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Formation and Properties of Peroxynitrite as Studied by Laser Flash Photolysis, High-Pressure Stopped-Flow Technique, and Pulse Radiolysis

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Flash photolysis of alkaline peroxynitrite solutions results in the formation of nitrogen monoxide and superoxide. From the rate of recombination it is concluded that the rate constant of the reaction of nitrogen monoxide with superoxide is $(1.9 \pm 0.2) \times 10^{10}$ M^{-1} s^{-1}. The pK_a of hydrogen oxoperoxonitrate is dependent on the medium. With the stopped-flow technique a value of 6.5 is found at millimolar phosphate concentrations, while at 0.5 M phosphate the value is 7.5. The kinetics of decay do not follow first-order kinetics when the pH is larger than the pK_a, combined with a total peroxynitrite and peroxynitrous acid concentration that exceeds 0.1 mM. An adduct between $ONOO^-$ and ONOOH is formed with a stability constant of $(1.0 \pm 0.1) \times 10^4$ M. The kinetics of the decay of hydrogen oxoperoxonitrate are not very pressure-dependent: from stopped-flow experiments up to 152 MPa, an activation volume of 1.7 ± 1.0 cm^3 mol^{-1} was calculated. This small value is not compatible with homolysis of the O−O bond to yield free nitrogen dioxide and the hydroxyl radical. Pulse radiolysis of alkaline peroxynitrite solutions indicates that the hydroxyl radical reacts with $ONOO^-$ to form $[(HO)ONOO]^{\bullet -}$ with a rate constant of 5.8×10^9 M^{-1} s^{-1}. This radical absorbs with a maximum at 420 nm ($\epsilon = 1.8 \times 10^3$ M^{-1} cm^{-1}) and decays by second-order kinetics, $k = 3.4 \times 10^6$ M^{-1} s^{-1}. Improvements to the biomimetic synthesis of peroxynitrite with solid potassium superoxide and gaseous nitrogen monoxide result in higher peroxynitrite to nitrite yields than in most other syntheses.

Introduction

Peroxynitrite [oxoperoxonitrate(1−) or nitrosodioxidanide] is an inorganic molecule of biological interest. Activated macrophages produce both superoxide and nitrogen monoxide; these react very rapidly to form peroxynitrite, with a rate constant of 6.7×10^9 M^{-1} s^{-1} (*1*), 4.3×10^9 M^{-1} s^{-1} (*2*), or 3.8×10^9 M^{-1} s^{-1} (*3*). The pK_a of peroxynitrous acid is near 7 (*4*-*8*), such that at physiological pH both the anion and the hydronated form are present, each with its own reactivity. While the single bond between N and the first peroxide oxygen has partial double-bond character, peroxynitrite and peroxynitrous acid can adopt cis and trans configurations (*9*, *10*). However, in solution the peroxynitrite anion is present in the cis configuration (*11*). Although production of peroxynitrite by macrophages is beneficial, its formation elsewhere must be considered harmful to the organism (*12*). The precise mechanism by which peroxynitrite and peroxynitrous acid exert their cytotoxicity is unknown: the high reduction potential [$E^{\circ\prime}(ONOOH, H^+/NO_2^{\bullet}, H_2O) = 1.4$ V at pH 7 (*13*)] is likely to play a role in the oxidation of thiols (*14*−*16*), nitration of tyrosines (*17*−*21*), and initiation of lipid peroxidation (*22*, *23*). One-electron oxidations and oxygen-transfer reactions have both been described (*24*, *25*). Carbon dioxide forms an adduct with peroxynitrite (*19*, *20*, *26*−*28*) with its own reactivity and which may be relevant to the toxicity of peroxynitrite in vivo. It is important to realize that reduction by one electron yields another oxidizing molecule, nitrogen dioxide: the reduction potential of the nitrogen dioxide/nitrite couple is 1.0 V (*29*, *30*). Peroxynitrous acid, but not peroxynitrite, isomerizes to nitrate at a rate of 1.3 s^{-1} at 25 °C (*13*); the mechanism of this reaction is still under scrutiny (*31*). Above the pK_a of peroxynitrous acid, about 6.8 dioxygen and nitrite are also formed (*32*). We therefore use the terms "isomerization" below the pK_a and "decay" above the pK_a.

We report here on a rate constant for the reaction of superoxide with nitrogen monoxide that is significantly higher than previously reported, on medium effects on the pK_a of peroxynitrous acid, on complex formation between peroxynitrite and peroxynitrous acid, and on the oxidation of peroxynitrite by hydroxyl radicals. We also address experimentally the possibility of homolysis of the O−O bond in peroxynitrite.[1]

Experimental Section

Chemicals. All reagents, except oxoperoxonitrate(1−), were purchased from Merck, Fluka, Riedel de Häen, or Aldrich and were analytical grade or better. Water was prepared by a Millipore Milli-Q unit from deionized water. Buffers were prepared from salts and acids, never from hydroxides, to avoid contamination with hydrogen carbonate(1−). Buffers were

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saturated with argon immediately before use. Freshly prepared oxoperoxonitrate(1−) solutions were used for every experiment.

Oxoperoxonitrate(1−) Preparation. Oxoperoxonitrate(1−) was prepared by a modification of our biomimetic synthesis of nitrogen monoxide with solid potassium superoxide (*33*). Briefly, 30% (mol) nitrogen monoxide was added with a gas burette to 100% (mol) solid potassium superoxide (ca. 0.3 g) that was stirred with sand under argon in a 100-mL Erlenmeyer flask. The addition takes place at reduced partial pressure of nitrogen monoxide, such that the reaction of nitrogen monoxide with potassium oxoperoxonitrate(1−), which leads to potassium nitrite and nitrogen dioxide, is avoided. The product was dissolved in 0.01 M potassium hydroxide, and 50−100 mg of manganese dioxide was added to destroy the hydrogen peroxide which resulted from the excess of superoxide. The solution was filtered through a G4 glass filter funnel and stored in a polyethylene bottle at −20 °C. Oxoperoxonitrate(1−) was determined at 302 nm ($\epsilon_{302} = 1.70 \times 10^3$ M^{-1} cm^{-1}) (*34*), and nitrite was measured at 354 nm [we determined $\epsilon_{354} = 24.6$ M^{-1} cm^{-1}, in agreement with the literature (*35*)], after isomerization of oxoperoxonitrate(1−) to nitrate with dihydrogen phosphate(1−) at pH 5.5 followed by addition of a potassium hydroxide solution until pH 12. It was further verified by mass balance that no other nitrogen-containing products were formed. Hydrogen peroxide was determined by the titanyl method and found to be below the detection limit of 5 μM (*36*).

Compared to the published procedure (*33*) the yield of oxoperoxonitrate(1−) relative to that of nitrite was improved by adding more sand and by mixing the manganese dioxide with the mixture of potassium oxoperoxonitrate(1−), potassium superoxide, and sand prior to adding the 0.01 M potassium hydroxide solution. The quality of the manganese dioxide plays a role: highly active fresh preparations from reduction of potassium permanganate must be avoided, and best results were obtained with a 90−95% aged product from Riedel de Häen. These improvements result in improved oxoperoxonitrate(1−) to nitrite ratios of ca. 8:1. The problem of contamination with nitrite is serious, since at low pH nitrite is in equilibrium with the nitrosyl cation, and nitrosation ascribed to oxoperoxonitrate(1−) may therefore be due to nitrite contamination. On the basis of a mass balance (nitrogen monoxide added, peroxynitrite and nitrite measured), we conclude that fresh preparations do not contain significant amounts (<1%) of nitrate. Our synthesis yields oxoperoxonitrate(1−) within 1 h with little nitrite at low ionic strength and does not suffer from contamination by reactants like most other methods (*33*, *37*). A synthesis of nitrite-free oxoperoxonitrate(1−) has been described by Bohle and co-workers (*34*, *37*).

Laser Flash Photolysis. Experiments were carried out with an Applied Photophysics LKS 50 instrument fitted with a YAG Laser (Quanta Ray DCR 3). Solutions were irradiated at 266, 355, and 532 nm with a pulse width (fwhm) of 10 ns. The bandwidth of the detection system is 300 MHz. Solutions of 0.1−1 mM oxoperoxonitrate(1−) and 10 mM potassium hydroxide were irradiated at ambient temperature (20−22 °C) in a closed cuvette. Traces were recorded every 5 nm between 220 and 500 nm. Kinetic analysis was initiated 1 μs after the pulse, since the kinetics during the first microsecond may be influenced by cage effects and exited states. For spectral analysis of products this restriction was not applied. Results from 20 flashes were typically averaged before analysis.

Stopped-Flow Spectrophotometry. Kinetic experiments at 25 °C and ambient pressure were carried out with an Applied Photophysics SX 17MV stopped-flow spectrophotometer operated in the symmetric mixing mode. The decay was initiated by mixing the oxoperoxonitrate(1−) solution in 0.01 M potassium hydroxide or sodium hydroxide with appropriate buffers to lower the pH. For the lowest buffer concentrations, matched amounts of perchloric acid were added to the buffer in order to neutralize most of the hydroxide. The pH after mixing was estimated by adding together equal volumes of oxoperoxonitrate(1−) and buffer solution in a small vessel and measuring the pH with a small-sized Ingold glass electrode. Additionally, a small glass cell holding the same glass electrode was mounted in the outlet of the stopped-flow apparatus; no significant differences in pH were found in both approaches.

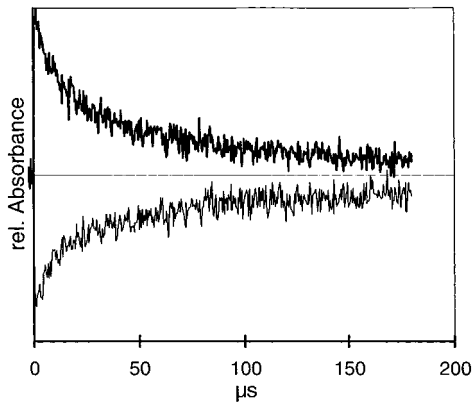


Figure 1. Absorbance changes of a 100 μM oxoperoxonitrate(1−) solution after irradiation with a 10-ns, 10-mJ laser pulse at 355 nm. At 300 nm (thin line) and 245 nm (thick line) changes in the oxoperoxonitrate(1−) and superoxide concentrations, respectively, were monitored. The traces show that the decay of superoxide and the appearance of oxoperoxonitrate(1−) are correlated. From this it is inferred that the other photolysis product is nitrogen monoxide.

In most experiments, the decay of oxoperoxonitrate(1−) was monitored at the absorption maximum of 302 nm. Time-dependent spectra were obtained by collecting multiple time traces at different wavelengths and by extracting time-dependent spectra from these data. A range from 250 to 450 nm with a resolution of 10 nm was covered. The time-dependent spectra were interpreted with the GLINT global analysis software package (Applied Photophysics).

High-pressure stopped-flow experiments were carried out with equipment that has previously been described (*38*) and with a nearly identical Hi-Tech HPSF56 stopped-flow. Absorbance changes were recorded at 302 nm, 15 °C, pH 6.2, and 23 °C, pH 5.6. The pH values represent the pH afterward (0.1 M phosphate buffer).

Pulse Radiolysis. Solutions were irradiated with a 2-MeV electron accelerator (Febetron 705, Hewlett-Packard) as described before (*39*). Only single-shot experiments were carried out. Aqueous solutions of oxoperoxonitrate(1−) at 21−24 °C and pH 12 were saturated with dinitrogen monoxide (24.4 mM) and then irradiated; the oxoperoxonitrate(1−) concentration was monitored by transmission of the solutions 1 ms before every pulse. Under these conditions over 90% of the radicals produced initially are hydroxyl and oxide(1−), $O^{\bullet-}$, radicals. For some experiments 1 mM potassium hexaoxo-μ-peroxodisulfate(2−) was used to scavenge hydrated electrons. These solutions at pH 12 also contained 0.1 mM oxoperoxonitrate(1−) and were saturated with argon. Upon irradiation about equal amounts of tetraoxosulfate(1−) and hydroxyl radicals were produced. Tetraoxosulfate(1−) is a very powerful one-electron oxidant, $E°(SO_4^{\bullet-}/SO_4^{2-}) = 2.4$ V (*30*). All stock solutions were kept on ice in the dark.

Results

Laser Flash Photolysis. Irradiation at 266 and 355 nm, but not at 532 nm, resulted in bleaching of oxoperoxonitrate(1−) and the appearance of "product 1" at 245 nm (Figures 1 and 2). Assuming a quantitative conversion from oxoperoxonitrate(1−) to product 1 and correcting for the contribution of oxoperoxonitrate(1−), we found an extinction coefficient of about 2.4×10^3 M^{-1} cm^{-1} at 245 nm for product 1, which is identical to that of superoxide. After the flash, product 1 decayed in a second-order process with a rate constant higher than 10^{10} M^{-1} s^{-1}, with simultaneous restoration of the oxoperoxonitrate(1−) absorption. The decay of product 1 and the regeneration of oxoperoxonitrate(1−) are strictly

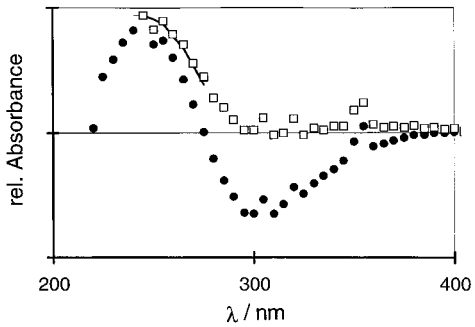


Figure 2. Kinetic spectra 500 ns after irradiation of an oxoperoxonitrate(1−) solution with a laser (355 nm, 10 ns, 5 mJ): ●, absorption differences relative to the unirradiated solution; □, same data but corrected for the bleaching of oxoperoxonitrate(1−) to give the spectrum of product 1 or superoxide. The peaks at 350 and 355 nm assigned to fluorescence artifacts disappear upon irradiation with 266-nm light. Line: spectrum of superoxide (*42*).

Table 1. Buffer Influence on the pK_a Determination of Hydrogen Oxoperoxonitrate, O=NOOH

	buffer					
	phosphate				ammonia,	borate,
	0.65 M	0.25 M	0.05 M	0.001 M	0.05 M	0.1 M
pK_a (±0.10)	7.33	7.12	6.71	6.55	7.11	8.59

correlated (Figure 1). Even after repeated flashing (800 laser flashes) of the same sample, oxoperoxonitrate(1−) was not degraded to any considerable extent, although very small gas bubbles could be seen in the measurement cell.

Stopped-Flow Determination of the pK_a of Hydrogen Oxoperoxonitrate. Since only the acid, but not the anion, decays rapidly to nitrate, the rate law for the decay includes K_a:

$$\frac{dc}{dt} = -\frac{[H^+]}{K_a + [H^+]} \times k_{iso} \times c \qquad (1)$$

where $c = [ONOO^-] + [ONOOH]$ and k_{iso} is the rate constant of decay of hydrogen oxoperoxonitrate. Thus

$$k_{obs} = \frac{[H^+]}{K_a + [H^+]} \times k_{iso} \qquad (2)$$

We evaluated K_a by fitting the $k_{obs}([H^+])$ (eq 2) function with the program proFIT (Quantum Soft) which is based on a Newton−Gauss algorithm to adapt nonlinear functions to data sets. k_{iso} was fixed to a value of 1.20 s^{-1} (25 °C), obtained from earlier determinations (11 times) at pH 3−4.5 at the same ionic strength. This is reasonable because k_{iso} does not depend on pH and, as $[H^+] \geq 100K_a$, the error in k_{iso} is not more than 1%, well below the accuracy of the kinetic determination. The restriction of k_{iso} to the well-established value reduces the problem to a one-parameter fit which is more easily handled by the algorithm. The pK_a was found to depend on the buffer type and its concentration, as shown in Table 1, for phosphate, borate, and ammonia buffers over the concentration range from 1 mM to 0.65 M. A plot of log k_{obs} versus pH (Figure 3) indicates that 0.1 M borate buffer, 0.05 M ammonia buffer, and 0.65 M phosphate buffer accelerate the decay compared to 0.05 M phosphate buffer.

Stopped-Flow Detection of an Oxoperoxonitrate(1−)−Hydrogen Oxoperoxonitrate Adduct. When the combined hydrogen oxoperoxonitrate and oxoperoxonitrate(1−) concentration is higher than 100 μM, and the pH is between 7 and 8.5, the decay reaction does not follow first-order kinetics (Figure 4, trace a). Only the first 3 s of such a kinetic trace can be fitted to a simple exponential function. In the second phase, the decay is slower due to a parallel reaction with the formation of an absorbing intermediate, "product 2". These unusual kinetics are absent at low oxoperoxonitrate(1−) concentrations (Figure 4, trace b) or pH values below the pK_a. Since simple first-order traces are geometrically similar, subtraction of the curve obtained at a lower concentration scaled to the amplitude of the higher one should yield a horizontal line, if the mechanism in both cases was the same. However, this is not the case here (Figure 4, trace d).

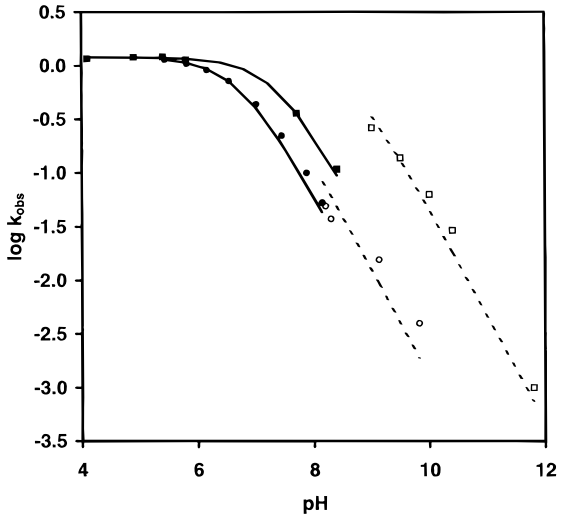


Figure 3. Dependence of log k_{obs} on the buffer: ●, 0.05 M phosphate buffer; ○, 0.05 M ammonia/ammonium nitrate buffer; ■, 0.65 M phosphate buffer; □, 0.1 M borate buffer; solid and dashed lines are calculated fits for eq 2 with $k_{iso} = 1.2$ s^{-1}.

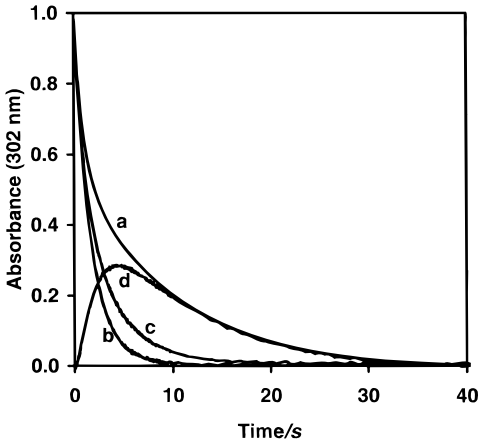


Figure 4. Anomalous kinetic behavior above pH 7. All traces were recorded at 302 nm and pH 7.9: a, $c_{tot} = 0.48$ mM; b, oxoperoxonitrate(1−) absorbance × 10, $c_{tot} = 0.048$ mM; c, oxoperoxonitrate(1−) absorbance × 10, $c_{tot} = 0.048$ mM, nitrite concentration = 4.2 mM; d, difference trace of traces a and b.

Stopped-Flow Study at High Pressure. If the isomerization reaction involves homolysis of the O−O bond of hydrogen oxoperoxonitrate, then one would expect high pressure to slow down the rate of reaction. Experiments at pressures up to 152 MPa (1500 atm) show that the rate of the isomerization reaction depends little on the pressure (Figure 5). From the two sets of results identical activation volumes of 1.5 ± 1.0 (solid line, pH 6.2) and 1.8 ± 1.1 (dashed line, pH 5.6) cm^3 mol^{-1} were determined. We conclude that the activation vol-

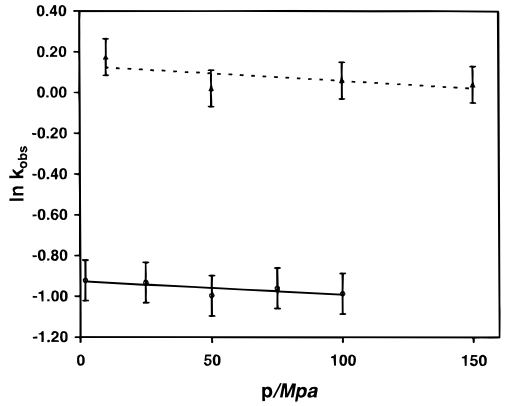


Figure 5. Pressure dependence of the decomposition reaction of hydrogen oxoperoxonitrate up to 1500 bar. The error bars are equal to 2 times the standard deviation. Solid line: $t = 15$ °C, $l = 302$ nm, pH = 6.2 (0.1 M phosphate buffer), $\Delta V^{\ddagger} = 1.5 \pm 1.0$ cm^3 mol^{-1}. Dashed line: $t = 23$ °C, $l = 302$ nm, pH = 5.6 (0.1 M phosphate buffer), $\Delta V^{\ddagger} = 1.8 \pm 1.1$ cm^3 mol^{-1}. Total peroxynitrite concentration: 50 μM.

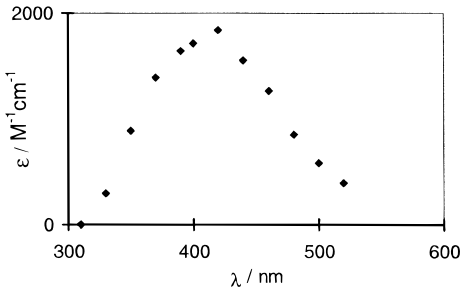


Figure 6. Spectrum of product 3, the product of the reaction of the hydroxyl radical with oxoperoxonitrate(1−).

ume of the isomerization of hydrogen oxoperoxonitrate to nitrate and a hydron has an activation volume of 1.7 ± 1.0 cm^3 mol^{-1}.

Pulse Radiolysis Studies. Solutions of 90−680 μM oxoperoxonitrate(1−) were irradiated with doses of 18−400 Gy. Such doses yield hydroxyl/oxide(1−) concentrations of 10−220 μM. Several processes were observed at wavelengths between 255 and 520 nm under pseudo-first-order conditions and with oxoperoxonitrate(1−) present in excess.

Directly after the pulse and at wavelengths between 287 and 330 nm, a very rapid decay of oxoperoxonitrate(1−) was detected. Under pseudo-first-order conditions with oxoperoxonitrate(1−) in excess, the absorption remained stable after approximately 2 μs. Concomitantly, between 370 and 520 nm the appearance of a strongly absorbing species, "product 3", with an absorption maximum at 420 nm and an extinction coefficient of 1.8×10^3 M^{-1} cm^{-1} was observed (Figure 6). The decay observed below 330 nm and the buildup seen above that wavelength are both pseudo-first-order, and within experimental error the corresponding first-order rate constants are equal and dependent on the oxoperoxonitrate(1−) concentration.

At times longer than 5 μs the absorbance remained stable between 295 and 330 nm. However, at higher wavelengths a second-order decay of product 3 was observed with a rate constant of approximately $(3-4) \times 10^6$ M^{-1} s^{-1} (Figure 7).

As discussed below, product 3 could either be ONOO$^{\bullet}$ or [(HO)ONOO]$^{\bullet-}$. To decide between these possibilities we oxidized oxoperoxonitrate(1−) with the tetraoxosulfate(1−) radical. Deaereated solutions containing 1 mM hexaoxo-μ-peroxodisulfate(2−), 0.1 mM oxoperoxonitrate(1−), and 10 mM potassium hydroxide were used to obtain more information about product 3. Upon irradiation about equal amounts of hydroxyl and tetraoxosulfate(1−) radicals are formed. Again product 3 was observed, but with the assumption of 1:1 conversion of oxoperoxonitrate(1−) to a single product, and the extinction coefficients derived were only one-half of those obtained previously.

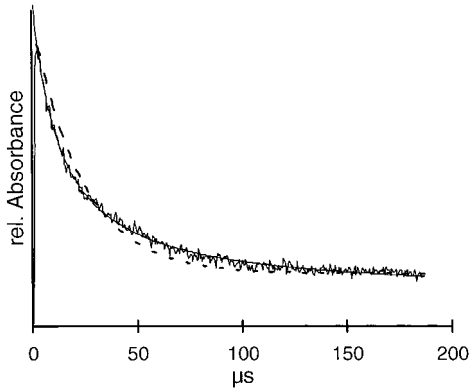


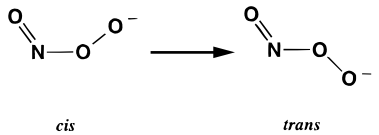
Figure 7. Absorbance change at 420 nm of a 600 μM solution of oxoperoxonitrate(1−) at pH 12 saturated with dinitrogen monoxide upon irradiation with 2-MeV electrons (noisy curve). Under these conditions mainly HO$^{\bullet}$ and O$^{\bullet-}$ are produced. The transient formed is the direct product of the reaction with oxoperoxonitrate(1−). Least-square fits for first-order (dashed line) and second-order (solid line) kinetics are given.

At high doses and wavelengths lower than 273 nm, a rapid appearance and a subsequent decay of an absorbing species were observed. Due to noise a detailed analysis was not attempted; however, the half-life of the decay was clearly dose-dependent and therefore not first-order. This absorbing species might be a photolysis product from the Cerenkov irradiation and could be product 1.

Discussion

Laser Flash Photolysis. In principle, the following reactions could take place.

(i) Isomerization: *cis*- and *trans*-oxoperoxonitrate(1−)



cis *trans* **(3)**

$$ONOO^- + h\nu \rightarrow NO_3^- \quad (4)$$

(ii) Photodissociation:

$$ONOO^- + h\nu \rightarrow NO_2^{\bullet} + O^{\bullet-} \quad (5)$$

$$ONOO^- + h\nu \rightarrow NO_2^- + O^{2\bullet}\ (O^{2\bullet} + H_2O \rightarrow 2^{\bullet}OH \text{ or } H_2O_2) \quad (6)$$

$$ONOO^- + h\nu \rightarrow NO^{\bullet} + O_2^{\bullet-} \quad (7)$$

$$ONOO^- + h\nu \rightarrow NO^- + O_2 \quad (8)$$

(iii) Photoionization:

$$ONOO^- + h\nu \rightarrow ONOO^{\bullet} + e_{aq}^{\bullet-} \quad (9)$$

As oxoperoxonitrate(1−) is reformed almost quantitatively in a second-order reaction, reactions 3−6 can be

neglected. Reaction 4 is essentially irreversible. Hydroxyl radicals produced in reactions 5 and 6 would be less likely to react with nitrogen dioxide than with oxoperoxonitrate(1−), which is in higher concentration, and would be expected to reduce further the absorbance due to oxoperoxonitrate(1−). Therefore reactions 5 and 6 would be irreversible, too. Both the forward and the reverse of reaction 3 would be first-order processes.

For spectroscopic reasons reactions 8 and 9 must be abandoned. The photoionization (9) would lead to a solvated electron, which absorbs strongly with a broad band at 720 nm (1.8×10^4 M^{-1} cm^{-1}) which was not observed (*40*). Nitrogen dioxide, oxide(1−) (reaction 5), and oxonitrate(1−) (reaction 8) do not have sufficient absorption at 245 nm. The absorption at 245 nm could be due to superoxide, reaction 7, as the observed spectrum is very similar to that reported for this radical (*41*, *42*).

Under the assumption that we observed the reverse of reaction 7,

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^- \qquad (-7)$$

the global analysis gives a rate constant of $(1.5-2.0) \times 10^{10}$ M^{-1} s^{-1}. Conventional second-order analysis at 25 different wavelengths and 4 different laser intensities resulted in a value of $(1.9 \pm 0.2) \times 10^{10}$ M^{-1} s^{-1}.

The rate constant for reaction −7 of 1.9×10^{10} M^{-1} s^{-1} is 3−4 times higher than those reported by other workers (*1*−*3*). These rate constants were measured under experimental designs that involve reaction cascades to arrive at the reactants. The rapid one-step production of superoxide and nitrogen monoxide reported herein has the advantage of allowing measurement of the recombination rate not complicated by other processes.

The high rate constant has important consequences for the generation of oxoperoxonitrate(1−) in vivo. Under normal in vivo conditions, the concentrations of nitrogen monoxide and superoxide dismutase are on the order of 1×10^{-8} and 5×10^{-6} M, respectively, whereby superoxide dismutase scavenges 98−99% of all superoxide, and consequently very little oxoperoxonitrate(1−) is formed. Near an activated macrophage the nitrogen monoxide and superoxide concentrations may be 100 times higher, and the ratio of superoxide reacting with nitrogen monoxide to superoxide reacting with superoxide dismutase becomes 3:2. While considerations based on homogeneous solution kinetics do not necessarily apply to the inhomogeneous milieu inside or outside a cell, they do indicate that oxoperoxonitrate(1−) formation is likely.

Stopped-Flow Determination of the pK_a of Hydrogen Oxoperoxonitrate. Earlier reported pK_a determinations for hydrogen oxoperoxonitrate vary over a wide range from 6 to 7.5 (*13*). We describe here the pK_a to be dependent on both buffer composition and concentration. In most buffers, an acceleration of the decay with increasing buffer concentration was observed, which we ascribe to general acid catalysis. Ammonium is presumably more effective than dihydrogen phosphate because it has a higher relative concentration above the "true" pK_a of hydrogen oxoperoxonitrate. Analogous interactions have been reported elsewhere, for example, hydronation of trioxonitrate(2−), $NO_3^{\bullet 2-}$ (*4*), dioxonitrate(2−), $NO_2^{\bullet 2-}$ (*43*), and trioxide(1−), $O_3^{\bullet -}$ (*44*), by dihydrogen phosphate(1−) in pulse radiolysis experiments. Solutions containing Lewis acids, of which borate buffer is a prime example, accelerate decay even better. Since the pK_a of boric acid is 9.14, there exists a significant amount of free boric acid even at pH 10, which results in an apparent pK_a of hydrogen oxoperoxonitrate of over 8. This effect has been observed earlier by Keith and Powell (*45*). They also found a correlation with the free boric acid concentration but interpreted the result as a consequence of peroxoborate formation. This would imply that the nitrosyl cation, NO^+, is released which subsequently forms nitrite as the sole nitrogen-containing product. However, this could not be confirmed. The Lewis acid mechanism we propose is thought to start with an addition of the peroxo moiety to the Lewis acid. Decay of the oxoperoxonitrate(1−)−Lewis acid adduct then occurs, but more slowly than for hydrogen oxoperoxonitrate.

The rate disappearance of oxoperoxonitrate(1−), or its hydronated form, may also be affected by hydrogen carbonate(1−) and carbon dioxide, which would involve both general acid catalysis and Lewis acid adduct formation. First, hydrogen carbonate(1−) and dihydrogen carbonate can act as proton donors. Second, carbon dioxide in equilibrium with dihydrogen carbonate is also a Lewis acid and forms an adduct with oxoperoxonitrate(1−) (*27*, *46*). As the acceleration of decay by ammonium is minor compared to that by boric acid, the carbon dioxide effect may rather be caused by carbon dioxide's ability to act as an Lewis acid, rather than by hydrogen carbonate(1−) donating a hydron, and it appears in general that Lewis acids have a greater influence on the rate of decay than direct hydron transfer by a weak acid. It seems that the essential step to induce the decay of oxoperoxonitrate(1−) is the partial or full neutralization of the negative charge on the peroxide group, which also means that the anion owes its stability to this rather localized charge. The consequence of the milieu dependence of the pK_a of hydrogen oxoperoxonitrate suggests that it varies also in vivo with the composition of the fluids found extracellularly and intracellularly.

Stopped-Flow Detection of an Oxoperoxonitrate(1−)−Hydrogen Oxoperoxonitrate Adduct. When the total concentration of oxoperoxonitrate(1−) exceeds 100 μM, the disappearance of oxoperoxonitrate(1−) deviates from first order in the pH range 7.5−8.5. Since the effect disappears upon dilution (Figure 4, trace b), it must be a bimolecular or higher order process. Because the decay seems to be delayed under the stated conditions, it would seem that some reaction parallel to the isomerization, possibly adduct formation, takes place. Potential reaction partners for hydrogen oxoperoxonitrate are hydrogen phosphate, nitrite (a minor contaminant in our preparation), or oxoperoxonitrate(1−). A quadratic dependence of the deviation from first-order behavior on the combined concentration of oxoperoxonitrate(1−) and hydrogen oxoperoxonitrate was found (Figure 8), which indicates that nitrite or oxoperoxonitrate(1−), but not the buffer, takes part in the reaction. If nitrite were responsible, the reduction of the delay observed upon dilution could be compensated for by adding appropriate amounts of nitrite. An 88-fold increased nitrite concentration restores the deviation from first-order kinetics somewhat (Figure 4, trace c) but clearly not to the original extent. The other argument against nitrite is the absence of the effect at pH values below the pK_a of hydrogen oxoperoxonitrate, where it should be more prominent because nitrite is still anionic but the oxoperoxonitrate(1−) mostly protonated. We therefore propose that oxoperoxonitrate(1−) reacts with hydrogen oxoperoxonitrate to stabilize it and inhibit decay. The decay goes to completion, which indicates that adduct formation is reversible, or that the

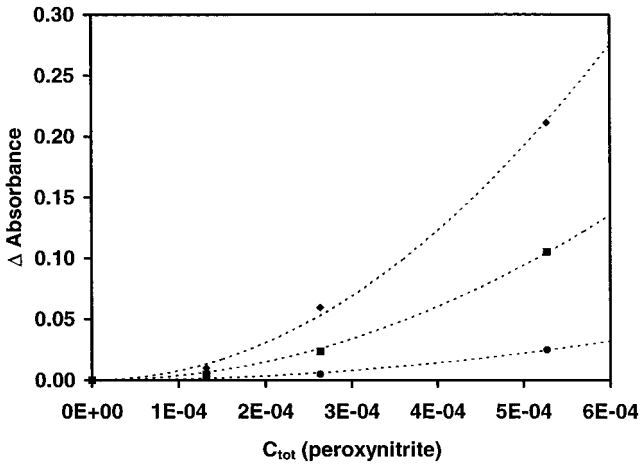


Figure 8. Deviation from first-order behavior as a function of the total concentration of $ONOO^- + ONOOH$: ●, at 0.5 s; ■, at 2 s; ♦, at 8 s of the kinetic record. Dashed lines: parabolic fits of the type $\Delta Abs = const \times c_{tot}^2$.

adduct itself decays. Two models can be formulated:

$$HOONO + ONOO^- \rightleftarrows X^- \qquad (A)$$

$$HOONO \rightarrow H^+ + NO_3^-$$

$$HOONO + ONOO^- \rightarrow X^- \qquad (B)$$

$$X^- \rightarrow \text{nonabsorbing products}$$

$$HOONO \rightarrow H^+ + NO_3^-$$

In both models, the normal decomposition of hydrogen oxoperoxonitrate takes place parallel to the other reactions. In model A it is the only pathway that leads to the final decomposition. It is also evident that the formation of X^- is not a fast process since the initial part of the kinetic trace is very close to first order, and a fit to this section yields the same rate constant as a trace from a more dilute solution under otherwise equal conditions where the full curve is used for fitting. The conclusion is that the reaction that leads to X^- is slower than the hydrogen oxoperoxonitrate decay. This reasoning excludes the ambiguity that is involved with consecutive processes, namely, the possibility of having either a weakly absorbing intermediate formed quickly or a strongly absorbing intermediate formed slowly (*47*). The parallel reaction of hydrogen oxoperoxonitrate decay serves as a measure for the formation rate of the intermediate. A rate constant for the intermediate formation numerically derived from the data that leads to an intermediate formation rate faster than this measure can be disregarded. Even more important, the above argument also holds for model B.

If model A were correct, then the dissociation must be slower than the association, or the delay in decay would not be observed. To determine the extent of adduct formation, spectral kinetic data were collected at pH 7.9. These data were first subjected to evolving factor analysis to estimate the number of spectral components involved. This was followed by singular value decomposition and a global fit for the unknown intermediate spectrum, a residual (background) spectrum, and the unknown rate constants. The GLINT program employs the well-established Newton−Gauss−Marquardt algorithm combined with Runge−Kutta integration for the fit. The measured spectra of oxoperoxonitrate(1−) and hydrogen

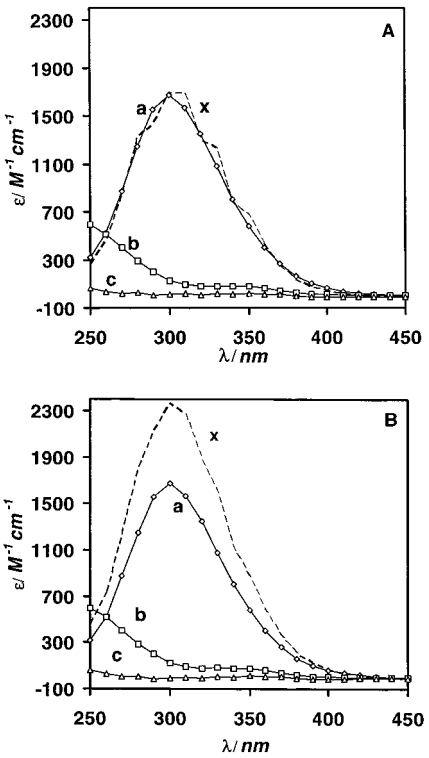
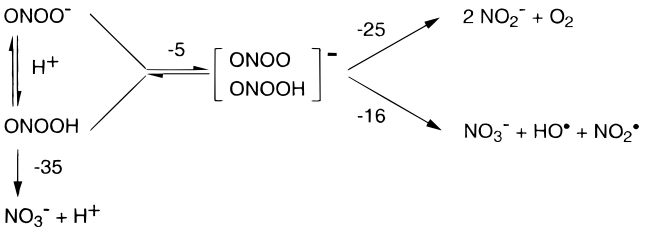


Figure 9. Absorption spectra relevant to the oxoperoxonitrate-(1−) decay at pH 7.9: a, oxoperoxonitrate(1−); b, hydrogen oxoperoxonitrate; c, background (mainly nitrate); x, product 2, the addition intermediate, $ONOOH \cdot ONOO^-$ (calculated with GLINT). A: Spectrum of product 2 based on the assumption that only free hydrogen oxoperoxonitrate isomerizes to nitrate. B: Spectrum of product 2 if both free hydrogen oxoperoxonitrate and the peroxynitrous acid in the complex isomerize to nitrate.

oxoperoxonitrate and $k_{iso} = 1.20\ s^{-1}$ for hydrogen oxoperoxonitrate were introduced as constants. The residual spectrum served as a diagnostic tool. When the residual spectrum shows little or no absorbance at various wavelengths, then the model is correct, but not necessarily unique.

The above treatment yielded a bimolecular rate constant of $(2.0 \pm 0.1) \times 10^3\ M^{-1}\ s^{-1}$ for the formation of the adduct and a unimolecular rate constant of $(0.20 + 0.01)\ s^{-1}$ for the dissociation. The equilibrium constant is therefore $(1.0 \pm 0.1) \times 10^4\ M^{-1}$. Additionally, the spectrum of the adduct was obtained (Figure 9A), which is similar to that of oxoperoxonitrate(1−) but appears to be shifted somewhat to greater wavelengths. Model B, which assumes that the adduct is not in equilibrium with its components but decays directly, was also tested. The time−concentration profile for the adduct is the same as for the first model, but the spectrum of the adduct has unusual high absorptivity values (Figure 9B). Given the strength of the interaction ($\Delta G^{\circ\prime} = -23\ kJ\ mol^{-1}$, based on $K = 1.0 \times 10^4\ M^{-1}$), one expects to a first approximation the spectrum of the adduct to be similar to that of oxoperoxonitrate(1−), as the hydrogen oxoperoxonitrate part of the adduct provides only little absorptivity. Therefore, the first model is more likely to be correct. In addition to dissociation into reactants, it is possible that the adduct yields dioxygen and nitrite, or even nitrate, nitrogen dioxide, and hydroxyl, as shown in Scheme 1. All reactions mentioned are thermodynamically possible, as indicated by negative Gibbs energy changes. Evidence for nitrite and dioxygen formation from peroxynitrite was presented by Pfeiffer et al. (*32*), where these products were observed under conditions of pH and concentration that are favorable to adduct formation. It is clear that

Scheme 1. Formation and Possible Decay Modes of the Oxoperoxonitrate(1−)−Hydrogen Oxoperoxonitrate Adduct[a]



[a] Gibbs energy changes are given in kJ mol^{-1}.

the oxoperoxonitrate(1−)−hydrogen oxoperoxonitrate interaction must be studied more thoroughly in the future over a range of pH values. Preliminary experiments showed that the biphasic curve can be obtained between pH 7.5 and 8.5. Above pH 8.5 and sufficient oxoperoxonitrate(1−) total concentration, the bimolecular pathway is probably the only one of significance. One conclusion can be drawn now: experiments at physiological pH and total oxoperoxonitrate(1−) concentrations higher than 100 μM must be interpreted cautiously.

A complex as described here between oxoperoxonitrate(1−) and hydrogen oxoperoxonitrate will not be formed under physiological conditions. However, the reaction between hydrogen peroxide and hydrogen oxoperoxonitrate is also slower than decay of the latter (*7*), which suggests an analogous interaction between the electrophilic nitrogen of hydrogen oxoperoxonitrate and the hydroperoxide ion. Such interactions may also occur with physiologically relevant molecules such as ascorbate (*48*).

Stopped-Flow Study at High Pressure. The activation volume, 1.7 ± 1.0 cm^3 mol^{-1}, is not consistent with a mechanism of isomerization in which homolysis of the peroxide bond to yield free hydroxyl and nitrogen dioxide radicals is rate-limiting. Such reactions typically have activation volumes of 10 cm^3 mol^{-1} (*49*, *50*). However, the observed activation volume may be compatible with (i) a lengthening of the O−O bond followed by isomerization as previously proposed (*13*), (ii) isomerization mainly as described under (i) with a small fraction isomerizing via homolysis, and, *possibly*, (iii) heterolysis into hydroxide and nitryl, in which case electrostriction would lower the activation volume of the heterolysis.

It is possible that adduct formation between hydrogen oxoperoxonitrate and oxoperoxonitrate(1−) affects high-pressure studies. Adduct formation could increase with pressure and therefore influence the rate of isomerization to nitrate at oxoperoxonitrate concentrations below 100 μM. For a solution with extensive dimerization, a large activation volume would be found for the isomerization because of the required dissociation of the adduct.

Pulse Radiolysis. The radiolysis experiments reported here were carried out at pH 12, near the pK of •OH (11.9), which makes it necessary to consider the different reactivities of both the hydroxyl (•OH) and oxide(1−) (O•$^-$) radicals: The protonated form is rather electrophilic, the deprotonated form rather nucleophilic. Furthermore, Coulombic repulsion between oxoperoxonitrate(1−) and oxide(1−) would be expected to slow the reaction.

To find out what product 3 (•OH + ONOO$^-$ → product 3) is, we attempted to fit several mechanisms. The following reactions were considered: •OH and ONOO$^-$ leading to an intermediate adduct, which subsequently (reaction 10) or immediately (reactions 11 and 12) fragments. Additionally, we considered an electron-transfer mechanism (reactions 13a,b).

$$^{\bullet}OH + ONOO^- \rightarrow [(HO)ONOO]^{\bullet -} \rightarrow \text{products} \quad (10)$$

$$^{\bullet}OH + ONOO^- \rightarrow HO_2^{\bullet} + NO_2^-,\ 2O_2^{\bullet -} + H_2O \rightarrow HO_2^- + O_2 + OH^- \quad (11)$$

$$^{\bullet}OH + ONOO^- \rightarrow HO_2^- + NO_2^{\bullet},\ 2NO_2^{\bullet} + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (12)$$

$$^{\bullet}OH + ONOO^- \rightarrow HO^- + ONOO^{\bullet} \quad (13)$$

$$ONOO^{\bullet} \rightarrow NO^{\bullet} + O_2 \quad (13a)$$

$$2ONOO^{\bullet} \rightarrow \text{products } (2NO_2^{\bullet} + O_2)? \quad (13b)$$

Because the decay of product 3 is second-order, we can exclude reactions 10 and 13a as main reaction channels. As hydrogen dioxide and superoxide radicals do not absorb at 420 nm, reaction 11 must be abandoned. Reaction 12 can be eliminated on the grounds that the extinction coefficient of nitrogen dioxide (see reaction 12) is about 8 times lower than that of product 3. By process of elimination, reaction 13 followed by reaction 13b is a possible reaction mechanism. Alternatively, [(HO)ONOO]•$^-$, the intermediate in reaction l0, could recombine in a second-order process instead of fragment. The experiments with potassium hexaoxo-μ-peroxodisulfate(2−) allow us to distinguish between these two reaction channels. When hydrated electrons are scavenged by potassium hexaoxo-μ-peroxodisulfate(2−), the resulting solution contains approximately equal amounts of two powerful oxidizing agents, namely, hydroxyl and tetraoxosulfate(1−) radicals. While hydroxyl radicals can react both by electron transfer and by adduct formation, tetraoxosulfate(1−) radicals react only by electron transfer. If both oxidants react by one-electron transfer, no change in the yield of product 3 should occur. However, we found that the yield was halved in the presence of potassium hexaoxo-μ-peroxodisulfate(2−). This result indicates that the hydroxyl radical adduct is formed, although a half-life of over 80 μs at low concentrations of oxoperoxonitrate(1−) seems long. This interpretation is supported by the fact that trinitrogen radicals formed from azide also do not react with oxoperoxonitrate(1−) to produce product 3.[2]

The chemistry of hydrogen oxoperoxonitrate near its pK_a is quite complex. An important caveat is that the outcome of experiments intended to be physiologically relevant is affected by conditions such as buffer composition and the total concentration of oxoperoxonitrate(1−).

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[2] G. Merényi, personal communication.

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