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The Complex Chemistry of Peroxynitrite Decomposition: New Insights[1]

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Abstract: The yield of hydroxyl radicals produced in the decomposition of peroxynitrous acid (HOONO) at room temperature in deoxygenated and bicarbonate free water at pH ~6.8 has been determined to be roughly 10%. This value rests on a detailed study of the decomposition of peroxynitrous acid in the presence of dimethyl sulfoxide with stopped-flow kinetics and product analyses by unequivocal methods. The $HO^{\bullet}$/DMSO reaction is known to yield methane sulfinic acid (MSA) and $CH_3^{\bullet}$ radicals with 91% efficiency. MSA was quantified by ^1H NMR and its measured yield was corrected to allow for its extensive further oxidation to methane sulfonic acid. Methyl radicals were quantified by trapping with a water-soluble, stable nitroxide. At low peroxynitrite concentrations these two techniques gave $HO^{\bullet}$ yields of ca. 8% and ca. 13%, respectively. We conclude that in water the main (ca. 90%) decomposition pathway for peroxynitrite involves a rearrangement to nitric acid via an in-cage collapse of the singlet $HO^{\bullet}/^{\bullet}NO_2$ radical pair which may, in part, be preceded by electron transfer to form an $HO^{-}/^{+}NO_2$ intimate ion pair. We emphasize that, in contrast to many earlier reports, a distinct pathway to hydroxyl radicals is present, which implies that a significant portion of the oxidative nature of peroxynitrite can stem from hydroxyl radical-induced chemistry.

In a 1990 landmark publication, Beckman et al.[5] suggested that two relatively unreactive, but biologically important, free radicals, superoxide and nitric oxide, would combine under physiological conditions to form peroxynitrite.[6] Reaction 1 appears to be diffusion controlled ($k_1 = 1.9 \times 10^{10}$ M^{-1} s^{-1})[7]

$$O_2^{\bullet -} + {}^{\bullet}NO \rightarrow {}^{-}OON{=}O \qquad (1)$$

and the anion is thermally stable. However, at physiological pH, the anion will be partially protonated (pK_a 6.8)[6−8] to form peroxynitrous acid, which has a half-life of just less than 1 s at 37 °C. Furthermore, Beckman et al.[5] reported that peroxynitrite decomposition[9] generated "a strong oxidant able to initiate many reactions currently used to implicate the action of $HO^{\bullet}$". These workers went on to make the exciting suggestion that this strong oxidant might actually be the hydroxyl radical (which is by far the most reactive of all biologically relevant "oxy radicals").

$${}^{-}OON{=}O \underset{-H^+}{\overset{H^+}{\rightleftharpoons}} HOON{=}O \rightarrow HO^{\bullet} + NO_2^{\bullet} \qquad (2)$$

Beckman et al.'s suggestion that peroxynitrite could undergo O−O bond homolysis, reaction 2, has had a difficult time because it has proved impossible to achieve consensus regarding the yield of freely diffusing $HO^{\bullet}$ radicals relative to the yield of the isomerization, *or in-cage collapse*, product, nitric acid.

$$H^+ + NO_3^- \leftarrow HOONO \underset{k_r}{\overset{k_f}{\rightleftharpoons}} [HO^{\bullet} + NO_2^{\bullet}]_{cage} \rightarrow H^+ + NO_3^- \qquad (3)$$

Thus, Beckman et al.[5] reported maximum "product yields (at infinite detector concentration relative to peroxynitrite) indicative of hydroxyl radical" at pH 6.0 of 7.0 ± 0.1% (based on malondialdehyde, MDA, produced by the oxidation of deoxyribose) and 24 ± 1.0% (based on formaldehyde produced by the oxidation of dimethyl sulfoxide, DMSO). The apparent yields of free $HO^{\bullet}$ radicals (or of a hydroxyl radical-like species) based both on MDA and formaldehyde production decreased at higher pH and there are conflicting explanations for these observations.[10,11] The yield of hydroxyl radicals (or, sometimes, of a hydroxyl radical-like species) from the decomposition of peroxynitrite has also been reported to be 40%,[10] 32%,[12] 25%,[11] 4%,[13] about 1%,[14] 0.9%[13] and zero[15] or probably zero.[7,16−19]

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However, many of these reports were published before the important role that CO_2 can play in peroxynitrite chemistry was known.[20,21] Adventitious CO_2 could have perturbed some of these measurements of $HO^\bullet$ yields.[22] Furthermore, some of the substrates used to measure the $HO^\bullet$ yields probably reacted directly with peroxynitrite. Additional uncertainties regarding the yield of $HO^\bullet$ radicals from peroxynitrite come from the variable effects of known hydroxyl radical scavengers on the yields of oxidation products from various substrates.[5,6,8,11,12,23]

The peroxynitrite story became even more confusing when Koppenol et al.[24] claimed that their thermodynamic calculations and kinetic measurements precluded the formation of $HO^\bullet$ from HOONO. The kinetic argument was based on the measured activation parameters for peroxynitrite decomposition, viz.,[24] $\Delta H^\ddagger = 18 \pm 1$ kcal mol^{-1} and $\Delta S^\ddagger = 3 \pm 2$ cal mol^{-1} K^{-1} (corresponding to $\log(A/\mathrm{s}^{-1}) = 13.9$). Although the calculated activation enthalpy for HOONO homolysis in water, viz.,[24] 17 kcal mol^{-1}, was in good agreement with the measured value, homolysis was discarded because the activation entropy was smaller than the ca. 12 cal mol^{-1} K^{-1} generally found for homolysis of the O−O bond in peroxides. The small magnitude of the activation entropy for peroxynitrite decomposition relative to the homolysis of other peroxides must indeed reflect "rigidity in the transition state" (or extensive in-cage return,[6] k_r, vide infra) if the former reaction is homolytic. However, the potential role of the water solvent in producing such "rigidity" appears not to have been considered until some years later.[6] Subsequent thermodynamic and kinetic arguments have both supported[25] and opposed[26] Koppenol et al.'s original conclusion that HOONO does not undergo homolysis in water. Most recently, Koppenol and Kissner[27] have admitted that homolysis is possible but, nevertheless, conclude that homolysis is unlikely.

Measurements of the rates of decay of peroxynitrite in the presence of, for example, thiols have shown that the reaction is first order in peroxynitrite and first order in thiol, which is consistent with a simple bimolecular reaction.[24,28] There are quite a number of other "substrates", however, which have been reported to react with kinetics that are first order in peroxynitrite but zero order in "substrate".[6] That is, the rate of decay of peroxynitrite is the same in the absence as in the presence of these substrates. (The occurrence of such reactions can, of course, only be revealed by product analyses.) One consequence of the thermochemical theories extant at the time was that these zero-order in substrate reactions were assumed to involve some high energy form of peroxynitrite that was not the $HO^\bullet$ radical. This high-energy form has been designated as HOONO* [6] and has often been assumed to be present together with ground-state HOONO in peroxynitrite solutions. Initially, the HOONO* was a "vibrationally excited" form of HOONO[11] but, as Pryor and Squadrito[6] have correctly pointed out, "vibrationally excited states generally are too short-lived (with lifetimes of $\sim 10^{-11}$ s) to participate in bimolecular reactions". As an alternative to the kinetically incompetent vibrationally excited state, Pryor and co-workers[6,8,16,19,29] have suggested that HOONO* is a high-energy, metastable form of HOONO that is present in steady state with ground-state peroxynitrous acid.

The foregoing is intended to provide some insight into the complexities of peroxynitrite chemistry and to set the stage for our own work. We limited our objective to determining to what extent, if at all, hydroxyl radicals are formed during the thermal decomposition of peroxynitrite at room temperature.

Results

Our work has been based on the following concepts: (1) use of a substrate that (i) is known to react with $HO^\bullet$ radicals at, or close to, the diffusion-controlled rate, (ii) is at least purported not to react with peroxynitrite, and (iii) is known to give a clearly defined product (or products) in its reaction with $HO^\bullet$; (2) study of the kinetics of the reactions (if any) of peroxynitrite with (i) the initial substrate, (ii) its $HO^\bullet$-derived product(s), and (iii) any secondary reagent added to "trap" any $HO^\bullet$-derived product; and (3) use of unequivocal analytical procedures to search for the "*expected* " $HO^\bullet$-derived product(s) in a manner that would allow their identification and quantification even if formed in a very low yield from the peroxynitrite.

Experimental Approach. Following in Beckman et al.'s footsteps,[5] we chose DMSO as the substrate to probe for $HO^\bullet$ radicals since this compound was not supposed to react with peroxynitrite.[5,11] (There is probably a direct reaction between peroxynitrite and DMSO but it is extremely slow, vide infra.) In water at ambient temperatures DMSO reacts extremely rapidly with hydroxyl radicals ($k_4 = 7 \times 10^9$ M^{-1} s^{-1}) to yield methane sulfinic acid (MSA) and methyl radicals with a ca. 91% efficiency.[30] In the event that peroxynitrite did yield hydroxyl radicals, an additional attraction of DMSO was the formation of two products which should permit two independent measurements of the $HO^\bullet$ yield.

$$HO^\bullet + \underset{\text{DMSO}}{(CH_3)_2SO} \rightarrow \underset{\text{MSA}}{CH_3S(O)OH} + CH_3^\bullet \qquad (4)$$

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[22] We did extensive earlier work on $HO^\bullet$ yields from peroxynitrite (which was written up and submitted!) before we appreciated (thanks to Prof. W. A. Pryor) the important role that CO_2 can play in peroxynitrite chemistry.[20,21] No precautions were taken in our earlier work to exclude CO_2 and although hydroxyl radicals were detected (using some of the methodology described herein) their yield was very much lower (ca. 0.4%). This low yield was probably due partly to the presence of CO_2 and partly to the fact that significantly higher peroxynitrite concentrations were used in the earlier work (vide infra).

[23] For a detailed listing of scavenger effects see: Goldstein, S.; Squadrito, G. L.; Pryor, W. A.; Czapski, G. *Free Radical Biol. Med.* **1996**, *21*, 965−974.

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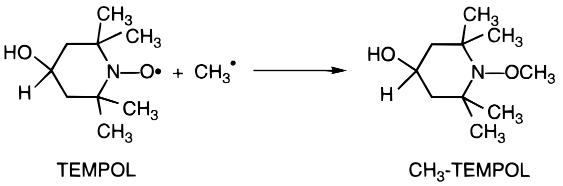
Peroxynitrite (≤75 mM) was synthesized at pH 12 by reaction of ozone with sodium azide following the procedure of Gleu and Roell[31] and of Pryor et al.[32] with rigorous exclusion of carbon dioxide[20−22,33] (see Experimental Section).

The kinetics of peroxynitrite decay were monitored at 302 nm at room temperature (23 ± 1 °C) in a stopped-flow apparatus.[5,8,10,16,19,24,25,28,29] The nitrogen-purged (deoxygenated and CO_2 free) pH 12 peroxynitrite solution was very rapidly mixed (∼1.3 ms) with known concentrations of substrate in nitrogen-purged (deoxygenated and CO_2 free) KH_2PO_4 buffer so that all subsequent reactions occurred at pH ∼6.8. (The pH was measured after every run.) In the absence of substrate, numerous measurements of the first-order rate constant for (2.6 mM) peroxynitrite decay, k_o, gave an average value of 0.95 ± 0.1 s^{-1} at room temperature. At higher peroxynitrite concentrations the decay appeared to be faster.[34] However, most of our product studies, and the *only* ones we consider to be reasonably reliable, were deliberately carried out with the minimum concentration of peroxynitrite (2.6 mM), which gave accurately measurable absolute yields of products by the techniques we employed. Even at 10.5 mM peroxynitrite the calculated $HO^•$ yields were dramatically lower than at 2.6 mM, vide infra. (The dependence of $HO^•$ yields on peroxynitrite concentrations both higher than 10.5 mM and lower than 2.6 mM is currently under investigation.)

Products were generated from DMSO under similar conditions in a separate rapid mixing (ca. 4 ms) apparatus with a glass collection vessel attached to the mixer. These products were analyzed by ^1H NMR.[35] Methyl radicals were trapped by 4-hydroxy-2,2,6,6-tetramethylpiperidin-*N*-oxyl, TEMPOL, which was added to the DMSO/buffer solution. This nitroxide is very soluble (>1 M) in the buffer, and the resultant *O*-methylhydroxylamine, CH_3-TEMPOL, was analyzed by GCMS with CD_3-TEMPOL (added postreaction) as an internal standard.



(5)

Kinetics. (1) DMSO. In contrast to earlier reports, there would appear to be a slow reaction between DMSO and peroxynitrite, which is first order in peroxynitrite **and** first order in DMSO, because the experimental first-order rate constant for peroxynitrite decay, k_{exptl}, increases as the DMSO concentration is increased (see Supporting Information). We believe that the most likely product from such a direct, bimolecular reaction would be methyl sulfone, reaction 6, and this compound was indeed observed by NMR product analysis (vide infra). If the

$$Me_2SO + {}^{-}OONO\ (HOONO) \rightarrow Me_2SO_2 + NO_2^{-} + (H^{+}) \quad (6)$$

kinetic data are plotted in the usual way according to $k_{exptl} = k_o + k_6[DMSO]$, where k_0 (=0.95 s^{-1}, vide supra) is the rate constant in the absence of DMSO, the derived value of k_6 is 2.1 $M^{-1}\ s^{-1}$.

(2) MSA. This acid was found to react fairly rapidly with peroxynitrite with kinetics that were first order in peroxynitrite and first order in MSA (see Supporting Information). We believe the most likely product of this reaction would be methane sulfonic acid (reaction 7) and this compound was also observed by NMR product analysis (vide infra). With use of

$$MeS(O)OH + {}^{-}OONO\ (HOONO) \rightarrow MeS(O)_2OH + NO_2^{-} + (H^{+}) \quad (7)$$

the equation $k_{exptl} = k_0 + k_7[MSA]$, with $k_o = 0.95\ s^{-1}$, the kinetic data yield $k_7 = 325\ M^{-1}\ s^{-1}$.

(3) TEMPOL. Rapid mixing of solutions to yield final concentrations of peroxynitrite of 10.5 mM and TEMPOL ranging from 5 to 25 mM showed that the decomposition of peroxynitrite was accelerated by the nitroxide (reaction 8), see

$$TEMPOL + {}^{-}OONO\ (HOONO) \rightarrow products \quad (8)$$

Supporting Information. According to $k_{exptl} = k_o + k_8[TEMPOL]$ the magnitude of k_8 was 44.5 $M^{-1}\ s^{-1}$.

Products. (1) ^1H NMR Product Analyses. Aliquots of the products formed by rapid mixing of peroxynitrite (concentrations 2.6 and 10.5 mM after mixing) and DMSO (concentration 105 mM after mixing) were analyzed by ^1H NMR (400 MHz). A typical spectrum is shown in Figure 1. In all samples four products could be detected: methyl sulfone, Me_2SO_2; methane sulfinic acid, MeS(O)OH (MSA); methane sulfonic acid, $MeS(O)_2OH$; and methanol (see Table 1). Unfortunately, the ^1H NMR peak due to $MeS(O)_2OH$ was not resolved from the peak due to DMSO at a DMSO concentration of 420 mM. Formaldehyde, a product expected on the basis of earlier work,[5,32] was not detected. However, blank experiments with $[CH_2O] = [CH_3OH] = 0.15$ mM in the buffer showed that formaldehyde could not be detected by ^1H NMR at this concentration.

Interestingly, the yields of $HO^•$ radical-derived products decreased dramatically relative to the initial peroxynitrite concentration as the latter was increased from 2.6 to 10.5 mM. We attribute this to other (higher order) reactions of peroxynitrite which appear to produce kinetic anomalies at higher peroxynitrite concentrations, vide supra,[34] and presumably reduce the fraction of peroxynitrite decaying by simple O−O bond homolysis.

The apparent yields of methyl sulfone in these peroxynitrite/DMSO reactions had to be corrected for the fact that this compound was present as a very minor impurity [(8×10^{-2})%] in the starting DMSO. Thus, in a typical experiment with postmixing [DMSO] = 105 mM and [peroxynitrite] = 2.6 mM,

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(31) Gleu, K.; Roell, E. *Z. Anorg. Allg. Chem.* **1929**, *179*, 233−266.

(32) 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.

(33) A referee of our earlier work pointed out that the ozone/azide route to peroxynitrite might not have been the best synthetic choice because nitrite is formed in only slightly lower concentration than peroxynitrite[32] and nitrite reacts with $HO^•$ radicals about as rapidly (see, e.g.: Logager, T.; Sehested, K. *J. Phys. Chem.* **1993**, *97*, 6664−6669) as does DMSO.[30] Thus, some $HO^•$ would be “lost” by reaction with nitrite. However, on repeating all our earlier work under CO_2-free conditions we opted for the simplicity of the ozone/azide method which can be justified by the fact that the DMSO concentration was always 10, or more, times greater than the concentration of peroxynitrite and, hence, than the concentration of nitrite.

(34) After this work was completed, ref 7 appeared, which reported that the decay of peroxynitrite was *slower* at 0.48 mM than at 0.048 mM [peroxynitrite]. Both of these concentrations are, unfortunately, below the minimum peroxynitrite concentration we felt we could employ for reliable quantitation of products.

(35) The popular analyses for MSA by its color-making reactions with diazonium salts such as Fast Garnet GBC[36] and Fast Blue BB[37] did not work in our DMSO/HOONO product mixtures, even when MSA was deliberately added! The culprit is residual azide (used in the synthesis of peroxynitrite), which destroys the diazonium salts even at very low concentrations.

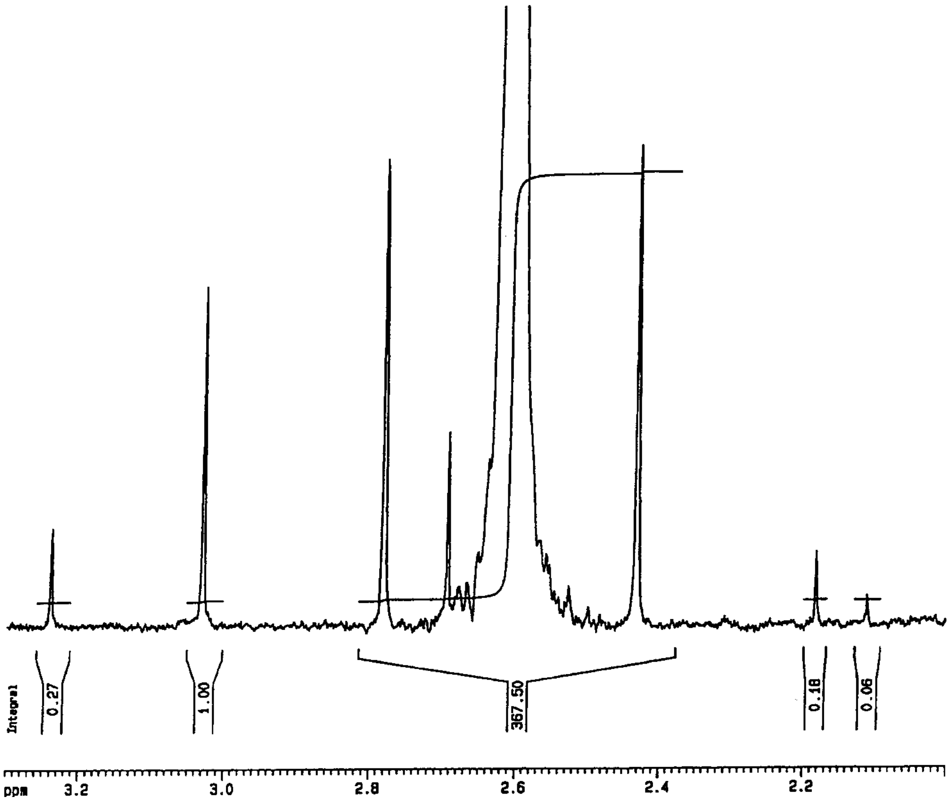

Figure 1. Typical ^{1}H NMR spectrum (400 MHz) of the products of reaction of 2.6 mM peroxynitrite with 105 mM DMSO. The peaks (ppm) are due to CH_3OH (3.24), Me_2SO_2 (3.03), $MeSO_3H$ (2.70), DMSO (2.61) with two ^{13}C satellites bands), $MeSO_2H$ (2.19), and an unknown impurity in the DMSO (2.12).

Table 1. Product Yields Measured by ^{1}H NMR (400 MHz) for the Decomposition of Peroxynitrite at pH 6.8 and 23 ± 1 °C in the Presence of DMSO

HOONO (mM)[a]	DMSO (mM)[a]	Me_2SO_2[b] (mM)	MeOH (mM)	$MeSO_2H$ (mM)	$MeSO_3H$ (mM)	$HO^•$ (%)[c]
2.6	105	0.20	0.14	0.04	0.26	$12._5$
2.6	105	0.21	0.16	0.10	0.21	13
2.6	105	0.14	0.13	0.06	0.22	12
2.6	105	0.15	0.14	0.10	0.24	14
2.6	105	0.18	0.16	0.12	0.18	$12._5$
10.5	105	0.13	0.06	0.06	0.06	1.2
10.5	105	0.24	0.14	0.12	0.22	$3._5$
2.6[d]	420[d]	0.36	0.17	0.19	e	f
2.6[d]	420[d]	0.30	0.14	0.16	e	f

[a] Concentration after mixing. There was also 100 mM KH_2PO_4 present after mixing. [b] Me_2SO_2 actually formed in the reaction. This compound is an impurity in the DMSO [(8.1×10^{-2}) %]. The measured concentrations have been corrected by subtracting (8.1×10^{-4})[DMSO] M. [c] Calculated as $100([MeSO_2H] + [MeSO_3H])/([HOONO]_0 \times 0.91)$, see text. [d] Representative run. [e] The peak due to $MeSO_3H$ was not resolved from that due to DMSO. [f] Impossible to calculate.

the measured concentration of methyl sulfone in the products was 0.18 mM. The correction for the sulfone impurity amounts to 0.08 mM and hence the corrected yield of sulfone is 0.10 mM, or 3.8% based on the initial concentration of peroxynitrite. If methyl sulfone were the only peroxynitrite/DMSO product (reaction 6) its relative yield would be given by:

$$[Me_2SO_2]/[^-OONO(HOONO)] = k_6[DMSO]/(k_0 + k_6[DMSO])$$

Taking $k_0 = 0.95$ s^{-1} and $k_6 = 2.1$ M^{-1} s^{-1} as measured in the stop-flow experiments (vide supra) this equation predicts that the yield of methyl sulfone should have been 18%. A possible reason for this discrepancy is that the measured kinetics of the reaction between peroxynitrite and DMSO is, in part, due to a solvent effect which enhances the apparent value of k_6 above its "proper" value.

The measured yields of MSA must be lower than the yields actually formed in the peroxynitrite/DMSO reaction because much of the sulfinic acid is oxidized to the sulfonic acid. For example, the first entry in Table 1 has [peroxynitrite] = 2.6 mM and [DMSO] = 105 mM (after mixing) and measured yields of MSA and $MeS(O)_2OH$ of 0.04 and 0.26 mM, respectively. If we make the assumption that MSA is formed by hydroxyl radical attack on DMSO (reaction 4) and the sulfonic acid by further oxidation of the MSA, the percentage yield of hydroxyl radicals from peroxynitrite is readily calculated to be $100(0.04 + 0.26)/(2.6 \times 0.91) = 12.5\%$, where the 0.91 refers to the efficiency of MSA formation in the $HO^•$/DMSO reaction.[30]

Interestingly, the extensive conversion of methane sulfinic acid into methane sulfonic acid cannot simply be attributed to its oxidation by peroxynitrite (reaction 7) despite the relatively high rate constant for this reaction (325 M^{-1} s^{-1}, vide supra). That is, if the measured concentration of MSA (0.04 mM in the cited experiment) were to correspond to a steady state (because it was generated in reaction 4 and consumed by reaction 7) then the first-order rate constant for peroxynitrite destruction via reaction 7 would be 4×10^{-5} M × 325 M^{-1} s^{-1} = 0.013 s^{-1}. Such a value is insignificant when compared with the rate constant for decay of peroxynitrite in the absence of substrate, $k_o = 0.95$ s^{-1}. Reaction 7, therefore, plays a negligible role in the conversion of the sulfinic acid to the sulfonic acid. Some other oxidizing species must, therefore, be present in the medium. One reasonable candidate is $NO_2^•$, reaction 9.

$$\mathrm{MeS(O)OH} + \mathrm{NO_2^{\bullet}} \rightarrow \mathrm{MeS(O)_2OH} + {}^{\bullet}\mathrm{NO} \qquad (9)$$

It should be borne in mind that although the peroxynitrite/DMSO reaction mixtures were immediately stored at low temperatures prior to their ^{1}H NMR analyses, further chemical change is not ruled out simply because all the peroxynitrite had decayed.

Another intriguing product is methanol, which would be most readily accounted for if the methyl radicals were trapped by dioxygen, and this was followed by the bimolecular self-reaction of the resulting methylperoxyl radicals:

$$\mathrm{CH_3^{\bullet}} + \mathrm{O_2} \rightarrow \mathrm{CH_3OO^{\bullet}} \qquad (10)$$

$$2\mathrm{CH_3OO^{\bullet}} \rightarrow \mathrm{CH_3OH} + \mathrm{CH_2O} + \mathrm{O_2} \qquad (11)$$

These two reactions are likely to be the origin of the formaldehyde observed in earlier product studies of the peroxynitrite/DMSO reaction since the solutions of the reactants were not deaerated.[5,32] In contrast, we rigorously excluded oxygen in our experiments ($<10^{-6}$ M). However, dioxygen (and nitrite) are formed during the decomposition of peroxynitrite both in the presence of hydrogen peroxide[12,38−40] and, more importantly, also in the absence of any deliberately added hydrogen peroxide.[39,40] Thus, Pfeiffer et al. [39] have reported that, in addition to nitrate, nitrite and dioxygen (ratio 2:1) are formed in appreciable yields during the decomposition of H_2O_2-free peroxynitrite, e.g., ca. 24% NO_2^- and 12% O_2 at 24 °C and pH 7.4. Roughly similar yields of oxygen were found by Kirsch et al.[40] The nitrite and oxygen yields are independent of the peroxynitrite concentration (0.01 to 1 mM),[39] they increase with an increase in temperature[39] and decrease with a decrease in pH.[39,40] Two different mechanisms for dioxygen and nitrite formation have been suggested[7,39] (see Discussion).

(2) CH_3-TEMPOL. The results summarized in Table 2 demonstrate that with 2.6 mM peroxynitrite and either 420 or 105 mM DMSO the yield of CH_3-TEMPOL is almost constant at ca. 8% even when the TEMPOL concentration was reduced from 210 mM to 3.3 mM, or even to 0.8 mM. Just as was found in the ^{1}H NMR product analyses, the yield of the $HO^{\bullet}$ radical-derived product, CH_3-TEMPOL, decreased relative to the initial peroxynitrite concentration as the latter was increased. Since bicarbonate destroys peroxynitrite[20−22] it was no surprise to find that the deliberate addition of 50 mM bicarbonate to the buffer reduced the yield of $HO^{\bullet}$ radical-derived products in both the ^{1}H NMR and TEMPOL experiments (results not shown).

Two sets of additional experiments were conducted to verify that the CH_3-TEMPOL was not being produced via slow secondary reactions. First, following the usual reaction procedure, the TEMPOL was added in three separate experiments about 10 s, 30 min and 240 min after the fast mixing of the peroxynitrite and DMSO solutions. About 20−30 s after these additions of TEMPOL, the solutions were extracted and analyzed. No CH_3-TEMPOL could be detected. Second, following the usual procedure, the TEMPOL was added about 10 s after the fast mixing and the solutions were allowed to stand for 30 or 240 min prior to extraction. Again, no CH_3-TEMPOL could be detected. It is clear, therefore, that CH_3-TEMPOL is a primary product of the peroxynitrite/DMSO reaction.

Table 2. Yields of CH_3-TEMPOL for the Decomposition of Peroxynitrite at pH 6.8 and 23 ± 1 °C in the Presence of DMSO and TEMPOL

HOONO (mM)[a]	DMSO (mM)[a]	TEMPOL (mM)[a]	CH_3-TEMPOL (mM)	$HO^{\bullet}$ (%)[b]
2.6	420	210	0.18	7.7
2.6	420	53	0.19	8.0
2.6	420	13	0.19	8.0
2.6	420	3.3	0.21	8.8
2.6	105	53	0.17	7.2
2.6	105	13	0.19	8.0
2.6	105	3.3	0.17	7.2
2.6	105	0.8	0.15	6.4
10.5	420	210	0.22	2.3
10.5	420	53	0.57	6.0
10.5	105	210	0.21	2.0
10.5	105	53	0.36	3.8
42	420	210	0.44	1.2
42	420	53	1.15	3.0
42	105	210	0.24	0.6
42	105	53	0.56	1.5

[a] Concentration after mixing. There was also 100 mM KH_2PO_4 present after mixing. [b] Calculated as $100[CH_3\text{-TEMPOL}]/([HOONO]_0 \times 0.91)$.

Discussion

Methanol Formation. There can be no doubt that methanol is formed in the peroxynitrite/DMSO reaction under our conditions (Table 1). Where does the oxygen come from? The yield of methanol is far too large for it to be formed by reaction with residual dioxygen in our nitrogen-purged reactant solutions. However, dioxygen is known to be formed during the decay of peroxynitrite, *vide supra*, and the reported yields[39,40] would provide sufficient oxygen to account for the formation of methanol via reactions 10 and 11 in the yields observed in the present work. Two mechanisms for the formation of oxygen have been proposed. First,[39] by the reaction sequence:

$$\mathrm{HOONO}\ (\mathrm{HOONO})^{*} \rightarrow \mathrm{HO^{\bullet}} + \mathrm{NO_2^{\bullet}} \qquad (12)$$

$$\mathrm{HO^{\bullet}} + {}^{-}\mathrm{OONO} \rightarrow \mathrm{HO^{-}} + {}^{\bullet}\mathrm{NO} + \mathbf{O_2} \qquad (13)$$

$$\mathrm{NO_2^{\bullet}} + {}^{\bullet}\mathrm{NO} \rightarrow \mathrm{N_2O_3} \qquad (14)$$

$$\mathrm{N_2O_3} + \mathrm{H_2O} \rightarrow \mathbf{2NO_2^{-}} + 2\mathrm{H^{+}} \qquad (15)$$

However, in our experiments the concentration of DMSO is very much greater than the concentration of peroxynitrite so that reaction 13 will not occur and dioxygen would not, therefore, be formed. Second,[7] by the reaction sequence:

$$\mathrm{HOONO} + {}^{-}\mathrm{OONO} \rightleftharpoons [\mathrm{HOONO/OONO}]^{-} \qquad (16)$$

$$[\mathrm{HOONO/OONO}]^{-} \rightarrow \mathbf{2NO_2^{-}} + \mathrm{H^{+}} + \mathbf{O_2} \qquad (17)$$

However, peroxynitrite decay gives mainly nitric acid via a kinetically first-order process (at least at relatively low concentration of peroxynitrite (2.6 mM)) and so this second mechanism would seem to imply that at a given pH the yield of oxygen would increase as the concentration of peroxynitrite increases. This appears not to be the case from 0.01 to 1 mM

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(36) Scaduto, R. C., Jr. *Free Radical Biol. Med.* **1995**, *18*, 271−277.

(37) Babbs, C. F.; Gale, M. J. *Anal. Biochem.* **1987**, *163*, 67−73. For related work, see: Babbs, C. F.; Griffin, D. W. *Free Radical Biol. Med.* **1989**, *6*, 493−503. Steiner, M. G.; Babbs, C. F. *Arch. Biochem. Biophys.* **1990**, *278*, 478−481. Fuku, S.; Hanasaki, Y.; Ogawa, S. *J. Chromatogr.* **1993**, *630*, 187−193.

(38) See, e.g.: Alvarez, B.; Denicola, A.; Radi, R. *Chem. Res. Toxicol.* **1995**, *8*, 859−864.

(39) Pfeiffer, S.; Gorren, A. C. F.; Schmidt, K.; Werner, E. R.; Hansert, B.; Bohle D. S.; Mayer, B. *J. Biol. Chem.* **1997**, *272*, 3465−3470.

(40) Kirsch, M.; Lomonosova, E. E.; Korth, H.-G.; Sustmann, R.; de Groot, H. *J. Biol. Chem.* In press, and private communication.

peroxynitrite.[39] Thus, neither of the proposed mechanisms for oxygen formation appear able to account for methanol formation via reactions 10 and 11 under our experimental conditions. Of course, if oxygen is actually formed by some third mechanism, then methanol (and formaldehyde) can readily be accounted for by these two reactions. However, there are other potential oxidants of the methyl radical which could yield methanol, of which the most likely would appear to be $NO_2^\bullet$ and, less probably, the peroxynitrite itself (assuming that $CH_3^\bullet$ is not oxidized to CH_3^+). We, therefore, suggest that methanol may be formed by reaction 18 and/or 19.

$$CH_3^\bullet + NO_2^\bullet \rightarrow CH_3ONO \xrightarrow{H_2O} CH_3OH + NO_2^- + H^+ \tag{18}$$

$$CH_3^\bullet + HOONO\ (^-OONO + H^+) \rightarrow CH_3OH + NO_2^\bullet \tag{19}$$

The Yield of Hydroxyl Radicals from Peroxynitrite. We will make the *assumption* that MSA and methyl radicals can only be formed from DMSO by its reaction with free hydroxyl radicals. We will also assume that *only* the data obtained at our lowest peroxynitrite concentration, 2.6 mM, are meaningful because the kinetic complexities in the decay of peroxynitrite at elevated concentrations, vide supra,[7,34] would appear to decrease the efficiency of $HO^\bullet$ radical formation. With these two assumptions, our quantitative analyses of the products of the peroxynitrite/DMSO reaction indicate that the thermal decomposition of peroxynitrite in water at ambient temperatures gives free hydroxyl radicals in yields of about 13% (^{1}H NMR analyses, Table 1) or about 8% (CH_3-TEMPOL analyses, Table 2). We do not understand why our two semiindependent approaches do not yield analytical data which are in better agreement. However, both sets of experimental data imply that free $HO^\bullet$ radicals are produced during the decomposition of 2.6 mM peroxynitrite in a significant yield that is probably best given as 10 ± 3%. We note with particular pleasure that this hydroxyl radical yield of 10 ± 3% is in excellent agreement with the 7% yield of malonaldehyde produced from deoxyribose reported by Beckman et al.[5] in their 1990 landmark paper and with the 8% yield of formaldehyde produced from DMSO reported by Pryor et al.[32]

Our confidence in our conclusion that ca. 10% free $HO^\bullet$ radicals are produced is bolstered by two other pieces of evidence. First, Beckman et al.[11] have shown that, in the presence of 50 mM DMSO, $NO_2^\bullet$ is formed in a yield of ca. 16% at pH 6.8.[43] Second, Butler et al.[44] have demonstrated, using ^{15}N CIDNP, that tyrosine nitration by ^{15}N-labeled peroxynitrite at pH 12 yields ^{15}N NMR signals from nitrate in enhanced absorption, which indicates a reaction of the caged $HO^\bullet/NO_2^\bullet$ *radical* pair, and from nitrite in emission, which indicates that nitrite is formed from *radicals* which escape the cage, i.e., *free radicals*. Unfortunately, these CIDNP experiments were done without exclusion of CO_2 so they are not definitive evidence for the formation of radicals from peroxynitrite under CO_2-free conditions.

Earlier claims for much lower hydroxyl radical yields (0–4%, see Introduction) probably arose from three main problems: (i) adventitious bicarbonate,[20–22] (ii) the use of high concentrations of peroxynitrite,[22,34] and (iii) direct, bimolecular reactions of peroxynitrite with the probe molecule or its expected hydroxyl radical-induced product. Unfortunately, the possibility of such direct reactions was not always explored. For example, low hydroxyl radical yields based on spin trapping (0.9%,[13] zero,[15] and probably zero[16–18]) would appear to be unreliable because Lemercier et al.[16] have shown that at least one of the spin traps employed (DMPO) reacts directly with peroxynitrite in a bimolecular reaction.[45] Other experimental results which have been used to argue against the simple homolysis of peroxynitrite to $HO^\bullet$ and $NO_2^\bullet$ radicals have included the lack of effect of viscosity on the rate of peroxynitrite decomposition.[19] However, in our opinion, it is doubtful that these measurements would actually be capable of clearly detecting a yield of free $HO^\bullet$ radicals of only ca. 10%. We hold the same doubts about Kissner et al.'s[7] report that favored a nonhomolytic isomerization of peroxynitrite to nitrate on the basis of a volume of activation for peroxynitrite decomposition which is considerably smaller than that for a "normal" bond homolysis, viz., 1.7 $cm^3\ mol^{-1}$ vs 10 $cm^3\ mol^{-1}$. However, these authors were careful to state the following: "the observed activation volume may be compatible with (i) a lengthening of the O–O bond followed by isomerization..., (ii) isomerization mainly as described under (i) *with a small fraction isomerizing via homolysis* (our italics), and, *possibly*, (iii) heterolysis into hydroxide and nitryl, in which case electrostriction would lower the activation volume of the heterolysis," vide infra. We therefore suggest that our experimental evidence for a ca. 10% yield of $HO^\bullet$ should be accepted. No doubt future work will refine this value and, we are confident, will confirm our assumption that peroxynitrite's powerful oxidant is, indeed, the $HO^\bullet$ radical and not some ill-defined "hydroxyl radical-like species".

We cannot explain all earlier claims for $HO^\bullet$ radical yields from peroxynitrite which are very much larger than 10% (see Introduction). However, a referee has pointed out that Crow et al.'s[11] calculated yield of $HO^\bullet$ radicals (25%) was based on the oxidation of 2,2′-azinobis(3-ethyl-1,2-dihydrobenzothiazoline 6-sulfonate) (ABTS) to its radical cation and that both $NO_2^\bullet$ (formed in 16% yield)[11] and $HO^\bullet$ (10 ± 3% yield) can perform this one-electron oxidation to give a combined oxidizing effect essentially equal to the reported 25%.

We tentatively attribute the low efficiency of free radical formation by peroxynitrite to a rather large cage effect induced by hydrogen bonding of peroxynitrous acid and its fragments by the surrounding water molecules. This idea was first considered seriously by Pryor and Squadrito.[6] These authors also pointed out that, in addition to the "stickiness" of the water molecules (caused by hydrogen bonding) that surround the $HO^\bullet/NO_2^\bullet$ singlet geminate radical pair, there could also be a pronounced polar effect that would further enhance cage return, i.e.,

$$\boxed{HO^\bullet\ \ NO_2^\bullet}_{\text{cage}} \longleftrightarrow \boxed{HO^-\ \ NO_2^+}_{\text{cage}}$$

However, the HO^-/NO_2^+ ion pair would appear to be more stable in water than the $HO^\bullet/NO_2^\bullet$ radical pair by about 9 kcal/

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[41] A much larger volume of activation (9.6 ± 1.0 $cm^3\ mol^{-1}$) has also been reported.[42] However, the experimental work was much more limited, including only one pressure different from atmospheric (vs 8),[7] one temperature (vs 2),[7] and one pH (vs 2).[7] The validity of Goldstein et al.'s[42] large volume of activation therefore remains uncertain.

[42] Goldstein, S.; Meyerstein, D.; van Eldik, R.; Czapski, G. *J. Phys. Chem. A* **1997**, *101*, 7114–7118.

[43] An earlier publication gives a somewhat higher yield of $NO_2^\bullet$, see: Zhu, L.; Gunn, C.; Beckman, J. S. *Arch. Biochem. Biophys.* **1992**, *298*, 452–457.

[44] Butler, A. R.; Rutherford, T. J.; Short, D. M.; Ridd, J. H. *J. Chem. Soc., Chem. Commun.* **1997**, 669–670.

[45] The unreliability of $HO^\bullet$ yields based on spin trapping is further emphasized by the fact that the $HO^\bullet$/DMPO spin adduct had already been shown to react directly with and be destroyed by peroxynitrite.[46] Other spin traps have also been shown to react directly with peroxynitrite.[17.]

mol.[47] This would make it difficult for the ion pair to generate the radical pair. Perhaps the ion pair is formed only slowly from the radical pair because this electron transfer reaction would involve a large solvent reorganization energy. A slow formation of the ion pair would allow time for some radicals to escape the solvent cage. Once formed, however, the ion pairs probably collapse to produce mainly (or solely) nitric acid.

The geminate radical pairs may be held in the positions in which they are produced by the surrounding water molecules so that most in-cage reactions yield peroxynitrite with only a small fraction of the radical pairs reorienting in-cage and collapsing to nitric acid.[48] (As Pryor and Squadrito[6] noted, a high ratio of in-cage return to give peroxynitrite relative to rearrangement to nitrite could explain the relatively low Arrhenius preexponential factor for peroxynitrite decomposition, vide infra.) Thus, our results suggest that ca. 90% of the peroxynitrite isomerizes to nitric acid by a rather inefficient in-cage reaction of the geminate $HO^{\bullet}/NO_2^{\bullet}$ radical pair with possibly some minor contribution from the geminate HO^-/NO_2^+ ion pair.[50] The remaining ca. 10% of the geminate radical pairs yield freely diffusing $HO^{\bullet}$ and $NO_2^{\bullet}$ radicals. The products derived from these free radicals depend on the presence and relative concentrations of compounds capable of reacting with them. Specifically, for the $HO^{\bullet}$ radical these may include ^-OONO [39] and NO_2^- [49b] but, in our experiments, the main sink for $HO^{\bullet}$ is DMSO (because of its high concentration relative to the two inorganic ions).

In support of the above picture we note that the measured activation enthalpy for peroxynitrite decomposition, viz.,[24] 18 ± 1 kcal mol^{-1}, lies in the range of calculated O−O bond dissociation energies (BDEs) which have been reported for HOONO in the gas phase, viz.[54] 16−23 kcal mol^{-1}. Our own thermochemical arguments (see Appendix A) also lead to BDE-[HO−ONO] = 18 ± 1 kcal mol^{-1}. An O−O BDE of 18 kcal mol^{-1} raises an intriguing question that others have wrestled with,[6,19,23,24,60,61] viz., why is peroxynitrous acid so long-lived in water? Gas-phase thermokinetic properties imply that the Arrhenius preexponential factor for unimolecular O−O bond cleavage of HOONO (k_f of reaction 3) should be ca. 6×10^{15} s^{-1}, which leads to a gas-phase rate constant for O−O bond homolysis of ca. 500 s^{-1} (see Appendix A). This is 500 times greater than the experimental rate constant of ca. 1 s^{-1} measured for peroxynitrite decay in water. Provided the activation enthalpy is unchanged at 18 kcal mol^{-1}, this means that the Arrhenius preexponential factor must be 500 times lower in water than in the gas phase, i.e., ca. 10^{13} s^{-1}.[62] The simplest explanation for this unexpectedly low *A* factor is that only 1 out of every 500 O−O bond homolysis events leads to products. That is, of every 500 geminate singlet $HO^{\bullet}/NO_2^{\bullet}$ radical pairs created in water there are 499 which undergo cage return to reform HOONO and only one which forms products and hence leads to a measurable loss of HOONO. Our results indicate that this 1 in 500 event yields nitrate and free $HO^{\bullet}$ (and $NO_2^{\bullet}$) radicals in a roughly 9:1 ratio. However, we do not claim that $HO^{\bullet}$ radical production from peroxynitrite is **necessarily** important in vivo. In the first place, the very formation of peroxynitrite in vivo has been seriously questioned[65] as has its role as a cytotoxic agent in the immune response.[65] Second, if peroxynitrite is, indeed, formed in vivo then some fraction of it will undoubtedly undergo direct, bimolecular reactions with the many available biological target molecules (e.g., thiols, ascorbate, and, particularly,[20,21,61] bicarbonate) rather than isomerizing to nitrate with a concomitant release of $HO^{\bullet}$ and $NO_2^{\bullet}$ radicals. Only future work will determine whether $HO^{\bullet}$ radical formation from peroxynitrite in vivo is biologically (and medically) significant.

Experimental Section

Materials. Compounds were the purest available commercially and were used as received: sodium azide, TEMPOL, methyl sulfone (Aldrich); DMSO (BDH Inc.); and methane sulfinic acid (Lancaster Synthesis Inc.).

Synthesis of Peroxynitrite. The procedure of Gleu and Roell[31] and Pryor et al.[32] was employed. To avoid any contamination with (bi)-carbonate from ambient air, the handling of the reagents and the synthesis of the peroxynitrite were performed inside a glovebag under a nitrogen atmosphere. Air inside the bag was removed by multiple flushings with nitrogen and the bag was then kept at slightly above

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(46) Augusto, O.; Gatti, R. M.; Radi, R. *Arch. Biochem. Biophys.* **1994**, *310*, 118−125. Augusto, O.; Radi, R.; Gatti, R. M.; Vásquez-Vivar, J. *Methods Enzymol.* **1996**, *269*, 346−353.

(47) Stanbury, D. M. *Adv. Inorg. Chem.* **1987**, *33*, 69−138. We are indebted to an unknown referee for bringing this important fact to our attention.

(48) It has been shown by pulse radiolysis that even free $HO^{\bullet}$ radicals can react with $NO_2^{\bullet}$ to form peroxynitrite by monitoring this reaction at 302 nm[49] where the peroxynitrite absorbs. Nitrate formation was not monitored and therefore the ratio of the yields of peroxynitrite to nitrate from freely diffusing $HO^{\bullet}$ and $NO_2^{\bullet}$ radicals remains to be established. However, if this ratio is large (as is probably the case: private communication from Professor W. H. Koppenol) then there is no longer any need to invoke a slow reorientation of the geminate $HO^{\bullet}/NO_2^{\bullet}$ radical pair due to hydrogen bonding from the surrounding water molecules to explain the large extent of in-cage return to peroxynitrite.

(49) (a) Grätzel, M.; Henglein, A.; Taniguchi, S. *Ber. Bunsen-Ges. Phys. Chem.* **1970**, *74*, 292−298. (b) Logager, T.; Sehested, K. *J. Phys. Chem.* **1993**, *97*, 6664−6669.

(50) A minor contribution to nitric acid formation from the ion pair is consistent with a recent report of 13% ^{18}O incorporation into the nitrate product when peroxynitrite was decomposed in CO_2-free $H_2{}^{18}O$ (pH 6.8) while 83% of the nitrate showed no ^{18}O incorporation.[51,52] That is, part of the ^{18}O-containing nitrate arises from the $NO_2^+ + H_2{}^{18}O$ reaction and the remainder from reaction of free $NO_2^{\bullet}$ radicals with the $H_2{}^{18}O$. Unfortunately, the experimental technique (Raman spectroscopy) did not allow determination of the ^{18}O content of the nitrite product.

(51) Bohle, D. S.; Hansert, B. *NITRIC OXIDE: Biol. Chem.* **1997**, *1*, 502−506.

(52) There is an earlier report that when peroxynitrite rearranges to nitrate none of the oxygen atoms come from water.[53] We are indebted to Professor W. A. Pryor for pointing out that this result is consistent with the “sticky cage” concept.

(53) Anbar, M.; Taube, H. *J. Am. Chem. Soc.* **1954**, *76*, 6243−6247.

(54) Individual BDE values are 16,[55] 16−18,[56] 20,[58] 21,[18] 22,[59] and 23[60] kcal mol^{-1}, with errors generally estimated as ±2−3 kcal mol^{-1}.

(55) McGrath, M. P.; Francl, M. M.; Rowland, F. S.; Hehre, W. J. *J. Phys. Chem.* **1988**, *92*, 5352−5357.

(56) Calculation at the B3LYP/6-311++G(d, p) level gives 12.3 kcal mol^{-1}.[57a] However, calculations at such a high level underestimate O−O bond strengths by 6−10 kcal mol^{-1} and O−H BDE’s by 3−7 kcal mol^{-1}.[57] Calculations at the lower B3LYP/6-31G* level give 20 kcal mol^{-1}.[57a.]

(57) (a) Korth, H.-G. Universität-GH Essen. Unpublished results. (b) van Scheppingen, W.; Dorrestijn, E.; Arends, I.; Mulder, P.; Korth, H.-G. *J. Phys. Chem. A* **1997,** *101*, 5404−5411.

(58) Burkholder, J. B.; Hammer, P. D.; Howard, C. J. *J. Phys. Chem.* **1987**, *91*, 2136−2144.

(59) Bach, R. D.; Ayala, P. Y.; Schlegel, H. B. *J. Am. Chem. Soc.* **1996**, *118*, 12758−12765.

(60) Tsai, H.-H.; Hamilton, T. P.; Tsai, J.-H. M.; van der Woerd, M.; Harrison, J.-G.; Jablonsky, M. J.; Beckman, J. S.; Koppenol. W. H. *J. Phys. Chem.* **1996**, *100*, 15087−15095.

(61) Houk, K. N.; Condroski, K. R.; Pryor, W. A. *J. Am. Chem. Soc.* **1997**, *119*, 2964. Correction: **1997**, *119*, 2964.

(62) Such a large reduction of the Arrhenius *A* factor for O−O bond homolysis on changing from the gas phase to a solvent that interacts with the product oxyl radicals is not without precedent. For O−O homolysis of di-*tert*-butyl peroxide the *A* factors are 7×10^{15} s^{-1} in the gas phase,[63] 4×10^{15} s^{-1} in THF,[64] 5×10^{14} s^{-1} in benzene,[64] but only 4×10^{12} s^{-1} in CH_3CN.[64]

(63) Lossing, F.; Tickner, A. W. *J. Chem. Phys.* **1952**, *20*, 907−914.

(64) Calculated from data given in the following: Reichardt, C. *Solvents and Solvent Effects in Organic Chemistry*; VCH: New York, 1988, Tables 5−14, p 187.

(65) Fukuto, J. M.; Ignarro, L. J. *Acc. Chem. Res.* **1997**, *30*, 149−152.

atmospheric pressure at all times. Doubly distilled water (100 mL) was purged with nitrogen, placed in the bag, and purged for a further 30 min. Sodium hydroxide (1 pellet, ca. 0.1 g, from a new container that was only opened in the bag) was added to the water, followed by sodium azide (1.3 g, 200 mM, taking the same precautions). This solution (pH ∼12) was poured into a nitrogen-containing 3-arm flask that was then sealed with septa, removed from the bag, placed in an ice bath and flushed with nitrogen through a hypodermic needle. A glass frit was immersed in the solution through which ozone was bubbled for 100 min. The ozone was made by passing oxygen (dried and passed over NaOH pellets) at a rate of ca. 0.3 mL/min through a Weisbach Laboratory Ozonator (Model T-816). Finally, the reaction mixture was again purged with nitrogen for more than 30 min to remove dissolved oxygen. The vessel ports were then sealed with septa and the flask was returned to the glovebag. The solution was divided into 5 mL sample vials which were then sealed, removed from the bag, and stored at −80 °C. The peroxynitrite concentration was calculated from the absorbance at 302 nm ($\epsilon = 1670\ \text{M}^{-1}\ \text{cm}^{-1}$)[66] after a 30-fold dilution with 0.1 N NaOH.[32] Samples were withdrawn from the 5 mL vials after thawing in the glovebag by using a hypodermic syringe within the bag.

In preliminary (monitoring) experiments we found that our concentration vs time plots for azide consumption (method of Herzog and Rudolf[67] with a Nicolet 20DXB FTIR spectrometer) and peroxynitrite formation were (surprisingly) almost indistinguishable from those shown in Figure 2 in Pryor et al's publication.[32] Although azide was below our detection limits in the final product is was present in sufficient concentration to make the determination of MSA yields by reaction with diazonium salts quite impossible.[35]

Preparation of 200 mM KH_2PO_4 Buffer. This solution was also made up in the nitrogen-filled glovebag. The correct amount of KH_2PO_4 was weighed out, transferred to the bag, and, after some time, added to a flask containing nitrogen-purged, doubly distilled water. Chelex was then added and the flask was sealed with a septum and stirred overnight in the bag. Samples of buffer were withdrawn in the glovebag with a hypodermic syringe.

Synthesis of CH_3-TEMPOL and CD_3-Tempol. Surprisingly, no physical properties have been reported for either of these hydroxylamines.[68] Into an oven-dried 50-mL three-necked flask was placed 0.2 g (1.16 mmol) of TEMPOL and 15 mL of anhydrous ether. The resulting solution was cooled to −78 °C and stirred under nitrogen, and methyllithium (1.66 mL of a 1.4 M solution in ether, 2.32 mmol) was added in one portion with a syringe. The reaction mixture was slowly warmed to room temperature and was held at this temperature for 1 h, during which time the solution turned pale orange. After the mixture was cooled to 0 °C, 5 mL of distilled water was added dropwise with continuous stirring. The aqueous phase was extracted with ether (3 × 10 mL) and the organic phases were combined, dried with $MgSO_4$, filtered, and concentrated by rotovap to yield 0.14 g (66%) of crude CH_3-TEMPOL. This was purified by column chromatography with ether as eluent and recrystallized from *n*-pentane at −78 °C: white crystals, mp 90−91 °C; ^1H NMR (300 MHz, $CDCl_3$, ppm) 3.94 (m, 1H), 3.60 (s, 3H), 1.79 (dd, $J = 11.6$, 3.0 Hz, 2H), 1.45 (dd, $J = 11.7$, 11.6 Hz, 2H), 1.26 (d, $J = 7.0$ Hz, OH), 1.21 (s, 6H), 1.13 (s, 6H).

The same procedure with CD_3Li yielded CD_3-TEMPOL: white crystals, mp 91−92 °C; ^1H NMR (300 MHz, $CDCl_3$, ppm) 3.94 (m, 1H), 1.79 (dd, $J = 11.7$, 3.0 Hz, 2H), 1.46 (dd, $J = 11.7$, 11.3 Hz, 2H), 1.25 (d, $J = 7.0$ Hz, OH), 1.21 (s, 6H), 1.13 (s, 6H).

Kinetics. The kinetics of peroxynitrite decay were monitored at 302 nm with a Biosequential SX-18MV stopped-flow reaction analyzer (Applied Photophysics). The stock peroxynitrite solution was, if needed, diluted with nitrogen-purged, doubly distilled water made to pH 12 in the glovebag as described above. The KH_2PO_4 buffer solution was either used directly or used after the addition of a known quantity of a substrate in the glovebag. Both solutions were then purged with nitrogen for 30 min before being drawn into gas tight syringes. The entire stop-flow apparatus was flushed with nitrogen for 30 min prior to each experiment. The syringes containing the two solutions were removed from the bag and attached to the stop-flow apparatus. Equal volumes of the two solutions were rapidly mixed (∼1.3 ms) and the decrease in absorbance at 302 nm was monitored. Reactions occurred at pH 6.8 (determined after each experiment) and at room temperature (23 ± 1 °C, automatically recorded).

Products. The products of the reaction of peroxynitrite with DMSO alone in the KH_2PO_4 buffer and with DMSO plus TEMPOL in the buffer were generated at room temperature (23 ± 1 °C) as in the above-described kinetic experiments but with a rapid mixing (∼ 4 ms) apparatus with no kinetic capabilities. A glass collection vessel (ca. 2 mL volume) was attached to the mixer. The collection vessel had stopcocks on both ends. It was pre-flushed with the mixed reactants before the products were collected for analyses.

The collected products from the peroxynitrite/DMSO reactions were diluted with 15% (v/v) D_2O and analyzed by ^1H NMR (400 MHz). The following compounds were identified and quantified relative to the known concentration of DMSO (2.61 ppm): CH_3OH (3.24 ppm), $(CH_3)_2SO_2$ (3.03 ppm), CH_3SO_3H (2.70 ppm) and CH_3SO_2H (MSA, 2.19 ppm).

For the peroxynitrite/(DMSO + TEMPOL) reactions, a 1 mL aliquot was removed from each collected product sample within 1 min of its generation. To this aliquot was added a known amount of CD_3-TEMPOL to serve as an internal standard. The aliquot was then extracted five times with ether (5 × 1 mL), the ether extract was dried, and the ether was removed by using a rotovap. After addition of a small quantity of ether, the yield of CH_3-TEMPOL (parent ion $m/e = 187$) was determined quantitatively relative to the CD_3-TEMPOL ($m/e = 190$) internal standard on a Hewlett-Packard 5790 Series gas chromatograph with a 5970 Series mass selective detector operated in the single ion monitoring (SIM) mode. Blank experiments with equal concentrations of CH_3-TEMPOL and CD_3-TEMPOL gave m/e peaks at 187 and 190 of equal intensity.

Appendix A

The reaction enthalpy ($\Delta_{20}H$) and reaction entropy ($\Delta_{20}S$) at 298 K can be derived as follows:

$$\text{HOONO} \underset{k_r}{\overset{k_f}{\rightleftarrows}} \text{HO}^\bullet + \text{NO}_2{}^\bullet \rightarrow \text{H}^+ + \text{NO}_3{}^- \qquad (20)$$

With a basis set containing $\Delta_fH°$ and $S°$ for CH_3OH, CH_3OOH, CH_3ONO, HONO, and H_2O_2,[69] the heat of formation of HOONO is computed according to group increment rules (replacing H by NO or H by OH) as −0.7 and −2 kcal mol^{-1}: mean value, $\Delta_fH°(\text{HOONO}) = -1 \pm 1$ kcal mol^{-1}. Accordingly, the entropy gain for H → NO amounts to 10.7 cal mol^{-1} K^{-1} and that for H → OH amounts to 10.2 cal mol^{-1} K^{-1}: thus the mean $S°(\text{HOONO}) = 68 \pm 1$ cal mol^{-1} K^{-1}. Combination with OH and NO_2 leads to the thermodynamic values for equilibrium 20 at 298 K of $\Delta_{20}H° = 18 \pm 1$ kcal mol^{-1}, $\Delta_{20}S° = 33 \pm 1$ cal mol^{-1} K^{-1}, and $\Delta_{20}G° = 8 \pm 1$ kcal mol^{-1}, which is in excellent agreement with Koppenol and Kissner's[27] value for $\Delta_{20}G°$ of 7.2 kcal mol^{-1}.

In a large number of studies pertaining to the gas phase[70] the rate constant for combination has been established to be $k_r = k_{-20} = 2.4 \times 10^{10}\ \text{M}^{-1}\ \text{s}^{-1}$. This is an average, high-pressure limit, value and is independent of the temperature. Combination of k_r ($=k_{-20}$) with the thermodynamic data given above yields

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the preexponential (A_{20}) at 298 K as follows:

$$A_{20}/\mathrm{s}^{-1} = A_{-20}(0.229T)^{-1}\exp(\Delta_{20}S^{\ddagger}/R) = (2.4 \times 10^{10})(1.5 \times 10^{-2})(1.64 \times 10^{7}) = 5.9 \times 10^{15}$$

Thus, with $\Delta_{20}H^{\ddagger} = \Delta_{20}H = 18$ kcal mol^{-1} and $\Delta_{20}S^{\ddagger} = 11.6$ cal mol K^{-1} (from $A_{20} = (5.66 \times 10^{10})T\exp(\Delta_{20}S^{\ddagger}R)$), the rate constant for homolysis of HOONO in the gas phase becomes $k_f(=k_{20}) = (5.9 \times 10^{15})\exp(-18/RT)$ s^{-1} = 465 s^{-1} at 298 K (where $R = 1.987 \times 10^{-3}$ kcal mol^{-1}).

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Supporting Information Available: Kinetic data for the reactions of peroxynitrite with DMSO, MSA, and TEMPOL (Tables 3−5, (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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