 10^6$	$(1.1 \pm 0.1) \times 10^6$
3-AP	$(9.6 \pm 0.6) \times 10^5$	$(1.5 \pm 0.6) \times 10^6$	ND	$(1.1 \pm 0.2) \times 10^6$
CHDO	$(5.0 \pm 0.5) \times 10^4$	$(6.7 \pm 0.5) \times 10^4$	ND	$(1.6 \pm 0.2) \times 10^5$
TPO	$(2.8 \pm 0.2) \times 10^7$	$(5.1 \pm 0.1) \times 10^7$	$(1.0 \pm 0.1) \times 10^8$	$(1.1 \pm 0.1) \times 10^8$
4-OH-TPO	$(3.3 \pm 0.2) \times 10^6$	$(5.4 \pm 0.2) \times 10^6$	$(4.4 \pm 0.1) \times 10^7$	$(2.7 \pm 0.3) \times 10^7$
4-amino-TPO	$(1.0 \pm 0.2) \times 10^6$	$(1.5 \pm 0.2) \times 10^6$	ND[b]	$<1 \times 10^7$
4-oxo-TPO	$(2.8 \pm 0.2) \times 10^5$	$(5.4 \pm 0.2) \times 10^5$	ND	$<6 \times 10^6$

[a] Calculated for comparison from the data in refs 16 and 27. [b] ND: not determined.

TABLE 3: Percent Yields of $ABTS^{\bullet-}$ ($100 \times [ABTS^{\bullet-}]/[ROO^{\bullet}]_{Total}$) Formed via Reaction 10

	t-BuOO$^{\bullet}$	$CH_3OO^{\bullet}$	$CH_2(OH)OO^{\bullet}$
4-amino-TPO	85 ± 2	98 ± 3^b	ND[c]
TPO	75 ± 3	85 ± 5^b	95 ± 5^b
4-OH-TPO	84 ± 5	90 ± 5^b	96 ± 9^b
4-oxo-TPO	$\geq 55^a$	75 ± 4^b	ND
3-CP	$\geq 47^a$	$\geq 65^a$	82 ± 8^b
3-AP	$\geq 52^a$	72 ± 8^b	ND
CHDO	$\geq 20^a$	$\geq 20^a$	ND

[a] The reaction was relatively slow and therefore relatively high concentrations of the nitroxides were used, which enables only the determination of a lower limit. [b] Determined from the intercept of the line obtained by plotting $1/[OD]_{416}$ vs [nitroxide] at constant [PB] and pulse intensity. [c] ND: not determined.

enabled only the determination of a lower limit value for $ABTS^{\bullet-}$ yields (Table 3). From the slope/intercept ratios we calculated k_9, for nitroxide reaction with $R^{\bullet}$, which are included in Table 1.

Kinetics of the Reaction of ROO$^{\bullet}$ with Hydroxylamines. The decay of about 5 μM $CH_3OO^{\bullet}$ or *t*-BuOO$^{\bullet}$ at pH 7 was unaffected by the presence of 2.6 mM 3-CP-H, TPO-H, or 4-OH-TPO or TPO-H indicating that the rate constant for this reaction cannot exceed 1×10^5 M^{-1} s^{-1}. Therefore, contamination of the nitroxide with the respective hydroxylamine should not have any effect on the kinetic results. Under γ-radiolysis conditions, the formation of characteristic absorption of the respective nitroxides was observed.

GOx Inactivation by ROO$^{\bullet}$ and the Effect of Nitroxides. The activity of 8−200 μg/mL GOx was determined prior and after γ-irradiation of solutions saturated with 50% N_2O and 50% O_2 containing 0.5 M *t*-BuOH or 0.5 M CH_3OH and 5 mM acetate buffer at pH 5.6. Phosphate buffer was replaced by acetate to slow down the base-catalyzed elimination of $HOO^{\bullet}$ from the peroxyl radical (reaction 8), which becomes the main decomposition pathway under steady-state radiolysis. Superoxide radicals are formed radiolytically ($G = 0.6$) and during the self-decomposition of *t*-BuOO$^{\bullet}$[39] and $CH_2(OH)OO^{\bullet}$ radicals (reaction 8). Therefore, the effect of superoxide radicals on the inactivation of GOx was tested upon γ-irradiation of solutions saturated with O_2 containing 0.1 M formate and 5 mM acetate at pH 5.6. Under these conditions, where all the primary radicals formed by the radiation are converted into superoxide radicals, the activity of GOx was not impaired. This was also the case with *t*-BuOO$^{\bullet}$, i.e., GOx maintained its full activity even when subjected to 90 min of irradiation ($[ROO^{\bullet}]_{Total} \sim 470$ μM), which could not be further increased due to the consumption of oxygen. The inactivation of GOx by $CH_2(OH)OO^{\bullet}$ increased upon increasing the dose and interestingly with increasing the dilution at a constant dose. A similar effect of dilution has been previously reported.[40,41] Within 90 min of irradiation, GOx (8 μg/mL) lost about 68% of its activity. We compared the protective effects of TPO, 3-CP and CHDO against peroxyl-mediated inactivation of GOx because k_{10} for TPO is about 10-fold higher than that for 3-CP. We also assumed that it is at least 100-fold higher than that for CHDO as is the case with *t*-BuOO$^{\bullet}$ and $CH_3OO^{\bullet}$ (see Table 2). The results in Table 4 show that nitroxides protected the enzyme in a concentration dependent manner, and that the efficacy is in the order TPO > 3-CP > CHDO. The results were unaffected by the presence of 30 μM diethylenetriaminepentaacetc acid (DTPA).

TABLE 4: Effect of Nitroxides on Inactivated GOx by $Ch_2(OH)OO^{\bullet}$ Radicals Expressed in Percentage of Remaining Activity

[nitroxide] (μM)	TPO	3-CP	CHDO
0	32	32	32
2	56	44	
5	93	77	35
10	96	90	
20		91	43
50		96	78

[a] GOx was exposed to 90 min of γ-irradiation in solutions saturated with 50% N_2O and 50% O_2 containing 0.5 M CH_3OH and 5 mM acetate at pH 5.6.

Discussion

The present results demonstrate that the reaction of nitroxide with peroxyl radicals forms mainly the corresponding oxoammonium cation, which oxidizes efficiently $ABTS^{2-}$. In the case of *t*-BuOO$^{\bullet}$, we identified spectrophotometrically the formation of the stable TPO^+ and the decomposition products of the relatively unstable 4-oxo-TPO^+. As previously reported for $HOO^{\bullet}$,[27] the reaction of ROO$^{\bullet}$ with the piperidine nitroxides was catalyzed by $H_2PO_4^-$. Hence, under physiological conditions (pH 7.4, 50 mM phosphate), the apparent rate constants for the piperidine nitroxides are somewhat higher than the values listed in Table 2 (see Figures 4 and 5). Furthermore, catalysis by $H_2PO_4^-$ implies that nitroxide reaction with peroxyl (Reaction 10) takes place via an inner-sphere electron-transfer mechanism, where the decomposition of the adduct, >NOOOR, can undergo general acid catalysis (reactions 11−13).

$$>NO^{\bullet} + ROO^{\bullet} \rightleftharpoons >NOOOR \quad (11)$$

$$>NOOOR \rightarrow >N^+{=}O + RO_2^- \quad (12)$$

$$>NOOOR + H_2PO_4^- \rightarrow >N^+{=}O + ROOH + HPO_4^{2-} \quad (13)$$

Clearly, in the absence of catalysis, an adduct formation still can take place but an outer-sphere electron-transfer mechanism (reaction 14) cannot be excluded.

$$>NO^{\bullet} + ROO^{\bullet} \rightarrow >N^+{=}O + ROO^- \quad (14)$$

The formation of an unstable amino trioxide was previously suggested using an *ab initio* thermochemical study.[42] The decomposition of this adduct can also take place via homolysis forming $>NOO^{\bullet}$ and $RO^{\bullet}$, where the alkoxyl radical is also expected to oxidize $ABTS^{2-}$. However, the homolysis mechanism is ruled out because the reaction is catalyzed by $H_2PO_4^-$. Also, we identified the formation of the stable TPO^+ and the decomposition products of the relatively unstable 4-oxo-TPO^+ in the case of *tert*-butanol, which is not oxidized by these oxoammonium cations. It was also suggested that >NOOOR decomposes into O_2 and >NOR and that the latter undergoes C−O bond cleavage forming the starting nitroxide and ROO$^{\bullet}$.[42] Our present results do not support this mechanism.

Table 2 demonstrates that the most efficient scavenger of ROO$^{\bullet}$ is TPO, which has the lowest oxidation potential among the studied nitroxides (Table 5). The rate constant values are in the order: $CH_2(OH)OO^{\bullet}$ > $CH_3OO^{\bullet}$ > *t*-BuOO$^{\bullet}$, which correlates well with the oxidation potentials of the peroxyl radicals (Table 5).

Contrary to our findings, Brede et al.[9] determined using pulse radiolysis the rate constant for the reaction of "some" alkyl peroxyl radicals with TPO and 4-OH-TPO to be lower than 1×10^5 M^{-1} s^{-1}. Brede et al.[9] used a giant dose between 100 and 200 Gy, but other important experimental conditions are not given. The effect of the nitroxide on the decay of ROO$^{\bullet}$ depends on the initial concentration of ROO$^{\bullet}$, $k(ROO^{\bullet} + ROO^{\bullet})$,

TABLE 5: Oxidation Potential E° (mV) vs NHE for the Redox Couples $>N^+=O/>NO^\bullet$ and $ROO^\bullet/ROO^-$

	$>N^+=O/>NO^\bullet$	$ROO^\bullet/ROO^-$
TPO	722,[15] 740 [47]	
4-OH-TPO	810,[15] 825 [47]	
4-NH_3^+-TPO	826,[15] 851,[15,51,52] 872[15,51,52]	
4-oxo-TPO	913[15]	
3-$CH_2NH_3^+$-proxyl	853[15]	
3-carbamoylproxyl	860−870[15,51,52]	
CHDO	900[15]	
R = *t*-Bu		710[53]
R = CH_3		770,[54] 780 [55]
R = CH_2OH		830[55]
R = H		750[43]

the concentration of the nitroxide and the rate constant for the reaction of the nitroxide with $ROO^\bullet$. We have no way to check if the reported upper-limit rate constant of 1×10^5 M^{-1} s^{-1} is correct because the concentration of the nitroxide is not given, the "other" $ROO^\bullet$ are not specified and it is not clear if these experiments were done in aqueous or nonaqueous solutions. In addition, we previously determined relatively high rate constants for the uncatalyzed reaction of $HOO^\bullet$ ($E^\circ(HOO^\bullet/HOO^-) = 750$ mV[43]) with the nitroxides, e.g., 2.8×10^7 and 1.1×10^8 M^{-1} s^{-1} for TPO and 4-OH-TPO, respectively,[16,27] which support our present results.

The results using γ-radiolysis also support the formation of the oxoammonium ion as the main product of reaction 10 as follows. TPO is consumed in the methanol but not in the *t*-BuOH system. In addition, we determined G(-TPO) $\approx$ $G(H_2O_2)$ $\approx$ $0.5G(CH_2O)$ $\approx$ 5.6. These results are in agreement with the ability of TPO^+ to oxidize only primary and secondary alcohols to aldehydes and ketons, respectively, forming the respective hydroxylamine,[44,45] which has no appreciable absorption around the absorption peak of TPO, i.e., reactions 15−17. We note that reaction 16 was suggested to take place through the formation of an adduct as an intermediate.[44,46]

$$\text{TPO} + \text{HOCH}_2\text{OO}^\bullet \xrightarrow{H^+} \text{TPO}^+ + \text{HOCH}_2\text{OOH} \quad (15)$$

$$\text{TPO}^+ + \text{CH}_3\text{OH} \rightarrow \text{TPO-H} + \text{CH}_2\text{O} + \text{H}^+ \quad (16)$$

$$\text{HOCH}_2\text{OOH} \rightarrow \text{CH}_2\text{O} + \text{H}_2\text{O}_2 \quad (17)$$

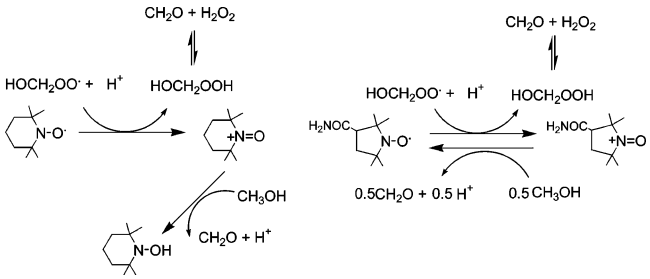
TPO can be regenerated by the reaction of TPO−H with TPO^+, but this reaction is too slow at pH 5.7 ($k = 2.6$ M^{-1} s^{-1}),[47] and does not compete efficiently with the reaction of TPO^+ with 0.5 M CH_3OH ($k_{11} = 0.5$ M^{-1} s^{-1}).[16] The reaction of 3-CP with $CH_2(OH)OO^\bullet$ is relatively fast, yet 3-CP is not consumed and $G(CH_2O) \approx G(H_2O_2) \approx 5.6$. We therefore assume that the reaction of 3-CP^+ with CH_3OH proceeds via the formation of an adduct, which reacts with another 3-CP before it decomposes into CH_2O and the respective hydroxylamine (reactions 18 and 19). An outer sphere electron-transfer mechanism forming 3-CP and $^\bullet CH_2OH$ is ruled out because we have previously shown that $O_2^{\bullet-}$ is not formed as an intermediate in this process.[16]

$$\text{3-CP}^+ + \text{CH}_3\text{OH} \rightarrow \text{adduct} + \text{H}^+ \quad (18)$$

$$\text{3-CP}^+ + \text{adduct} \rightarrow \text{3-CP} + \text{3-CP} + \text{CH}_2\text{O} + \text{H}^+ \quad (19)$$

4-oxo-TPO^+ is even a better oxidant than 3-CP^+, but it is relatively unstable,[10,12] and most probably its self-decomposition competes efficiently with its reaction with *t*-BuOH. The overall mechanisms for TPO and 3-CP in the methanol system are summarized in Scheme 2.

SCHEME 2



Peroxyl radicals derived from methanol inactivated GOx whereas those derived from *t*-BuOH were practically inactive demonstrating that in this system *t*-BuOH can serve as a useful $^\bullet OH$-scavenger without the formation of secondary toxic radicals. The inactivation of GOx by $CH_2(OH)OO^\bullet$ was inhibited in the presence of nitroxides. The extent of inhibition correlated well with the respective rate constant, i.e., the efficacy being in the order TPO > 3-CP > CHDO. In the present system, the nitroxide provided protection at extremely low concentrations most probably because it is recycled either via the reaction of 3-CP^+ with CH_3OH (Scheme 2) or via the reaction of TPO^+ with CH_3OH followed by the reaction of TPO-H with the peroxyl radical. Under physiological conditions, the nitroxides could detoxify peroxyl radicals because recycling of the nitroxide is expected through its reaction with common biological reductants such as thiols.[48,49]

Conclusions

Many controversies in the literature exist regarding the reaction of nitroxides with peroxyl radicals. The present results demonstrate that the reaction of nitroxide with peroxyl radicals forms mainly the corresponding oxoammonium cation. The most efficient peroxyl radical scavenger is TPO, which has the lowest oxidation potential among the studied nitroxides. The rate constants are in the order $CH_2(OH)OO^\bullet > CH_3OO^\bullet >$ *t*-$BuOO^\bullet$, which correlates with the oxidation potential of these peroxyl radicals, and for TPO it varies between 2.8×10^7 and 1.0×10^8 M^{-1} s^{-1}. The inactivation of GOx by $CH_2(OH)OO^\bullet$ was inhibited in the presence of nitroxides. The extent of inhibition correlated well with the respective rate constant, i.e., the efficacy being in the order TPO > 3-CP > CHDO. Nitroxides may provide protection at extremely low concentrations most probably because they are recycled through the reaction of the respective oxoammonium cations with common biological reductants.

Acknowledgment. This research was supported by grant from the Israel Science Foundation of the Israel Academy of Sciences.

References and Notes

(1) Mitchell, J. B.; Samuni, A.; Krishna, M. C.; DeGraff, W. G.; Ahn, M. S.; Samuni, U.; Russo, A. *Biochemistry* **1990**, *29*, 2802−2807.

(2) Samuni, A.; Godinger, D.; Aronovitch, J.; Russo, A.; Mitchell, J. B. *Biochemistry* **1991**, *30*, 555−561.

(3) Reddan, J. R.; Sevilla, M. D.; Giblin, F. J.; Padgaonkar, V.; Dziedzic, D. C.; Leverenz, V.; Misra, I. C.; Peters, J. L. *Exp. Eye Res.* **1993**, *56*, 543−554.

(4) Miura, Y.; Utsumi, H.; Hamada, A. *Arch. Biochem. Biophys.* **1993**, *300*, 148−156.

(5) Howard, B. J.; Yatin, S.; Hensley, K.; Allen, K. L.; Kelly, J. P.; Carney, J.; Butterfield, D. A. *J. Neurochem.* **1996**, *67*, 2045−2050.

(6) Chatterjee, P. K.; Cuzzocrea, S.; Brown, P. A. J.; Zacharowski, K.; Stewart, K. N.; Mota-Filipe, H.; Thiemermann, C. *Kidney Int.* **2000**, *58*, 658−673.

(7) Asmus, K. D.; Nigam, S.; Willson, R. L. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **1976**, *29*, 211−219.
(8) Bowry, V. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1992**, *114*, 4992−4996.
(9) Brede, O.; Beckert, D.; Windolph, C.; Gottinger, H. A. *J. Phys. Chem. A* **1998**, *102*, 1457−1464.
(10) Samuni, A.; Goldstein, S.; Russo, A.; Mitchell, J. B.; Krishna, M. C.; Neta, P. *J. Am. Chem. Soc.* **2002**, *124*, 8719−8724.
(11) Augusto, O.; Bonini, M. G.; Amanso, A. M.; Linares, E.; Santos, C. C. X.; De Menezes, S. L. *Free Radical Biol. Med.* **2002**, *32*, 841−859.
(12) Goldstein, S.; Samuni, A.; Russo, A. *J. Am. Chem. Soc.* **2003**, *125*, 8364−8370.
(13) Goldstein, S.; Samuni, A.; Merenyi, G. *Chem. Res. Toxicol.* **2004**, *17*, 250−257.
(14) Samuni, A.; Krishna, C. M.; Riesz, P.; Finkelstein, E.; Russo, A. *J. Biol. Chem.* **1988**, *263*, 17921−17924.
(15) Krishna, M. C.; Grahame, D. A.; Samuni, A.; Mitchell, J. B.; Russo, A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5537−5541.
(16) Goldstein, S.; Merenyi, G.; Russo, A.; Samuni, A. *J. Am. Chem. Soc.* **2003**, *125*, 789−795.
(17) Krishna, M. C.; Samuni, A.; Taira, J.; Goldstein, S.; Mitchell, J. B.; Russo, A. *J. Biol. Chem.* **1996**, *271*, 26018−26025.
(18) Bar-On, P.; Mohsen, M.; Zhang, R.; Feigin, E.; Chevion, M.; Samuni, A. *J. Am. Chem. Soc.* **1999**, *121*, 8070−8073.
(19) Antosiewicz, J.; Bertoli, E.; Damiani, E.; Greci, L.; Popinigis, J.; Przybylski, S.; Tanfani, F.; Wozniak, M. *Free Radical Biol. Med.* **1993**, *15*, 203−208.
(20) Noguchi, N.; Damiani, E.; Greci, L.; Niki, E. *Chem. Phys. Lipids* **1999**, *99*, 11−19.
(21) Damiani, E.; Castagna, R.; Greci, L. *Free Radical Biol. Med.* **2002**, *33*, 128−136.
(22) Damiani, E.; Paganga, G.; Greci, L.; Riceevans, C. *Biochem. Pharmacol.* **1994**, *48*, 1155−1161.
(23) Nilsson, U. A.; Carlin, G.; Bylundfellenius, A. C. *Chem.-Biol. Interact.* **1990**, *74*, 325−342.
(24) Antosiewicz, J.; Popinigis, J.; Wozniak, M.; Damiani, E.; Carloni, P.; Greci, L. *Free Radical Biol. Med.* **1995**, *18*, 913−917.
(25) Barton, D. H. R.; Le Gloahec, V. N.; Smith, J. *Tetrahedron Lett.* **1998**, *39*, 7483−7486.
(26) Offer, T.; Samuni, A. *Free Radical Biol. Med.* **2002**, *32*, 872−881.
(27) Goldstein, S.; Merenyi, G.; Samuni, A. *J. Phys. Chem. A.* **2006**, *110*, 3679−3685.
(28) Nash, T. *Biochem. J.* **1953**, *55*, 416−421.
(29) Allen, A. O.; Hochanadel, C. J.; Ghormley, J. A.; Davies, T. W. *J. Phys. Chem.* **1952**, *56*.
(30) Flyunt, R.; Leitzke, A.; von Sonntag, C. *Rad. Phys. Chem.* **2003**, *67*, 469−473.
(31) Mallard, W. G.; Ross, A. B.; Helman, W. P. *NIST Standard References Database 40, Version 3.0*; NIST: Washington, DC, 1998.
(32) Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041−1100.
(33) von Sonntag, C.; Schuchmann, H. P. In *Peroxyl Radicals*; Alfassi, Z. B., Ed.; Wiley: Chichester, U.K., 1997.
(34) Goldstein, S.; Lind, J.; Merenyi, G. *J. Phys. Chem. A* **2004**, *108*, 1719−1725.
(35) Ilan, Y.; Rabani, J.; Henglein, A. *J. Phys. Chem.* **1976**, *80*, 1558−1562.
(36) Bothe, E.; Schuchmann, M. N.; Schultefrohlinde, D.; Vonsonntag, C. *Photochem. Photobiol.* **1978**, *28*, 639−644.
(37) Nigam, S.; Asmus, K. D.; Willson, R. L. *J. Chem. Soc. Faraday Trans. 2* **1976**, 2324 -2340.
(38) Zhou, X. L.; Lee, Y. N. *J. Phys. Chem.* **1992**, *96*, 265−272.
(39) Merenyi, G.; Lind, J.; Goldstein, S. *J. Am. Chem. Soc.* **2002**, *124*, 40−48.
(40) Walter, D. M. *Br. J. Radiobiol.* **1943**, *16*, 338−339.
(41) Laser, H. *Proc. Symp. Biol. Effects Ionizing Radiation Mol. Level Brno* **1962**, 161−174.
(42) Stipa, P. *J. Chem. Soc., Perkin Trans. 2* **2001**, 1793−1797.
(43) Stanbury, D. M. *Adv. Inorg. Chem.* **1989**, *33*, 69−138.
(44) Bobbitt, J. M.; Flores, C. L. *Hetrocycles* **1988**, *27*, 509−517.
(45) Bobbitt, J. M. *J. Org. Chem.* **1998**, *63*, 9367−9374.
(46) Ganiev, M.; Suvorkina, E. S.; Igoshina, A. V.; Kabal'nova, N. N.; Imashev, U. B.; Tolstikov, G. A. *Russ. Chem. Bull.* **2002**, *51*, 982−985.
(47) Israeli, A.; Patt, M.; Oron, M.; Samuni, A.; Kohen, R.; Goldstein, S. *Free Radical Biol. Med.* **2005**, *38*, 317−324.
(48) Liu, Y.; Zhang, F. *Huaxue Xuebao* **1989**, *47*, 411−416.
(49) Maeda, H.; Wu, H.-Y.; Yamauchi, Y.; Ohmori, H. *J. Org. Chem.* **2005**, *70*, 8338−8343.
(50) Goldstein, S.; Lind, J.; Merenyi, G. *J. Chem. Soc. Dalton Trans.* **2002**, 808−810.
(51) Morris, S.; Sosnovsky, B.; B.; H.; Huber, C. O.; Rau, N. U. M.; Swartz, H. M. *J. Pharm. Sci.* **1991**, *80*, 149−152.
(52) Baur, J. E.; Wang, S.; Brandt, M. C. *Anal. Chem.* **1996**, *68*, 3815−3821.
(53) Das, T. N.; Dhanasekaran, T.; Alfassi, Z. B.; Neta, P. *J. Phys. Chem. A* **1998**, *102*, 280−284.
(54) Merenyi, G.; Lind, J.; Engman, L. *J. Chem. Soc., Perkin Trans. 2* **1994**, 2551−2553.
(55) Jonsson, M. *J. Phys. Chem.* **1996**, *100*, 6814−6818.