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Cage-Escape of Geminate Radical Pairs Can Produce Peroxynitrate from Peroxynitrite under a Wide Variety of Experimental Conditions[1]

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Abstract: The spontaneous and CO_2-catalyzed decomposition of peroxynitrite yields $HO^{\bullet}$ and $^{-}CO_3^{\bullet}$ radicals, respectively, together with $^{\bullet}NO_2$. The geminate $HO^{\bullet}/^{\bullet}NO_2$ and $^{-}CO_3^{\bullet}/^{\bullet}NO_2$ pairs undergo competitive in-cage collapse to nitrate and diffusive separation. Free $HO^{\bullet}$ and $^{-}CO_3^{\bullet}$ radicals react with H_2O_2 and, in the presence of O_2, suitable alcohols or formate to produce superoxide, which is trapped by the $^{\bullet}NO_2$ to form peroxynitrate. The formation of peroxynitrate may influence the rate of change in optical density at 302 nm, the wavelength normally employed to monitor peroxynitrite decay, leading to misleading kinetic traces. Tetranitromethane (TNM) was used as a colorimetric probe for superoxide to quantify the yield of free $HO^{\bullet}$ (27−28%) and free $^{-}CO_3^{\bullet}$ (32−33%). The yields of both of these free radicals are in excellent agreement with other recent estimates. Superoxide was also detected in some oxygenated aldehyde-catalyzed peroxynitrite decompositions both by peroxynitrate formation and by its reaction with TNM. Superoxide yields, measured with TNM, were aldehyde (RCHO) dependent ($R = {}^{-}O_2CC_6H_4$, CH_3, CH_3CH_2, $(CH_3)_3C$ and $HOCH_2CHOH$; yields were 15, 9, 0.8, 0, and 30%, respectively).

Introduction

In 1990, Beckman and co-workers[3] proposed that two endogenous, but relatively unreactive, free radicals, superoxide and nitrogen monoxide, could undergo a rapid combination reaction ($k = 1.9 \times 10^{10}$ M^{-1} s^{-1})[4] to yield, in vivo, the powerful oxidant peroxynitrite.[5] The peroxynitrite anion is thermally stable; however, peroxynitrous acid ($pK_a = 6.55$ and 7.33 in 1 and 650 mM phosphate buffer, respectively)[4] spontaneously decays with a rate constant of 1.20 s^{-1} at 25 °C.[4] A highly reactive oxidizing species is formed during the spontaneous decay of the peroxynitrous acid, which is probably the hydroxyl radical.[6−10] The peroxynitrite anion reacts rapidly with CO_2 ($k = 3 \times 10^4$ M^{-1} s^{-1})[11] to yield an adduct, $^{-}O_2COONO$, which can undergo subsequent reactions with added substrates and/or spontaneously decay at a higher rate than the peroxynitrous acid.[11−13] The concentrations of CO_2 in vivo are relatively high (bicarbonate in intracellular and interstitial fluids of 12 and 30 mM, respectively),[14] and hence most peroxynitrite will be trapped by CO_2. Therefore, the subsequent reactions of the peroxynitrite/CO_2 adducts are of key importance in understanding the effects of peroxynitrite in vivo.

Alvarez and co-workers have reported that hydrogen peroxide, mannitol, or ethanol decreases the rate of peroxynitrite decomposition in a concentration-dependent manner, following a hyperbolic function.[15,16] Addition of 100 mM hydrogen peroxide, mannitol, or ethanol reduced the spontaneous rate of peroxynitrite decomposition by ca. 50, 34, and 25%, respectively. It was tentatively proposed that these reagents hydrogen-bonded to peroxynitrite and thereby slowed its rate of decomposition. The possibility of endogenous compounds (e.g., glucose) associating themselves with peroxynitrite and altering its reactions and reactivity could have wide-ranging biological implications and could influence the results of in vivo studies.

The abilities of water and simple primary alcohols to act as hydrogen bond donors, as described by their a^{H} values of 0.353 and 0.328, respectively,[17] are very similar, with water being the slightly better hydrogen bond donor. Analogously, the abilities of water and ethanol to act as hydrogen bond acceptors, as described by their β^{H} values of 0.38 and 0.41, respectively,[18] are also very similar, with ethanol being the slightly better

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hydrogen bond acceptor. It therefore appeared unlikely to us that 100 mM alcohol could compete with 55 M water as a hydrogen bond acceptor and/or donor with peroxynitrite. The possibility that molecular products of formal reactions involving the peroxynitrite anion were responsible for the observations of Alvarez et al.[15,16] was virtually eliminated using spectroscopic methods.[19] We report herein our observations on the effects of alcohols, hydrogen peroxide, and some other additives which, under certain conditions, can induce complexities in the measured kinetics of peroxynitrite decay.

Results

Kinetic experiments were carried out in a stopped-flow apparatus at 24 °C using a 1.4 mM aqueous stock solution of argon-purged (and therefore dioxygen-free) peroxynitrite (pH 12) in one syringe and buffer (purged or otherwise) in the other syringe to reduce the pH to 6.3 or 8.5 after mixing. All concentrations given below are those obtained after mixing equal volumes from the two syringes; i.e., in the kinetic experiments the peroxynitrite concentration was 0.7 mM. The rigorous exclusion of adventitious CO_2 was achieved as described previously.[7] Optical density (OD) refers to ODs measured at 302 nm unless otherwise stated.

In 1998, Alvarez et al.[16] reported that, "when contaminating carbon dioxide was eliminated through bubbling freshly prepared (air-equilibrated) solutions with argon for 3 h, the slowing effect (produced by mannitol and ethanol on the rate of peroxynitrite decay) was observed". In our initial attempts to duplicate the results of Alvarez et al.,[15,16] we therefore employed rigorously CO_2-free conditions. Under such conditions (pH 6.3, dioxygen free), neither ethanol nor hydrogen peroxide (both up to 150 mM) had any readily observable effect on the rate of peroxynitrite decay, and certainly not effects comparable to those which had been reported.[15,16] A later, very careful reexamination of these additives under exactly the same conditions confirmed that 150 mM ethanol had no effect on the measured rate constant for peroxynitrite decay but revealed that 150 mM hydrogen peroxide actually did reduce this rate constant by about 10% (from 0.76 to 0.68 s^{-1}, see Note Added in Proof). Alvarez et al. used "air-equilibrated" solutions in their experiments with ethanol,[16] and, although the possible role of CO_2 was not addressed in their earlier experiments with hydrogen peroxide,[15] we assume that these solutions were also air-equilibrated. We therefore turned to "air-equilibrated" buffer solutions while keeping the alkaline peroxynitrite solutions free of dioxygen and CO_2. Under these conditions, both ethanol and hydrogen peroxide reduced the rate of decrease in OD as peroxynitrite decayed, just as had been reported.[15,16]

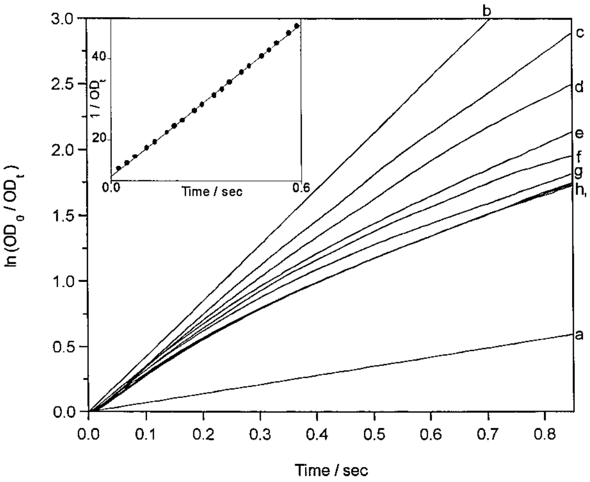

Figure 1. Change in OD at 302 nm on mixing equal volumes of peroxynitrite (1.4 mM, argon-purged and CO_2-free) with phosphate buffer (200 mM, dioxygen-purged) containing either no added reagents (trace a) or 2 mM sodium bicarbonate (added 2−5 min prior to use) and 0, 10, 20, 50, 100, 200, 300, 400, or 600 mM 2-propanol (traces b−j, respectively). Final pH 6.3 and 24 °C. Inset: Alternative plot of the data from trace j.

It appeared likely that the rate-retarding effects of hydrogen peroxide and ethanol on decay of the 302-nm absorption were dependent on the presence of CO_2, which is known to catalyze the decomposition of peroxynitrite.[11−13] The critical role of CO_2 was simple to demonstrate. The addition of sodium bicarbonate to deoxygenated solutions allowed the rate-retarding effect of added hydrogen peroxide to be observed, but ethanol did not retard the rate unless the solutions contained dioxygen. Rate retardation was also induced by methanol and 2-propanol in the presence of both CO_2 and O_2. No rate retardation was induced by *tert*-butyl alcohol, 2,2,2-trifluoroethanol, pure *tert*-butyl hydroperoxide,[21] tetrahydrofuran, benzoate, or *p*-toluenesulfonate under any experimental conditions explored. The effects of aldehydes are described below.

The rate of peroxynitrite decay is enhanced by the addition of CO_2, the increase in rate being directly proportional to the CO_2 concentration.[11] At low CO_2 concentrations (e.g., 0.05−0.1 mM), the addition of XOH (where XOH = methanol, ethanol, 2-propanol, or hydrogen peroxide) slowed the loss in OD as peroxynitrite decayed in a concentration-dependent manner, provided O_2 was also present for the three alcohols. These systems become "saturated" in XOH before the accelerating effect of the CO_2 was completely suppressed. At these low CO_2 concentrations, the decrease in OD always appeared to follow first-order kinetics, regardless of the XOH concentration (up to 1 M). However, with 1 mM CO_2, although there was an analogous slowing of the decrease in OD on addition of XOH, there was a deviation from first-order kinetics (Figure 1), and, at high XOH, the results could be fitted to second-order kinetics (see inset in Figure 1). The observed kinetic effects were the same whether the XOH was premixed in the peroxynitrite anion syringe or in the phosphate buffer syringe.

The apparent shift to second-order kinetics shown in the Figure 1 inset is very misleading, since the rate of OD change at a given OD is dependent on the starting peroxynitrite concentration (see Figure 2). The kinetic traces shown in Figure 2 would be consistent with either the loss of a reagent or the build-up of an unstable intermediate.[22,23] To distinguish between these two possibilities, the CO_2 concentration was increased to increase the rate of peroxynitrite decay and hence increase the rate of formation of any intermediate. At relatively high CO_2 concentrations, it became immediately apparent that the unusual

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(19) (i) The UV−Vis spectrum of the peroxynitrite anion (0.7 mM, pH = 12) was unaltered upon the addition of ethanol (up to 750 mM). (ii) The Raman spectrum of an aqueous solution (pH 12) containing peroxynitrite anion (1 M) and ethanol (4 M) was identical to the sum of the spectra for the same concentrations of these two species measured separately: the bands were not shifted, nor were any new bands formed. (The Raman spectrum of the peroxynitrite anion (1 M, pH 12) matched that previously reported by Tsai et al.[20]) (iii) The ^{14}N NMR spectrum of the peroxynitrite anion (1 M, pH 12) showed a very broad band (590 Hz) at 173 ppm (relative to nitromethane at zero ppm), which was not shifted, nor were any new bands observed between −60 and 280 ppm (scan width 9800 Hz) on addition of ethanol (up to 1 M).

(20) Tsai, J.-H. M.; Harrison, J. G.; Martin, J. C.; Hamilton, T. P.; van der Woerd, M.; Jablonsky, M. J.; Beckman, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 4115−4116.

(21) Commercial *tert*-butyl hydroperoxide showed a rate-retarding effect on the CO_2-catalyzed decomposition of peroxynitrite, but this was found to be due to the presence of the impurity, H_2O_2.

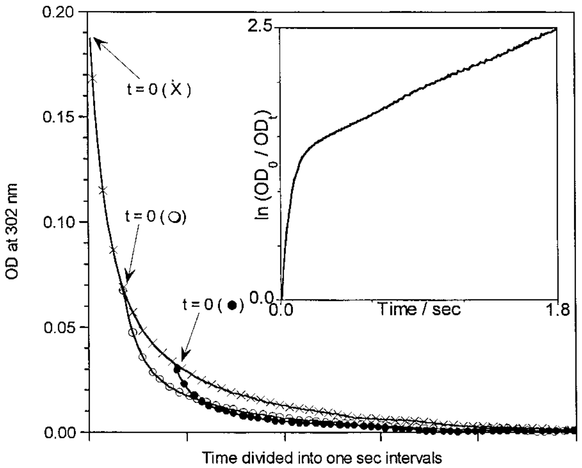

Figure 2. Change in OD at 302 nm on mixing equal volumes of peroxynitrite (3.4, 1.7, and 0.85 mM; ×, ○, and ● respectively) with phosphate buffer (200 mM, dioxygen-purged) containing ethanol (700 mM) and bicarbonate (2 mM, added 2−5 min prior to use). Final pH 6.3 and 24 °C. For the two lower peroxynitrite concentrations, the times have been offset so that the differences between the decay traces for equal concentration of "fresh" peroxynitrite and "partially decayed" peroxynitrite can be clearly seen. Inset: Change in OD at 302 nm on mixing equal volumes of peroxynitrite (1.4 mM, argon-purged and CO_2-free) with phosphate buffer (200 mM, air-purged) containing ethanol (700 mM) and sodium bicarbonate (10 mM, added 2−5 min prior to use). Final pH 6.3 and 24 °C.

kinetics arose from the build-up of an unstable intermediate which absorbs at the 302 nm wavelength used to monitor peroxynitrite decay. With 5 mM CO_2 and, for example, 350 mM ethanol (using an air-saturated buffer solution), the time scales for peroxynitrite decay and for decay of the intermediate became well separated, both following first-order kinetics (see inset in Figure 2). Another important observation was that the rate of decay of the intermediate (i.e., the slow, second stage of reaction) was identical, within the accuracy of our kinetic measurements, for all (active) XOH reagents. This implies that the same intermediate is formed in all active XOH, peroxynitrite, CO_2 (and O_2), systems.

As a further probe into the origin of the unstable intermediate, we added tetranitromethane (TNM, 0.2−2.5 mM) to peroxynitrite/bicarbonate (buffer dioxygen-saturated) systems. The nitroform anion (λ_{max} 350 nm, $\epsilon = 15\,000$ M^{-1} cm^{-1})[26] was not observed in the absence of XOH. However, the nitroform anion was readily identified upon the addition of all (active) XOH. It is, of course, produced by the one-electron reduction of TNM, a compound which has been widely employed as a probe for the superoxide anion radical, reaction 1, in various biomimetic and chemical systems[27] (to which we can now add the peroxynitrite/CO_2/XOH(O_2) systems).[28]

$$O_2^{\bullet-} + C(NO_2)_4 \longrightarrow O_2 + {}^-C(NO_2)_3 + {}^\bullet NO_2 \qquad (1)$$

We believe that the unstable intermediate formed during the decay of peroxynitrite in the presence of CO_2 and active XOH (+O_2) is *peroxynitrate*, as was first proposed by Goldstein and Czapski[30] (see below). Their mechanism for its formation from active alcohols and dioxygen is shown in reactions 2−8.

$$^-OONO + CO_2 \longrightarrow {}^-O_2COONO \qquad (2)$$

$$^-O_2COONO \longrightarrow CO_2 + NO_3^- \qquad (3)$$

$$^-O_2COONO \longrightarrow CO_3^{\bullet-} + {}^\bullet NO_2 \qquad (4)$$

$$CO_3^{\bullet-} + R_2CHOH \longrightarrow HCO_3^- + R_2\dot{C}OH \qquad (5)$$

$$R_2\dot{C}OH + O_2 \longrightarrow R_2C(OO^\bullet)OH \qquad (6)$$

$$R_2C(OO^\bullet)OH \longrightarrow R_2\overset{+}{C}OH + O_2^{\bullet-} \qquad (7)$$

$$O_2^{\bullet-} + {}^\bullet NO_2 \longrightarrow {}^-OONO_2 \qquad (8)$$

It seemed likely to us that peroxynitrate might also be produced when CO_2-free peroxynitrite was decomposed in the presence of aliphatic aldehydes since Pryor and co-workers[31] have shown that these compounds react rapidly with the peroxynitrite anion and, like CO_2, catalyze its decomposition. The homolytic portion of the proposed mechanism[31] is given by reactions 9 and 10. Using aldehydes in place of CO_2 offered

$$^-OONO + RCHO \xrightarrow{H^+} RCH(OH)OONO \qquad (9)$$

$$RCH(OH)OONO \longrightarrow [RCH(OH)O^\bullet\ {}^\bullet NO_2]_{cage} \longrightarrow RCH(OH)O^\bullet + {}^\bullet NO_2 \qquad (10a)$$

$$[RCH(OH)O^\bullet\ {}^\bullet NO_2]_{cage} \longrightarrow RCHO + NO_3^- + H^+ \qquad (10b)$$

the potential advantage of working at higher pH's, where the fraction of peroxynitrite present as peroxynitrous acid would be small, and consequently the spontaneous decay of peroxynitrite would be slow.[32] The reactions described below were performed at pH 8.5 using dioxygen-saturated, CO_2-free pyrophosphate buffer (100 mM).[33] Trimethylacetaldehyde increased

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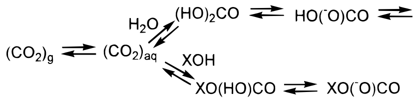
[22] Since [XOH] ≫ [CO_2], the traces shown in Figure 1 could also be consistent with a reduction in the free CO_2 concentration because of complex formation between XOH and CO_2, i.e.,



However, such an interaction can be ruled out because the strong, asymmetric stretch IR band of $(CO_2)_{aq}$ centered at 2344 cm^{-1} had neither its intensity nor its position affected by the addition of ethanol (up to 200 mM).

[23] At [CO_2] < [$^-$OONO], the addition of tyrosine[24] or nitrite[25] does not affect the initial rate of decay of peroxynitrite, but the first-order plots are curved because the reaction between CO_2 and peroxynitrite is catalytic in CO_2 and the tyrosine or nitrite reduces the efficiency of re-formation of CO_2, which, of course, lowers the CO_2 concentration as the reactions proceed.[24,25] Since we worked at [CO_2] > [$^-$OONO], a similar explanation for our results can be ruled out.

[24] Zhang, H.; Squadrito, G. L.; Pryor, W. A. *Nitric Oxide: Biol. Chem.* **1997**, *1*, 301−307.

[25] Pryor, W. A.; Lemercier, J.-N.; Zhang, H.; Uppu, R. M.; Squadrito, G. L. *Free Radical Biol. Med.* **1997**, *23*, 331−338.

[26] Bielski, B. H. J.; Allen, A. O. *J. Phys. Chem.* **1967**, *71*, 4544−4549.

[27] See, e.g.: McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049−6055. Ingold, K. U.; Paul, T.; Young, M. J.; Doiron, L. *J. Am. Chem. Soc.* **1997**, *119*, 12364−12365.

[28] Although strongly reducing radicals (such as $MeC^\bullet(OH)_2$, see below) also react rapidly with TNM to form the nitroform anion,[29] this reaction can be ignored because our experiments were done in dioxygen-saturated (1.4 mM) solutions (both buffer and peroxynitrite). Most of the carbon-centered radicals formed will react with dioxygen rather than with (0.2 mM) TNM.

[29] Schuchmann, M. N.; von Sonntag, C. *J. Am. Chem. Soc.* **1988**, *110*, 5698−5701.

[30] Goldstein, S.; Czapski, G. *J. Am. Chem. Soc.* **1998**, *120*, 3458−3463.

[31] Uppu, R. M.; Winston, G. W.; Pryor, W. A. *Chem. Res. Toxicol.* **1997**, *10*, 1331−1337.

[32] In fact, the anion dissociates slowly but reversibly to give $NO^\bullet$ and $^-O_2^\bullet$.[9a] The rate of anion decay can be accelerated by adding TNM to remove the superoxide.[9a]

[33] Buffers made from borate are known to accelerate the decay of peroxynitrite.[4,34]

[34] Hughes, M. N.; Nicklin, H. G. *J. Chem. Soc. (A)* **1968**, 450−452.

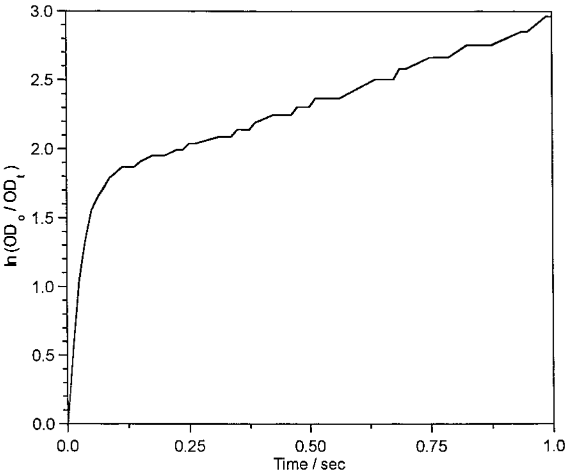


Figure 3. Change in OD at 280 nm on mixing equal volumes of peroxynitrite (1.4 mM, argon-purged and CO_2-free) with pyrophosphate buffer (100 mM, dioxygen-purged and CO_2 free) containing acetaldehyde (350 mM). Final pH 8.5 and 24 °C.

the rate of peroxynitrite decay in a concentration-dependent manner. These reactions followed pseudo-first-order kinetics for up to seven half-lives, with dramatic increases in the peroxynitrite decay rate; e.g., with 100 mM trimethylacetaldehyde (from a near saturation 200 mM in the buffer), the half-life was reduced from ~35 to 0.06 s. Peroxynitrate formation was not observed, and when the reaction was repeated with added TNM[35] (0.1 or 2.5 mM) the nitroform anion was not detected.

More interestingly, while acetaldehyde also accelerated the decomposition of peroxynitrite (in a concentration-dependent manner), there was a build-up and subsequent decay of an unstable intermediate (Figure 3). The decay of this intermediate followed essentially the same kinetics[36] as that of the intermediate formed in the peroxynitrite/CO_2/(active) $XOH(O_2)$ systems, from which we conclude that it is peroxynitrate (see Figure 3 and compare with inset in Figure 2). The addition of 0.05 mM TNM reduced the yield of peroxynitrate, while 0.2 mM TNM prevented any build-up of peroxynitrate and gave, instead, the nitroform anion.[28]

In the presence of 4-carboxybenzaldehyde, the decay of peroxynitrite and possible build-up and decay of peroxynitrate could not be monitored reliably because of a strong background absorbance below 320 nm. However, upon the addition of TNM (0.2 mM) to carboxybenzaldehyde/peroxynitrite systems at pH 8.5, the absorbance due to the nitroform anion grew in at a rate directly proportional to the aldehyde concentration (25, 50, and 100 mM). In the presence of TNM, the propionaldehyde- and glyceraldehyde-catalyzed decomposition of peroxynitrite also gave the nitroform anion.

Discussion

After this work had been initiated, we became aware of Goldstein and Czapski's[30] very thorough and careful study of the effect of XOH on the kinetics of decay of peroxynitrite in the presence of CO_2 for XOH = methanol, 2-propanol, hydrogen peroxide, and formate. Our present results serve to further confirm both their observations and mechanistic conclusions and to extend the conditions under which peroxynitrate formation can be observed.

Briefly, under appropriate conditions (viz., high CO_2 concentrations), Goldstein and Czapski[30] observed that peroxynitrite decay monitored at 302 nm showed two consecutive first-order processes (similar to the decay trace shown in the inset in our Figure 2). They clearly identified the intermediate responsible for the slow, second stage of the decay as peroxynitrate formed in a maximum yield of 30−33%. (The peroxynitrate anion absorbs at 302 nm,[30,37,38] whereas peroxynitric acid, pK_a = 5.9,[30,37,38] does not absorb above 280 nm[30,37,39]). Their proposed mechanism for the formation of peroxynitrate in the presence of methanol and 2-propanol (and ethanol) is shown in reactions 2−8. The critical steps in the generation of peroxynitrate include hydrogen atom abstraction from the alcohol by the carbonate radical anion[40] (reaction 5), subsequent formation of the superoxide radical anion (via reactions 6 and 7), and its trapping by the $^{\bullet}NO_2$ radical (reaction 8) which is, itself, formed, together with the carbonate radical anion, in the initial homolysis of $^{-}O_2COONO$ (reaction 4).

Our finding that neither *tert*-butyl alcohol, 2,2,2-trifluoroethanol, nor *tert*-butyl hydroperoxide influence the observed decay kinetics of peroxynitrite (in the presence of CO_2 and O_2) further supports the Goldstein and Czapski mechanism. That is, *tert*-butyl alcohol has no α-hydrogen atom available for abstraction by the carbonate radical anion. Although trifluoroethanol has α-hydrogen atoms which might be abstracted, any $CF_3CH(OO^{\bullet})$-OH radicals formed would not undergo heterolytic fragmentation to superoxide and a carbocation (reaction 7) because the carbocation would be destabilized by the electron-withdrawing CF_3 group. Radical attack on *tert*-butyl hydroperoxide would not yield superoxide.

Dioxygen is required for formate to exhibit its effect on the apparent kinetics of peroxynitrite decay[30] (reaction 11), a result we have confirmed. However, dioxygen is not required with H_2O_2 because the superoxide-forming reactions 5, 6, and 7 are replaced by reactions 12 and 13.[30]

$$CO_3^{\bullet-} + HCO_2^- \longrightarrow HCO_3^- + CO_2^{\bullet-} \xrightarrow{O_2} CO_2 + O_2^{\bullet-} \tag{11}$$

$$CO_3^{\bullet-} + H_2O_2 \longrightarrow HCO_3^- + HOO^{\bullet} \tag{12}$$

$$HOO^{\bullet} \rightleftharpoons H^+ + O_2^{\bullet-} \tag{13}$$

It will be obvious that any powerful one-electron oxidant produced during the decay of peroxynitrite *should* yield superoxide (and hence peroxynitrate, reaction 8) from suitable substrates. There is now strong evidence that peroxynitrous acid decomposes to give the hydroxyl radical in significant yields under conditions where CO_2 has been rigorously excluded (reaction 14).[6−14] *Peroxynitrate should therefore be formed from peroxynitrite in the presence of suitable substrates in the complete absence of* CO_2. This raises the following question: Why has peroxynitrate been observed only (or, probably only, in view of the argon-bubbling experiments of Alvarez et al.[16]) in the presence of CO_2? The answer lies in the general usage

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[35] There is a direct reaction between aldehydes and TNM, but it is insignificant on the 1 s time scale of our experiments.

[36] The decay is actually slightly faster at pH 8.5 than at pH 6.3. This increase in rate can be quantitatively accounted for by the decreased ratio of (relatively stable) peroxynitric acid (pK_a 5.9) to (short-lived) peroxynitrate anion.

[37] Logager, T.; Sehested, K. *J. Phys. Chem.* **1993**, *97*, 10047−10052.

[38] Goldstein, S.; Czapski, G. *Inorg. Chem.* **1997**, *36*, 4156−4162.

[39] Appelman, E. H.; Gosztola, D. J. *Inorg. Chem.* **1995**, *34*, 787−791.

[40] There is now overwhelming evidence that the peroxynitrite/CO_2 reaction yields the carbonate radical anion.[41−43]

[41] Bonini, M. G.; Radi, R.; Ferrer-Sueta, G.; Ferreira, A. M. Da C.; Augusto, O. *J. Biol. Chem.* **1999**, *274*, 10802−10806.

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[43] Meli, R.; Nauser, T.; Koppenol., W. *Helv. Chim. Acta* **1999**, *82*, 722−725.

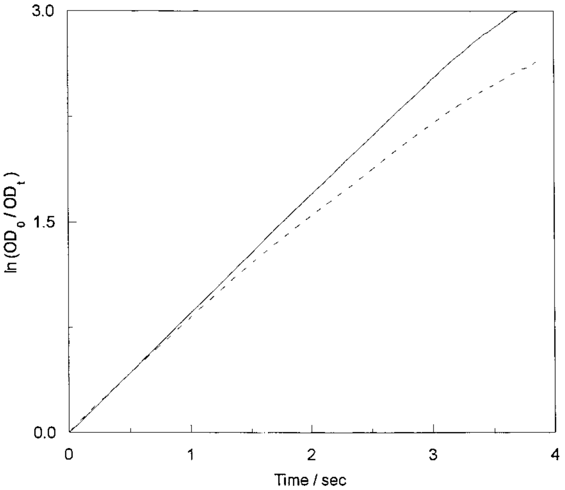
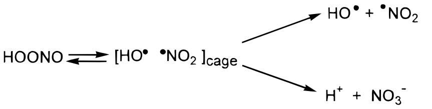


Figure 4. Change in OD at 280 nm on mixing equal volumes of peroxynitrite (1.4 mM, argon-purged and CO_2-free) with phosphate buffer (200 mM, dioxygen-purged and CO_2-free) containing either no (—) or 700 mM (- - -) ethanol. Final pH 6.3 and 24 °C.



(14)

(15)

of 302 nm to monitor peroxynitrite decay and the kinetics of decay of peroxynitrite and peroxynitrate at the pH's commonly employed in such studies (e.g., 6.3 in the present work). That is, the peroxynitrite anion ($\lambda_{max} = 302$ nm) is remarkably stable, but peroxynitrous acid (pK_a = 6.5−7.3, depending on buffer,[4] see Introduction) decays with a half-life, $\tau_{1/2} = 0.58$ s at 25 °C.[4] In contrast, peroxynitric acid (pK_a ∼ 5.9)[30,37,38] is more stable ($\tau_{1/2}$ = 16.5 min)[37] than its anion ($\tau_{1/2}$ ∼ 0.7s).[37] Moreover, although the peroxynitrate anion has a broad absorption band centered at 280 nm, peroxynitric acid does not absorb appreciably above 280 nm,[30,37,39] making it "invisible" at 302 nm.

With the above information, it becomes obvious that peroxynitrate formation should become kinetically observable at 280 nm if the pH is reduced slightly from 6.3. This will increase the rate of decay of peroxynitrite and decrease the rate of decay of peroxynitrate. However, this kinetic advantage has to be balanced against the reduction in detection sensitivity due to protonation of the peroxynitrate anion. We therefore chose a pH of 5.5. As expected, the 280 nm trace for peroxynitrite decay followed perfect first-order kinetics for CO_2-free, O_2-saturated solutions in the absence of ethanol, but in the presence of ethanol the rate of loss of the 280 nm absorbance decreased with time (see Figure 4 and compare with Figure 1). The slower, second stage of these reactions can be attributed to decay of peroxynitrate formed during peroxynitrite decay. In these CO_2-free systems, reactions 2, 4, and 5 are replaced by reactions 14 and 16.

$$HO^{\bullet} + MeCH_2OH \longrightarrow H_2O + Me\dot{C}HOH \qquad (16)$$

Peroxynitrate formation was also demonstrated by studying the decay of CO_2-free peroxynitrite in two stages. In the first stage, under CO_2-free conditions, the peroxynitrite anion (pH 12) was rapidly mixed with an equal volume of oxygen-saturated phosphate buffer (200 mM)/HCl (100 mM) containing either hydrogen peroxide (80 mM) or ethanol (300 mM) to give a solution with a final pH of 3.3. Under these conditions, the peroxynitrous acid would decay completely in a few seconds, whereas the peroxynitric acid would decay much more slowly.[44] In the second stage, this solution was rapidly mixed with an equal volume of aqueous sodium hydroxide (50 or 100 mM) to increase the pH to 7.6 or 9.3, respectively. The OD at 280 nm increased instantly and then decayed with first-order kinetics.[46] When the ethanol experiment was repeated using a peroxynitrite solution which had been allowed to decompose by standing at room temperature for 72 h, there was no transient peroxynitrate absorbance at 280 nm. However, peroxynitrate was detected when hydrogen peroxide was used, which can be attributed to the in situ formation of peroxynitrite via reaction 17.

$$H_2O_2 + HNO_2 \longrightarrow HOONO + H_2O \qquad (17)$$

It is obvious that CO_2 is not required in order to form peroxynitrate from peroxynitrite in the presence of active XOH (and dioxygen). However, except under certain carefully chosen conditions, peroxynitrate will not be observed because it decays at a rate similar to or even faster than that of the peroxynitrite. The CO_2 aids in the "visualization" of peroxynitrate because it increases the rate of peroxynitrite decay without affecting the decay of peroxynitrate, which thus becomes easier to observe. Addition of CO_2 also slightly increases (vide infra) the yield of the one-electron oxidizing agent needed to generate the superoxide form XOH (viz., ${}^{-}CO_3^{\bullet}$ vs ${}^{\bullet}OH$). It is important to realize that peroxynitrate will almost certainly also be formed in any CO_2-free or CO_2-containing systems in which peroxynitrite is generated in situ from a source of superoxide and nitric oxide (because ${}^{\bullet}NO_2$ will be produced during peroxynitrite decay and will compete with the ${}^{\bullet}NO$ for the available superoxide).

TNM (2 mM) was used to measure the yields of superoxide formed during the decomposition of peroxynitrite/XOH (24 °C, pH 6.3) in the absence and presence of CO_2. In the absence of CO_2, the superoxide was generated from H_2O_2 and EtOH/O_2 but not from formate/O_2 because CO_2 would also be produced (reaction 18). In the presence of CO_2, all three superoxide-generating systems were employed. The measured yields of superoxide decreased as the peroxynitrite concentration was increased (Figure 5 and Supporting Information), presumably because peroxynitrite and its nitrite contaminant compete with the substrate for the available oxidizing radicals. This was most noticeable with H_2O_2[47] because only 50 mM could be employed (in order to limit its background reaction with TNM), whereas 500 mM EtOH and formate were used. The yields of superoxide, measured as the nitroform anion, when extrapolated to zero peroxynitrite concentration can be equated to the yields of cage-escaped $HO^{\bullet}$ and ${}^{-}CO_3^{\bullet}$ radicals (see Table 1). The yields for these two radicals are independent of the superoxide-generating system employed (although a small correction is necessary for the $HO^{\bullet}$/EtOH system, see footnote *a* in Table 1) and are in outstanding agreement with what appear to be the best literature

$$HO^{\bullet} + HCO_2^{-} \longrightarrow H_2O + CO_2^{\bullet -} \xrightarrow{O_2} CO_2 + O_2^{\bullet -} \qquad (18)$$

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(44) Under the conditions used, the peroxynitric acid had a half-life of about 1 min (rather than 16.5 min)[37] because of its reaction with nitrous acid (pK_a 3.4) present from the peroxynitrite synthesis.[45]

$$HOONO_2 + HNO_2 \rightarrow 2\ H^+ + 2\ NO_3^- \ (k = 12 \pm 2\ M^{-1}\ s^{-1})^{37}$$

(45) Pryor, W. A.; Cueto, R.; Jin, X.; Koppenol, W. H.; Ngu-Schwemlein, M.; Squadrito, G. L.; Uppu, P. L.; Uppu, R. M. *Free Radical Biol. Med.* **1995**, *18*, 75−83.

(46) For reasons we did not explore, the rate constant for peroxynitrate decay was about 50% larger than the value found in all other experiments.

(47) Nitroform anion yields were, indeed, reduced by the addition of nitrite or decomposed peroxynitrite to H_2O_2-containing systems.

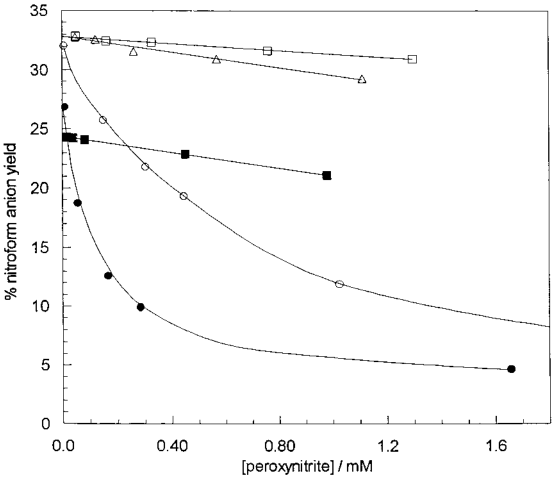

Figure 5. Yields of nitroform anion upon mixing equal volumes of TNM (4 mM, phosphate buffer, dioxygen saturated) containing H_2O_2 (●/○, 50 mM), EtOH (■/□, 500 mM), or $^-HCO_2$ (△, 500 mM) with various concentrations of peroxynitrite (dioxygen-saturated). The filled symbols are for data acquired under strictly CO_2-free conditions, and the open symbols are for data acquired with 12 mM bicarbonate added to the TNM solution 2−5 min prior to mixing (under these conditions, >99% of the peroxynitrite will react with the CO_2). Final pH 6.3 and 24 °C.

Table 1. Estimated Yields of Cage-Escaped $HO^•$ and $^-CO_3^•$ Free Radicals Produced in the Spontaneous and CO_2-Catalyzed Decomposition of Peroxynitrite, Respectively[a]

$O_2^{\bullet -}$ producing substrate	% Yields of Free Radicals	
	$HO^\bullet + NO_2^\bullet$	$CO_3^{\bullet -} + NO_2^\bullet$
EtOH	28.1[b]	32.8
H_2O_2	27.2	32.0
$^-HCO_2$	*c*	33.0
Yields from literature (ref)	28,[48] 32[6,49]	30-33,[30,53] 35[54]

[a] These yields are based on superoxide formation in the presence of appropriate substrates, as quantified by nitroform anion formation from TNM and extrapolated to zero peroxynitrite concentration (see Figure 5). Final pH 6.3 and 24 °C. [b] Abstraction of hydrogen atoms from EtOH by $HO^•$ occurs from the CH_3 (13.2%), CH_2 (84.3%), and OH (2.5%) positions; see: Asmus, K. D.; Mockel, H.; Henglein, A. *J. Phys. Chem.* **1973**, *77*, 1218−1221. Superoxide is formed from $CH_3C^•HOH$, which requires abstraction from the CH_2 or OH group (86.8%). The nitroform anion yield extrapolated to zero peroxynitrite concentration (24.4%) was therefore divided by 0.868. [c] Not measured because of CO_2 formation, see text.

values for yields of cage-escaped $HO^•$ [6,48,49] and $^-CO_3^•$ [30,53,54] radicals measured by quite different procedures.[55]

The formation of superoxide (and, hence, of peroxynitrate) in the reaction of oxygenated peroxynitrite with all the aldehydes examined except trimethylacetaldehyde is intriguing.[56] These different results can be understood by a consideration of the likely reactions of the cage-escaped hydroxyalkoxyl radical, $RCH(OH)O^•$, formed in reaction 10a. This radical will undergo two principal competing reactions, *β*-scission (reaction 19) and a rearrangement to give the carbon-centered radical, $RC^•(OH)_2$, via the well-known, water-mediated, 1,2-hydrogen atom shift[58−60] (reaction 20). In water, these electron-rich, carbon-centered radicals are known to react with dioxygen to form superoxide and a carbocation[29,61] (reaction 21).

$$RCH(OH)O^\bullet \xrightarrow{\beta} R^\bullet + HCO_2H \qquad (19)$$

$$RCH(OH)O^\bullet \xrightarrow{H_2O} R\overset{\bullet}{C}(OH)_2 \qquad (20)$$

$$R\overset{\bullet}{C}(OH)_2 + O_2 \longrightarrow \longrightarrow R\overset{+}{C}(OH)_2 + O_2^{\bullet -} \qquad (21)$$

The 1,2-H atom shift for analogous rearrangements of $RCH_2O^•$ radicals is believed to occur more rapidly when R is allyl[59] or phenyl[60] than when R is an alkyl group because of the increased thermodynamic driving force provided by resonance stabilization of the product $RC^•H(OH)$ radical. Reaction 20 will therefore be particularly fast in the peroxynitrite/carboxybenzaldehyde system. Moreover, in this system the *β*-scission with loss of the carboxyphenyl radical will be extremely slow for thermodynamic reasons (to judge from the extensive data available for structurally analogous alkoxyl radicals).[62] Carboxybenzaldhyde and other benzaldehydes would therefore be expected to give a molecule of superoxide for every $RCH(OH)O^•$ radical which escapes from the solvent cage. In contrast, reaction 20 is expected to be somewhat slower when carboxybenzaldehyde is replaced by trimethylacetaldehyde, whereas *β*-scission, with loss of the *tert*-butyl radical, is expected to be extremely fast.[62] Thus, the oxygenated peroxynitrite/trimethylacetaldehyde system was not expected to give superoxide in significant yield, and, in fact, none could be detected as peroxynitrate or with TNM.

Superoxide yields were quantified with TNM (2 mM) in aldehyde/peroxynitrite/dioxygen systems at pH 8.5 under CO_2-free conditions and with aldehyde concentrations high enough that >98% of the peroxynitrite underwent aldehyde-catalyzed decay. These superoxide yields (which were independent of the peroxynitrite concentration, 0.02−1.0 mM) were as follows: 15%, 4-carboxylbenzaldehyde (100 mM); 9%, acetaldehyde (35

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(48) Gerasimov, O. V.; Lymar, S. V. *Inorg. Chem.* **1999**, *38*, 4317−4321.

(49) A highly reactive oxidant which was said not to be the $HO^•$ radical was found to be formed in a yield of 40%,[50] which was later revised to 32%.[51] Still later[9b] it was admitted that this oxidant was the $HO^•$ radical (though its yield was still given as 40%). The yield of free $HO^•$ from these experiments[50,51] should be considered to be 32%.[52]

(50) Goldstein, S.; Czapski, G. *Inorg. Chem.* **1995**, *34*, 4041−4048.

(51) Goldstein, S.; Czapski, G. *Nitric Oxide: Biol. Chem.* **1997**, *1*, 417−422.

(52) Goldstein, S. Private communication.

(53) Goldstein, S.; Czapski, G. *Inorg. Chem.* **1997**, *36*, 5113−5117.

(54) Lymar, S. V.; Hurst, J. K. *Inorg. Chem.* **1998**, *37*, 294−301.

(55) We estimated previously that $HO^•$ radicals were formed in a yield of ca. 10%,[7] which was based on their trapping with dimethyl sulfoxide. Although we proved by product identification that $HO^•$ radicals were undoubtedly produced, their yield was clearly underestimated for reasons we do not yet understand.

(56) The reaction of peroxynitrite with pyruvate shows many parallels with its reactions with aldehydes,[57] viz.,

$$CH_3COCO_2^- + {}^-OONO \longrightarrow CH_3CO_2^- + CO_2^{\bullet -} + {}^\bullet NO_2$$

(57) Vásquez-Vivar, J.; Denicola, A.; Radi, R.; Augusto, O. *Chem. Res. Toxicol.* **1997**, *10*, 786−794.

(58) Gilbert, B. C.; Laue, H. A. H.; Norman, R. O. C. *J. Chem. Soc., Perkin Trans. 2* **1976**, 1040−1044. Dobbs, A. J.; Gilbert, B. C.; Laue, H. A. H.; Norman, R. O. C. *J. Chem. Soc., Perkin Trans. 2* **1976**, 1044−1047. Gilbert, B. C.; Holmes, R. A. G.; Laue, H. A. H.; Norman, R. O. C. *J. Chem. Soc., Perkin Trans. 2* **1976**, 1047−1052.

(59) Elford, P. E.; Roberts, B. P. *J. Chem. Soc., Perkin Trans. 2* **1996**, 2247−2256.

(60) Konya, K. G.; Paul, T.; Lin, S.; Lusztyk, J.; Ingold, K. U. *J. Am. Chem. Soc.*, submitted.

(61) Bothe, E.; Schuchmann, M. N.; Schulte-Frohlinde, D.; von Sonntag, C. *Photochem. Photobiol.* **1978**, *28*, 639−644. Bothe, E.; Schulte-Frohlinde, D.; von Sonntag, C. *J. Chem. Soc., Perkin Trans. 2* **1978**, 416−420.

(62) Howard, J. A.; Scaiano, J. C. In *Radical Reaction Rates in Liquids*; Fischer, H., Ed.; Landolt-Börnstein, New Series; Springer-Verlag: Berlin, 1984; Vol. 13d. Lusztyk, J. In *Radical Reaction Rates in Liquids*; Fischer, H., Ed.; Landolt-Börnstein, New Series; Springer-Verlag: Berlin, 1997; Vol. 18d-1.

mM); 0.8%, propionaldehyde (50 mM); and 0%, trimethylacetaldehyde (30 mM). We attribute the decrease in the yield of superoxide along this series of aldehydes to the undoubted increase in the importance of β-scission[62] (reaction 19) relative to the 1,2-H atom shift (reaction 20). That β-scission plays a significant role even in the acetaldehyde/peroxynitrite reaction has been unequivocally demonstrated by Nakao, Ouchi, and Augusto.[63] These workers spin-trapped the methyl radical in yields of about 10% (based on peroxynitrite), which paralleled (as they should) the yields of formate (8% at pH 6.7 and 13% at pH 9.1). Comparing our measured yield of superoxide (9% at pH 8.5) with Augusto and co-workers' [63] yields of methyl radicals and formate indicates that the rates of reactions 19 and 20 are roughly equal when R = CH_3. Furthermore, the total yield of cage-escaped radicals in the acetaldehyde/peroxynitrite system must be ca. 9% + (8−13%), i.e., ca. 19%. Acetate is, of course, formed concomitantly with superoxide, i.e., reaction 22. However, our 9% yield of superoxide is lower than the reported yields of acetate for this system, viz., 17% (pH 6.7),[63] 19% (pH 7.0),[31] and 20% (pH 9.1),[63] which implies that there must be another, previously unrecognized, route to acetate. We suggest that this is an in-cage disproportionation (or electron transfer) involving the initial geminate radical pair (reaction 23). That is, this caged radical pair undergoes *three* competing forward reactions: reactions 10a (R = CH_3), 10b (R = CH_3), and 23.

$$O_2 + CH_3\dot{C}(OH)_2 \longrightarrow O_2^{\bullet-} + CH_3\overset{+}{C}(OH)_2 \longrightarrow CH_3CO_2H + H^+ \quad (22)$$

$$\left[CH_3CH(OH)O^\bullet \ {}^\bullet NO_2\right]_{cage} \longrightarrow CH_3CO_2H + HNO_2 \ (\longrightarrow H^+ + NO_2^-) \quad (23)$$

The reaction of glyceraldehyde (50 mM) with oxygenated peroxynitrite and TNM was studied because it appeared likely that the hydroxyalkoxyl radical formed in reaction 10 would be able to yield superoxide both via the "normal" 1,2-H atom shift and from the *hydroxyalkyl* radical formed by β-scission (reactions 24−27). The yield of superoxide (measured as the nitroform anion) with glyceraldehyde was 29.7%, a result which is very comparable to the yields of superoxide (cage-escaped free radicals) from HOONO (∼28%) and $^-O_2COONO$ (∼33%).

The result with glyceraldehyde raises two intriguing possibilities: (i) different aldehydes give very different yields of cage-escaped radicals and (ii) different aldehydes give similar yields of cage-escaped radicals. If (ii) is correct, then it would seem probable that aldehyde/peroxynitrite reactions should give a ca. 30% yield of cage-escaped radicals and that our lower values for 4-carboxybenzaldehyde (15%) and acetaldehyde (19%) are a consequence of as-yet unrecognized "other" reactions of $RCH(OH)O^\bullet$ radicals. If (i) is correct, then there are some fascinating structure/activity relations waiting to be explored. Further work is planned.

$$HOCH_2CH(OH)CHO + {}^-OONO \xrightarrow{H^+} HOCH_2CH(OH)CH(OH)O^\bullet + {}^\bullet NO_2 \quad (24)$$

$$HOCH_2CH(OH)CH(OH)O^\bullet \xrightarrow[O_2]{1,2\text{-H shift}} HOCH_2CH(OH)CO_2H + H^+ + O_2^{\bullet-} \quad (25)$$

$$HOCH_2CH(OH)CH(OH)O^\bullet \xrightarrow{\beta} HOCH_2\dot{C}H(OH) + HCO_2H \quad (26)$$

$$HOCH_2\dot{C}H(OH) + O_2 \longrightarrow HOCH_2CHO + H^+ + O_2^{\bullet-} \quad (27)$$

Conclusions

We have provided additional evidence in support of the Goldstein and Czapski[30] mechanism(s) by which the decomposition of peroxynitrite in the presence of CO_2 and H_2O_2 or dioxygen plus formate or a suitable alcohol yields peroxynitrate, the formation of which can affect apparent peroxynitrite/CO_2 decay kinetics monitored at 302 nm. We have also shown that peroxynitrate can be a confounding factor during the decay of peroxynitrite in the presence of H_2O_2 or a suitable alcohol plus dioxygen in the complete absence of CO_2. Peroxynitrate is also formed in the presence of certain aldehydes and dioxygen. The formation of peroxynitrate under so many different conditions certainly raises the question of its biological significance. Finally, we have used tetranitromethane to determine the yields of superoxide formed from peroxynitrite under a variety of conditions. These superoxide yields can be used to quantify the yields of freely diffusing $^\bullet NO_2$ and oxyl radicals for peroxynitrous acid, 27−28% $HO^\bullet$, and for the peroxynitrite/CO_2 adduct, 32−33% $^-CO_3^\bullet$, both percentages being in excellent agreement with recent literature data.[6,30,48,49,53,54] Not all peroxynitrite/aldehyde adducts homolyze to hydroxyalkoxyl radicals which quantitatively rearrange and react with dioxygen to form superoxide. However, our data suggest that freely diffusing $^\bullet NO_2$ and oxyl radicals are formed in yields of 15% from carboxybenzaldehyde, 30% from glyceraldehyde, and (when combined with data from the literature)[63] ca. 19% from acetaldehyde.

Acknowledgment. We sincerely thank Drs. O. Augusto, G. Czapski, S. Goldstein, W. H. Koppenol, S. V. Lymar, and G. Merenyi and an anonymous reviewer for their comments, criticisms, and suggestions regarding an earlier version of this paper. We also thank Drs. T. Paul and J. Lusztyk for helpful discussions, Dr. C. A. Talk for the Raman spectra, D. Leek for the ^{14}N NMR spectra, and the National Foundation for Cancer Research for partial support of this work.

Note Added in Proof: Dr. Sara Goldstein has demonstrated by chemical kinetic simulation that in the absence of nitrite (a contaminant in our peroxynitrite) the reduction is the peroxynitrite's apparent rate of decay induced by 150 mM H_2O_2 in the absence of CO_2 would be ca. 210%, rather than the 10% we measured.

Supporting Information Available: Full experimental details and a table of nitroform anion yields in the absence and presence of CO_2 using various superoxide generating systems (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA991077U

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[63] Nakao, L. S.; Ouchi, D.; Augusto, O. *Chem. Res. Toxicol.* **1999**, *12*, 1010−1018.