J|A|C|S
ARTICLES
Published on Web 06/28/2002

Kinetics and Mechanism of Hydroxyl Radical and OH-Adduct Radical Reactions with Nitroxides and with Their Hydroxylamines

Amram Samuni,*,‡ Sara Goldstein,§ Angelo Russo,† James B. Mitchell,† Murali C. Krishna,† and Pedatsur Neta⊥

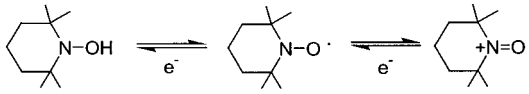
Contribution from the Radiation Biology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, Department of Molecular Biology, The Hebrew University of Jerusalem—Hadassah Medical School, Jerusalem 91120, Israel, Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel, and Physical and Chemical Properties Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899

Received November 21, 2001

Abstract: Stable nitroxide radicals are potent antioxidants and are among the most effective non-thiol radioprotectants, although they react with hydroxyl radicals more slowly than typical phenolic antioxidants or thiols. Surprisingly, the reduced forms of cyclic nitroxides, cyclic hydroxylamines, are better reductants yet have no radioprotective activity. To clarify the reason for this difference, we studied the kinetics and mechanisms of the reactions of nitroxides and their hydroxylamines with •OH radicals and with OH-adducts by using pulse radiolysis, fluorimetric determination of phenolic radiation products, and electron paramagnetic resonance spectrometric determination of nitroxide concentrations following radiolysis. Competition kinetics with phenylalanine as a reference compound in pulse radiolysis experiments yielded rate constants of $(4.5 \pm 0.4) \times 10^9$ M^{-1} s^{-1} for the reaction of •OH radical with 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TPO), 4-hydroxy-TPO (4-OH-TPO), and 4-oxo-TPO (4-O-TPO), $(3.0 \pm 0.3) \times 10^9$ M^{-1} s^{-1} for deuterated 4-O-TPO, and $(1.0 \pm 0.1) \times 10^9$ M^{-1} s^{-1} for the hydroxylamine 4-OH-TPO-H. The kinetic isotope effect suggests the occurrence of both •OH addition to the aminoxyl moiety of 4-O-TPO and H-atom abstraction from the 2- or 6-methyl groups or from the 3- and 5-methylene positions. This conclusion was further supported by final product analysis, which demonstrated that •OH partially oxidizes 4-O-TPO to the corresponding oxoammonium cation. The rate constants for the reactions of the nitroxides with the OH-adducts of phenylalanine and terephthalate have been determined to be near 4×10^6 M^{-1} s^{-1}, whereas the hydroxylamine reacted at least 50 times slower, if at all. These findings indicate that the reactivity toward •OH does not explain the differences between the radioprotective activities of nitroxides and hydroxylamines. Instead, the radioprotective activity of nitroxides, but not of hydroxylamines, can be partially attributed to their ability to detoxify OH-derived secondary radicals.

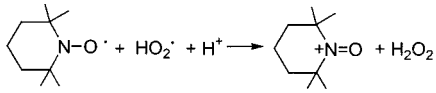
Introduction

Nitroxide radicals are not only potent antioxidants but also among the most effective non-thiol radioprotectants.[1] Nitroxides can be reduced through cellular metabolic processes and nonenzymatic reactions to their respective hydroxylamines, and oxidized to oxoammonium cations, as shown for 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TPO) in eq 1.

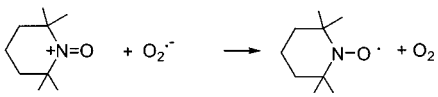


(1)

The antioxidative activity of nitroxides is attributable to several reaction pathways and primarily to the catalytic removal of superoxide through reactions 2 and 3,[2,3]



(2)



(3)

and to oxidation of redox-active transition metals through reaction 4,[4,5]

* Address correspondence to Amram Samuni, Molecular Biology, Faculty of Medicine, The Hebrew University of Jerusalem, Hadassah Medical School, Jerusalem 91120, Israel. Phone: 972-2-6758244. Fax: 972-2-6758944. E-mail: samuni@cc.huji.ac.il.

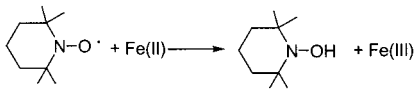
† National Cancer Institute, National Institutes of Health.

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

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

⊥ National Institute of Standards and Technology.

(1) Mitchell, J. B.; DeGraff, W.; Kaufman, D.; Krishna, M. C.; Samuni, A.; Finkelstein, E.; Ahn, M. S.; Hahn, S. M.; Gamson, J.; Russo, A. *Arch. Biochem. Biophys.* **1991**, *289*, 62−70.



(4)

which pre-empts the Fenton reaction. However, these mechanisms are less likely to account for the radioprotective activity of nitroxides. Generally, chelators such as desferrioxamine or DTPA, which detoxify redox-active (denoted also as chelatable, catalytic, or "free") metal ions, demonstrate poor radioprotective activity. Similarly, superoxide dismutase (SOD) and SOD mimics, which remove superoxide, are not effective radioprotectants. The concentration at which nitroxides provided radioprotection to cells in vitro[1] and to laboratory animals[6] is in the millimolar range, a concentration similar to that required for protection by aminothiols—the "gold standards" for comparison. It is unlikely that antioxidants in general protect cells from radiation damage by directly reacting with •OH radicals. In particular, nitroxide reactivity toward •OH is lower than that of most phenolic antioxidants and thiols.[7] Moreover, cyclic hydroxylamines, such as 4-OH-TPO-H (the reduced form of 4-hydroxy-TPO, 4-OH-TPO), which are more effective reductants, lack radioprotective activity.[1] The present study focuses on the reaction kinetics of nitroxides and their hydroxylamines with •OH radicals and OH-adducts using pulse radiolysis and γ-radiolysis techniques. The results indicate that direct reaction of either nitroxides or their corresponding hydroxylamines with •OH does not provide an explanation for their strikingly different radioprotective activities. Instead, the radioprotective activity of nitroxides, but not of hydroxylamines, most likely results from their detoxifying OH-radical-derived secondary radicals.

Experimental Section

Materials. The nitroxides TPO, 4-OH-TPO, and 4-oxy-TPO (4-O-TPO), benzoic acid, terephthalic acid (TA), and phenylalanine (PA) were purchased from Aldrich. Potassium ferricyanide was obtained from BDH. The cyclic hydroxylamine 4-OH-TPO-H was 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.[8] Water was distilled and further purified with a Millipore Super Q system. Fresh solutions of 4-OH-TPO-H were prepared immediately before each experiment to minimize oxidation to the nitroxide radical. Synthetic hydroxyterephthalate (HTA) for calibration was prepared by a published procedure.[9] Unless stated otherwise, all solutions contained 10 mM phosphate buffer (PB).

Steady-State γ-Irradiation. γ-Irradiation was carried out at room temperature using a ^{60}Co source with a dose rate of 39 Gy min^{-1} as determined by Fricke dosimetry.

Pulse Radiolysis. Pulse radiolysis experiments were carried out with a Varian 7715 linear accelerator with 5-MeV electron pulses of 0.1−1.5 μs duration. The dose per pulse was 5−12 Gy as determined by thiocyanate dosimetry in N_2O-saturated water.[10] The spectrophotometric detection system utilized a Hamamatsu 75 W xenon lamp, a shutter and optical cutoff filters to prevent photolysis of the solution, an irradiation cell of 2 cm optical path length, a Jobin-Yvon monochromator, a Hamamatsu R955 photomultiplier, and a Tektronix 420A digitizing oscilloscope. Reaction rate constants were reported with their estimated uncertainties. In some experiments, solutions were prepared in 1- and 4-cm quartz cells sealed with a rubber septum. The cells were pulse-irradiated, and the spectrum was immediately recorded using a HP 8453 diode array spectrophotometer. All experiments were carried out at room temperature.

Fluorimetric Assay. A Perkin-Elmer LS50B spectrofluorimeter was used for the fluorimetric assays. The irradiated solutions were sampled and diluted 1:25, 1:50, or 1:125 before the fluorescence measurements. The assays were conducted at the dynamic range of concentration, as judged from the linear dependence of fluorescence intensity on the concentration of the fluorescent probe. Control experiments confirmed that the optical absorption of added solutes, under such experimental conditions (TA, nitroxide, or hydroxylamine) at the selected excitation and emission wavelength range, was negligible and had no quenching effect on fluorescence intensity.

Electron Paramagnetic Resonance (EPR) Measurements. To determine nitroxide concentration, samples were drawn into a gas-permeable, 0.8-mm-i.d. Teflon capillary. The capillary was inserted into a quartz tube open at both ends and then placed within the EPR spectrometer cavity. The EPR spectra were recorded on a Varian spectrometer working at X band with center field set at 3370 G, 100 kHz modulation frequency, 1 G modulation amplitude, and 10 mW incident microwave power. The nitroxide concentration was calculated from the EPR signal intensity using standard solutions of the nitroxide.

Bulk Electrolysis. A homemade electrochemical reactor, similar to that previously described,[11] was used. The cell consisted of a working electrode of graphite packed inside a porous Vycor glass tube (5 mm i.d.), through which the test solutions were pumped (4 mL min^{-1}) through a 1-cm flow optical quartz cell under anoxia. An outer glass cylinder, with separate electrolyte (4 mM phosphate, pH 7), contained the platinum auxiliary electrode. The voltage was controlled by a homemade power supply. The spectrum of the eluent was measured with a HP 8453 diode array spectrophotometer.

Results

Reaction with •OH. Pulse radiolysis was used to determine the rate constants for the reaction of •OH radical with nitroxides and their corresponding hydroxylamines. The solutions were saturated with N_2O, which served to convert the hydrated electrons into •OH radicals. The rate constant of reaction 5 for several scavengers (S) including TPO, 4-OH-TPO, 4-O-TPO,

$$\text{•OH} + \text{S} \rightarrow \text{products} \qquad (5)$$

and 4-OH-TPO-H was determined using competition kinetics against phenylalanine as a reference solute.[12]

$$\text{•OH} + \text{PA} \rightarrow \text{HO-PA}^{\bullet} \qquad k_6 = 6.5 \times 10^9\ \text{M}^{-1}\ \text{s}^{-1}\ ^7 \qquad (6)$$

The yield of HO-PA• was determined by monitoring the

(2) Samuni, A.; Krishna, C. M.; Riesz, P.; Finkelstein, E.; Russo, A. *J. Biol. Chem.* **1988**, *263*, 17921−17924.
(3) Krishna, M. C.; Grahame, D. A.; Samuni, A.; Mitchell, J. B.; Russo, A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5537−5541.
(4) Krishna, M. C.; Samuni, A.; Taira, J.; Goldstein, S.; Mitchell, J. B.; Russo, A. *J. Biol. Chem.* **1996**, *271*, 26018−26025.
(5) Bar-On, P.; Mohsen, M.; Zhang, R.; Feigin, E.; Chevion, M.; Samuni, A. *J. Am. Chem. Soc.* **1999**, *121*, 8070−8073.
(6) Hahn, S. M.; Tochner, Z.; Krishna, C. M.; Glass, J.; Wilson, L.; Samuni, A.; Sprague, M.; Venzon, D.; Glatstein, E.; Mitchell, J. B. *Cancer Res.* **1992**, *52*, 1750−1753.
(7) Buxton, G. V.; Greenstock, C. L.; Helman, W. P.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1988**, *17*, 513−886.
(8) Zhdanov, R. I.; Komarov, P. G. *Free Radical Res. Commun.* **1990**, *9*, 367−377.
(9) Mason, T.; Lorimer, J. P.; Bates, D. M.; Zhao, Y. *Ultrason. Sonochem.* **1994**, *1*, S91−S95.
(10) Schuler, R. H.; Patterson, L. K.; Janata, E. *J. Phys. Chem.* **1980**, *84*, 2088−2089.
(11) Miner, D. J.; Kissinger, P. T. *Biochem. Pharmacol.* **1979**, *28*, 3285−3290.
(12) Willson, R. L. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **1972**, *21*, 401−403.

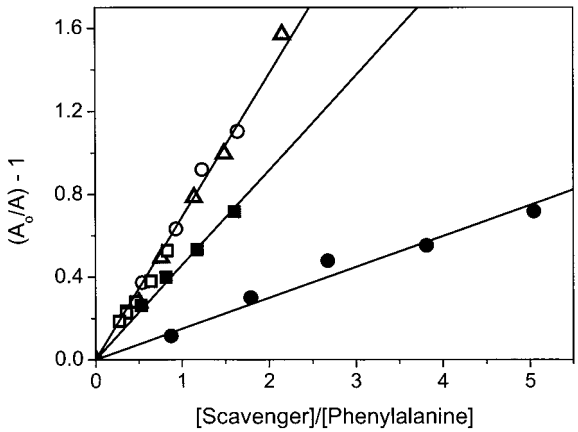


Figure 1. Competition kinetics using phenylalanine as a reference solute. The yield of phenylalanine OH-adduct was measured at 325 nm upon pulse irradiation of N_2O-saturated solution containing 1.1 mM phenylalanine and 10 mM PB, pH 7.4, in the absence (A_o) and in the presence (A) of various concentrations of TPO (△), 4-OH-TPO (○), 4-O-TPO (□), d-4-O-TPO (■), and 4-OH-TPO−H (●).

absorbance at 325 nm immediately after the pulse in the absence (A_o) and in the presence of various scavenger concentrations (A) at pH 7.4. When only reactions 5 and 6 take place, eq 7 is obtained, and plots of (A_o/A −1) vs [S]/[PA] were found to be

$$A_o/A = 1 + k_5[\mathrm{S}]/k_6[\mathrm{PA}] \qquad (7)$$

linear (Figure 1). The value of k_5 was calculated from the slope of these lines using the recommended value for $k_6 = 6.5 \times 10^9$ M^{-1} s^{-1} [7] and found to be $(4.5 \pm 0.4) \times 10^9$ M^{-1} s^{-1} for TPO, 4-OH-TPO, and 4-O-TPO, $(3.0 \pm 0.3) \times 10^9$ M^{-1} s^{-1} for deuterated 4-O-TPO, and $(1.0 \pm 0.1) \times 10^9$ M^{-1} s^{-1} for the hydroxylamine 4-OH-TPO-H. The value $(4.5 \pm 0.4) \times 10^9$ M^{-1} s^{-1} agrees with the previously reported ones.[12−14] Similar experiments with thiocyanate as a competing solute resulted in considerably higher values as a result of the fast reaction between the nitroxide and $(SCN)_2^{\bullet-}$, and possibly also with its radical precursor $SCN^{\bullet}$, which makes thiocyanate unsuitable as a competing compound.

The isotope effect on the reaction kinetics shows that the rate constant is reduced by a factor of 1.5 when H atoms in 4-O-TPO are replaced by D atoms (Figure 1). The rate of the addition of $^{\bullet}OH$ to the aminoxyl moiety should not be affected by this replacement. Conversely, the rate of the H atom abstraction by $^{\bullet}OH$ can be significantly lower for the deuterated nitroxide but only if the rate constant for the reaction is significantly lower than the diffusion-controlled limit, which is close to 1×10^{10} M^{-1} s^{-1}. To further examine the possibility that $^{\bullet}OH$ adds to the aminoxyl moiety in 4-O-TPO,[12−14] eventually leading to the formation of the oxoammonium cation,[12] we generated the oxoammonium cation via electrolytic oxidation of the nitroxide[3] and compared its spectrum with that formed from the reaction of $^{\bullet}OH$ with 4-O-TPO. Figure 2A displays the spectrum of 0.1 mM oxoammonium cation of 4-O-TPO formed electrochemically under anoxia at pH 7.0, which has two absorption maxima at 249 nm ($\epsilon = 7400 \pm 700$ M^{-1} cm^{-1}) and at 650 nm ($\epsilon =$ ca. 14 M^{-1} cm^{-1}). The extinction coefficient at 650 nm could not

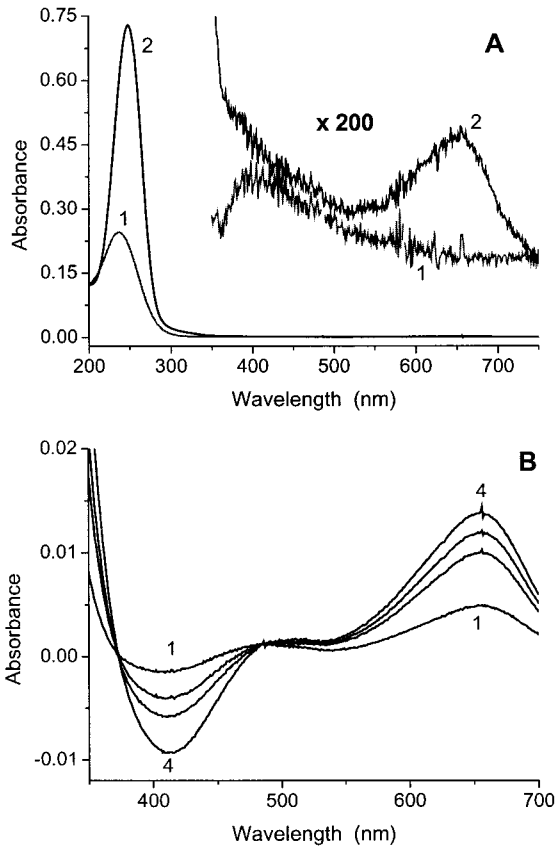


Figure 2. Electrolysis of 4-O-TPO. (A) The respective spectra of 0.1 mM 4-O-TPO in 4 mM PB at pH 7 before (trace 1) and after (trace 2) electrolysis at 0.85 V ($I = 6$ mA). (B) Partial electrolysis of 3.66 mM 4-O-TPO in 4 mM PB at pH 7.0 under anoxia at $E = 1.4$ V ($I = 25$ mA). Four differential spectra collected after 2 (trace 1), 10, 15, and 22 min (trace 4) of electrolysis are presented. The optical path length was 1 cm.

be determined accurately due to the very small absorption, and therefore we show in Figure 2B the differential spectra obtained upon partial electrolytic oxidation of 3.66 mM 4-O-TPO at $\lambda > 350$ nm. The latter shows the bleaching at 417 nm of the nitroxide and the formation at 650 nm of the corresponding oxoammonium cation. As previously reported,[3] this nitroxide was almost fully recovered when the polarity of the electrodes in the electrochemical reactor was reversed and the oxidized solution was subjected to reduction. Figure 3 (trace 1) displays the spectrum obtained upon repetitive pulsing of N_2O-saturated solutions containing 0.5 mM 4-O-TPO and 4 mM PB at pH 7.0. The results demonstrate that the oxoammonium cation is produced from the reaction between $^{\bullet}OH$ and 4-O-TPO, and its yield was calculated to be 32 ± 7% of the total $^{\bullet}OH$ radicals produced by the radiation. To further quantify the yield of the oxoammonium cation, we studied the reaction of 4-O-TPO with azide radical, which is more selective than $^{\bullet}OH$ radical and, therefore, is expected to form the oxoammonium cation stoichiometrically.

Reaction with $^{\bullet}N_3$. The azide radical is formed in N_2O-saturated solutions by reaction 8:

$$^{\bullet}OH + N_3^- \rightarrow HO^- + {}^{\bullet}N_3 \qquad k_8 = 1.4 \times 10^{10}\ M^{-1}\ s^{-1}\ [7] \qquad (8)$$

At $[N_3^-]$/[nitroxide] ≥ 10, reaction 8 competes efficiently with

(13) Fielden, E. M.; Roberts, P. B. *Int. J. Radiat. Biol.* **1971**, *20*, 355−362.
(14) Asmus, K. D.; Nigam, S.; Willson, R. L. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **1976**, *29*, 211−219.

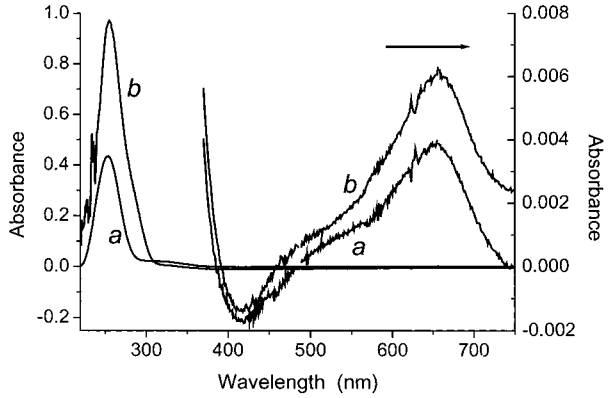


Figure 3. Spectrum of the oxoammonium cation of 4-O-TPO formed by pulse radiolysis. Differential spectra obtained after delivering 30 pulses (10.3 Gy/pulse) into N_2O-saturated solutions containing 0.5 mM 4-O-TPO and 4 mM PB at pH 7.0 in the absence (trace a) and in the presence (trace b) of 5 mM azide, $l = 1$ cm. The differential spectra at 370−750 nm were measured in a 4-cm optical cell.

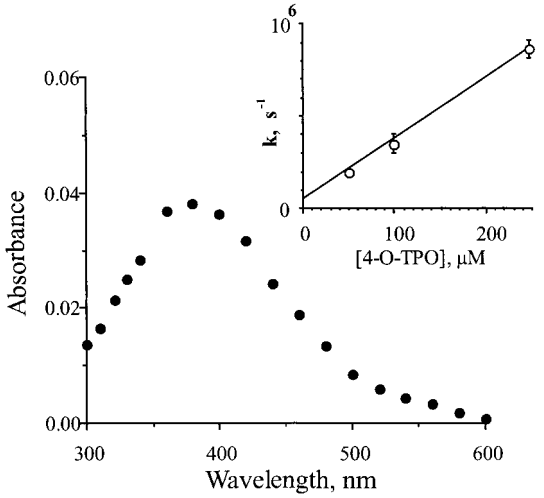


Figure 4. Reaction of 4-O-TPO with azide radical. The transient absorption spectra were produced upon pulse radiolysis of N_2O-saturated solution containing 0.46 mM 4-O-TPO, 10 mM azide, and 2 mM PB at pH 6.8. (Inset) The dependence of the observed pseudo-first-order rate constant of the formation of the absorption at 380 nm on [4-O-TPO].

reaction 5, and the $^{\bullet}N_3$ radical thus formed reacts with 4-O-TPO:

$$^{\bullet}N_3 + \text{4-O-TPO} \rightarrow \text{products} \qquad (9)$$

When the N_2O-saturated solution containing 4-O-TPO was pulse-irradiated in the presence of azide, the formation of a transient spectrum with a maximum at 380 nm was observed (Figure 4). The buildup of the absorption was followed at 380 nm in the presence of various concentrations of 4-O-TPO and found to obey first-order kinetics. The observed first-order rate constant was linearly dependent on [4-O-TPO], resulting in $k_9 = (3.4 \pm 0.4) \times 10^9\ M^{-1}\ s^{-1}$ (inset to Figure 4). This absorption rapidly decayed with a rate that increased upon increasing the pH and did not obey simple first- or second-order kinetics. Figure 3 (trace b) displays the spectrum obtained upon repetitive pulsing of N_2O-saturated solutions containing 0.5 mM 4-O-TPO, 5 mM azide, and 4 mM PB at pH 7.0, which demonstrates the formation of the oxoammonium cation in this system. The yield of the oxoammonium cation in the presence of azide is 2.3 times higher than that obtained in the absence of azide (compare traces 1 and 2 in Figure 3). If azide radical oxidizes the nitroxide stoichiometrically, then about 43% of the $^{\bullet}OH$ radicals add to the aminoxyl group in 4-O-TPO to yield eventually the oxoammonium cation. This value is somewhat higher than the value determined above, i.e., 32 ± 7%. Our results also suggest that $^{\bullet}N_3$ radical adds to the aminoxyl group, forming an adduct whose spectrum is displayed in Figure 4, which eventually decomposes to yield the oxoammonium cation.

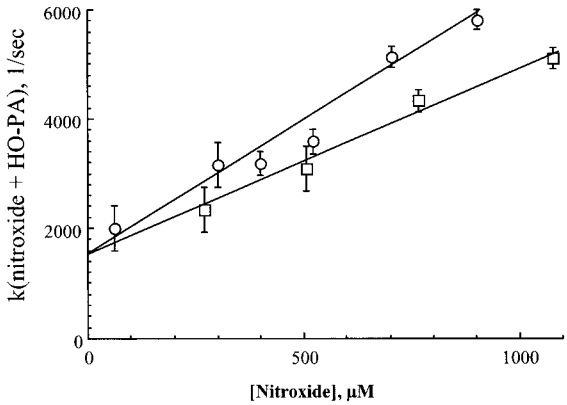


Figure 5. Kinetics of nitroxides reaction with phenylalanine OH-adduct. The phenylalanine OH-adduct was formed by pulse irradiation of N_2O-saturated solution of 1 mM phenylalanine in 10 mM PB, pH 7.4, in the absence and in the presence of various concentrations of nitroxide. The kinetics of the decay of the absorption at 325 nm were determined, and the observed first-order rate constant was plotted vs [4-OH-TPO] (○) and [4-O-TPO] (□).

Nitroxide Reaction with the OH-Adducts. Since the difference (4-fold) between the reactivities of $^{\bullet}OH$ with nitroxides and hydroxylamine is not large, we compared the kinetics of their reaction with the OH-adduct of phenylalanine (HO-PA$^{\bullet}$).

$$\text{nitroxide} + \text{HO-PA}^{\bullet} \rightarrow \text{products} \qquad (10)$$

The HO-PA$^{\bullet}$ adduct was produced by pulse irradiation of N_2O-saturated solution containing 1 mM PA and various concentrations of nitroxide at pH 7.4. In the absence of nitroxide, the transient absorption at 325 nm decayed through a second-order process, with $2k_{decay}/\epsilon l = 2.9 \times 10^4\ s^{-1}$. In the presence of nitroxide, the decay obeyed first-order kinetics. At [nitroxide] < 0.1 mM, the decay was not first order but rather a mixed order. The values of the first-order rate constants (k_{obs}) evaluated from analysis of a pure exponential decay were plotted *vs.* [4-OH-TPO] and [4-O-TPO] in Figure 5. From the linear plots, the rate constants k_{10} for 4-OH-TPO and 4-O-TPO were determined to be $(4.8 \pm 0.5) \times 10^6$ and $(3.5 \pm 0.4) \times 10^6\ M^{-1}\ s^{-1}$, respectively. However, 6.6 mM 4-OH-TPO-H had no detectable effect on the decay of HO-PA$^{\bullet}$, and hence an upper limit of $k_{10} < 1 \times 10^5\ M^{-1}\ s^{-1}$ can be estimated for 4-OH-TPO-H.

The nitroxide reaction with the OH-adduct was studied by also using terephthalate (TA), another aromatic target molecule, which is widely used as a dosimeter for radiation and ultrasound studies[15−18] and as a sensitive assay for $^{\bullet}OH$ production in

(15) McLean, J. R.; Mortimer, A. J. *Ultrasound Med. Biol.* **1988**, *14*, 59−64.
(16) Candeias, L. P.; Stratford, M. R.; Wardman, P. *Free Radical Res.* **1994**, *20*, 241−249.
(17) Montgomery, J.; Ste, M. L.; Boismenu, D.; Vachon, L. *Free Radical Biol. Med.* **1995**, *19*, 927−933.

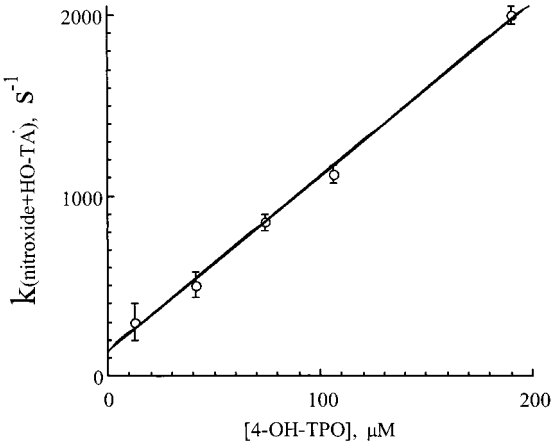
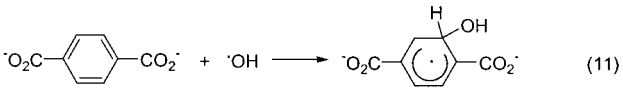


Figure 6. Reaction of 4-OH-TPO with the OH-adduct of terephthalate. The OH-adduct of terephthalate was formed by pulse irradiation of N_2O-saturated solution containing 1 mM terephthalate and 0 mM PB, pH 7.4, in the presence of various concentrations of 4-OH-TPO. The decay of the absorption was followed at 350 nm, and the observed first-order rate constant was plotted vs [4-OH-TPO]. Error bars are shown when greater than the symbol.

diverse cell-free[9,19−22] and cellular systems.[23,24] In this case, reaction 11 is the main one, and the hydroxycyclohexadienyl



(11)

radical (HO-TA•) thus formed is eventually oxidized to yield a single phenolic end product (unlike the case of salicylate or phenylalanine, to which •OH adds at different ring sites). In the present study, the HO-TA• was formed by pulse irradiation of N_2O-saturated solution containing 0.4−1 mM TA at pH 7.4. The decay of HO-TA• was followed at 350 nm in the absence and in the presence of various concentrations of nitroxide. In the absence of nitroxide, HO-TA• decayed via a second-order process with $2k_{decay}/\epsilon l = 4.7 \times 10^3\ s^{-1}$. In the presence of 4-OH-TPO, the decay of HO-TA• obeyed first-order kinetics, and k_{10} was determined from the dependence of k_{obs} on [4-OH-TPO] to be $(3.5 \pm 0.4) \times 10^6\ M^{-1}\ s^{-1}$ (Figure 6). No effect on the decay of HO-TA• was detected, even in the presence of 21 mM 4-OH-TPO-H, and therefore the rate constant for the reaction of the hydroxylamine with HO-TA• does not exceed $5 \times 10^4\ M^{-1}\ s^{-1}$.

The Fate of the OH-Adducts. Product analysis following the nitroxide reaction with the OH-adducts can assist in clarifying the reaction mechanism. N_2O-saturated solutions containing TA at pH 7.4 were subjected to various doses of continuous radiation, and the fluorescence intensity of the accumulating hydroxyterephthalic acid (HTA) was monitored (λ_{ex} = 315 nm, λ_{em} = 425 nm). The yield of HTA was calculated (using a standard curve) and plotted against the dose,

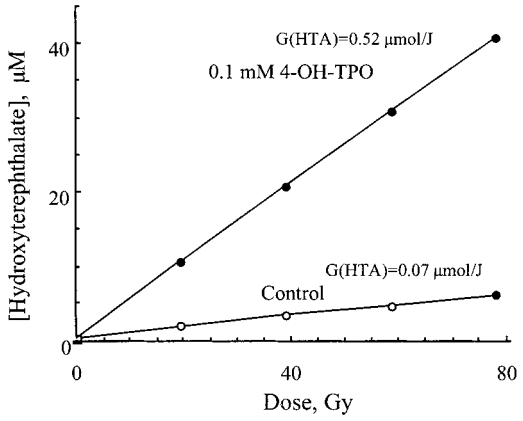


Figure 7. Effect of 4-OH-TPO on the radiation-induced hydroxylation of terephthalate. Fluorimetry was used to monitor (λ_{ex} = 315 nm, λ_{em} - 425 nm) the yield of hydroxyterephthalic acid (HTA) accumulated during γ-irradiation of N_2O-saturated solution of 5 mM terephthalate in 10 mM PB, pH 7.4, in the absence (○) and in the presence (●) of 0.1 mM 4-OH-TPO. The *G* values were evaluated from the slopes of the lines.

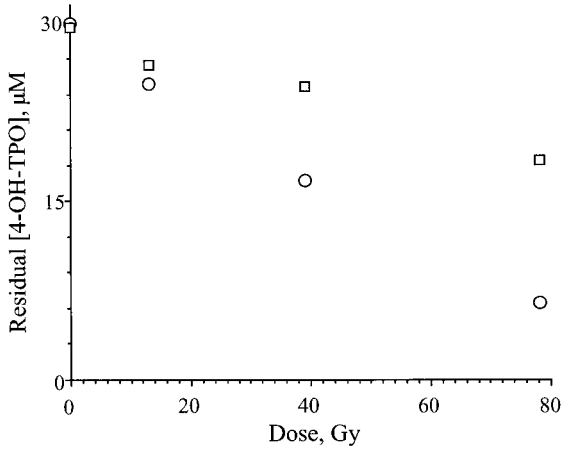
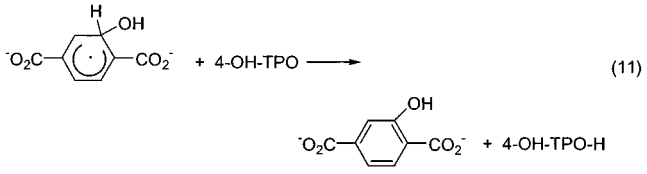


Figure 8. Decrease in 4-OH-TPO concentration upon reaction with radiolytically formed OH-adducts. The residual EPR signal measured immediately (○) and 48 h after (□) irradiation of 1 mM terephthalate and 30 μM 4-OH-TPO in 10 mM PB at pH 7.4. Addition of 1 mM ferricyanide after irradiation restored the signal.

and the *G*(HTA) values were determined from a linear least-squares fit (Figure 7). 4-OH-TPO at concentrations as low as 10 μM induced practically complete oxidation, i.e., a full yield of hydroxylated products. TPO and 4-O-TPO had a similar effect (data not shown).

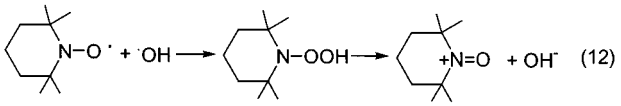
The Fate of the Nitroxide. During γ-irradiation of TA in the presence of 4-OH-TPO, the nitroxide was depleted in a dose-dependent manner (Figure 8). This depletion was evident from EPR scanning of the irradiated samples. A value of *G*(4-OH-TPO) = 0.45 μmol J^{-1} was evaluated from the initial dependence of the spin-loss on the dose. Repeating the EPR scan 48 h following aeration of the samples showed recovery of the signal. Similarly, addition of 1 mM ferricyanide after irradiation restored the EPR signal of 4-OH-TPO. Both the effect of ferricyanide and the restoration of the signal under aerobic conditions showed that the spin loss of the nitroxide resulted from one-electron reduction of the aminoxyl moiety rather than ring modification. This indicates that the oxidation of the OH-adduct to a phenolic product is accompanied by a reduction of the nitroxide to its respective hydroxylamine (reaction 11).

(18) Fang, X. W.; Mark, G.; von Sonntag, C. *Ultrason. Sonochem.* **1996**, *3*, 57−63.
(19) Buxton, G. V.; Langan, J. R.; Smith, J. R. L. *J. Phys. Chem.* **1986**, *90*, 6309−6313.
(20) Barreto, J. C.; Smith, G. S.; Strobel, N. H.; McQuillin, P. A.; Miller, T. A. *Life Sci.* **1995**, *56*, 89−96.
(21) Saran, M.; Summer, K. H. *Free Radical Res.* **1999**, *31*, 429−436.
(22) Yang, X. F.; Guo, X. Q. *Analyst* **2001**, *126*, 928−932.
(23) Qu, X.; Kirschenbaum, L. J.; Borish, E. T. *Photochem. Photobiol.* **2000**, *71*, 307−313.
(24) Kirschenbaum, L. J.; Qu, X.; Borish, E. T. *J. Cosmet. Sci.* **2000**, *51*, 161−181.



Discussion

The water-derived e_{aq}^- and other reducing species, such as $H^\bullet$ and $CO_2^{\bullet-}$ radicals, react with the aminoxyl moiety of the nitroxide and reduce it to the respective cyclic hydroxylamine.[14,25] Analogously, it was anticipated that $^\bullet$OH radicals would oxidize nitroxides (reaction 5) to the oxoammonium cation via formation of an intermediate adduct (mechanism 12).[12,14,26]



The present results demonstrate that, at least in the case of 4-O-TPO, reaction 12 takes place in parallel to H atom abstraction. This is most probably the case for other similar nitroxides as well. The contribution of reaction 12 is about 40%, as evident from the comparison of the respective yields of the oxoammonium cation formed via the reaction of the 4-O-TPO with the azide and hydroxyl radicals as well as with the respective spectrum formed electrochemically. The kinetic isotope effect for the reaction of OH radicals with 4-O-TPO was found to be about 1.5, which is similar to that determined for the reaction of $^\bullet$OH with C_2H_5OH and C_2D_5OH, i.e., 1.9×10^9 and 1.2×10^9 M^{-1} s^{-1}, respectively.[27] The measured factor of 1.5 suggests that the rate of the H atom abstraction is about 2×10^9 M^{-1} s^{-1}. In other words, the kinetic isotope effect suggests that about half of the $^\bullet$OH radicals abstract H atoms and is in good agreement with our conclusion derived from the results obtained spectrophotometrically.

The relatively small difference between the reactivities of $^\bullet$OH toward 4-OH-TPO and 4-OH-TPO-H (Figure 1) cannot explain why nitroxides are effective radioprotectants whereas the hydroxylamine provides no radioprotection. We conclude, therefore, that the radioprotective activity of nitroxides is not related to the scavenging of hydroxyl radicals. This conclusion is true in general, given the high reactivity of $^\bullet$OH radicals with most cellular components and the relatively high concentrations of such components. For the same reasons, it is unlikely that any antioxidant added exogenously would protect the cell by successfully competing for radiolytically formed $^\bullet$OH radicals.

Nitroxides but Not Hydroxylamines Detoxify Secondary Radicals. 4-OH-TPO greatly differs (at least 50-fold) from 4-OH-TPO-H in terms of its reactivity with the OH-adduct. In converting OH-adduct into phenolic end product (reaction 11), the nitroxide actually acts as an oxidant. Thus, when present at millimolar concentration range, it can compete with dioxygen, which yields peroxyl radicals. It might appear paradoxical, but nitroxides, which are effective radioprotectants and antioxidants in general, are oxidizing the OH-adduct while providing radioprotection. In contrast, the corresponding hydroxylamine, such as 4-OH-TPO, is essentially not reactive with OH-adduct, and it would not be anticipated to provide radioprotection.

Conclusions

The overall rate constant for the reaction of $^\bullet$OH with TPO, 4-OH-TPO, and 4-O-TPO has been determined to be $(4.5 \pm 0.4) \times 10^9$ M^{-1} s^{-1}, whereas for the hydroxylamine 4-OH-TPO-H the rate constant is reduced ca. 4.5 times, i.e., $k = (1.0 \pm 0.1) \times 10^9$ M^{-1} s^{-1}. The present results demonstrate that, at least in the case of 4-O-TPO, about 40% of the $^\bullet$OH radicals add to the aminoxyl moiety and the rest abstract H atoms. This is most probably the case for other similar nitroxides as well. The hydroxycyclohexadienyl radicals of phenylalanine are oxidized by 4-OH-TPO and 4-O-TPO, with rate constants of $(4.8 \pm 0.5) \times 10^6$ and $(3.5 \pm 0.4) \times 10^6$ M^{-1} s^{-1}, respectively, and the hydroxycyclohexadienyl radical of terephthalate is oxidized by 4-OH-TPO with a rate constant of $(3.5 \pm 0.4) \times 10^6$ M^{-1} s^{-1}. In these reactions, the phenolic products are formed while the nitroxides are reduced to the respective hydroxylamines. 4-OH-TPO-H is much less (at least 2 orders of magnitude less) reactive toward the hydroxycyclohexadienyl radicals. Thus, the radioprotective effect does not result from scavenging $^\bullet$OH radicals per se. Instead, the radioprotective activity of nitroxides, but not of hydroxylamines, results, at least in part, from detoxifying OH-derived secondary radicals and most likely peroxyl radicals[28] as well.

Acknowledgment. This research was partially supported by Grant 95-00287 from the USA–Israel Binational Science Foundation (BSF) and from the Israel Science Foundation of the Israel Academy of Sciences. We thank Dr. Louis J. Kirschenbaum (University of Rhode Island) for supplying us the synthetic HTA. The mention of commercial equipment or material does not imply recognition or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified are necessarily the best available for the purpose.

JA017587H

(25) Madden, K. P. *Radiat. Res.* **1997**, *147*, 335–341.
(26) Nigam, S.; Asmus, K. D.; Willson, R. L. *J. Chem. Soc., Faraday Trans. 1* **1976**, 2324–2340.
(27) Anbar, M.; Meyerstein, D.; Neta, P. *J. Phys. Chem.* **1966**, *70*, 2660–2662.
(28) Offer, T.; Samuni, A. *Free Radical Biol. Med.* **2002**, *32*, 872–881.