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Vol. 327, No. 2, March 15, pp. 335–343, 1996
Article No. 0131

Acceleration of Peroxynitrite Oxidations by Carbon Dioxide

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Received December 29, 1995

Stopped-flow kinetic studies of the isomerization of peroxynitrite to give nitrate have been performed in carbonate-enriched buffers using pH jump and carbonic anhydrase as probes. The data are consistent with the reaction of CO_2 and the peroxynitrite anion rapidly forming an unstable nitrosoperoxycarbonate anion adduct, $O{=}N{-}OOCO_2^-$ (1). The CO_2 catalysis of the isomerization of peroxynitrite is not accompanied by the formation of nitrite, hydrogen peroxide, or other hydroperoxidic material like peroxycarbonate. The reaction proceeds via the transient formation of an oxidant or oxidants that is (are) capable of promoting electrophilic nitration reactions. We propose that $O{=}N{-}OOCO_2^-$ rearranges to give a nitrocarbonate anion, $O_2N{-}OCO_2^-$ (2) which, in turn, may serve as the proximal oxidant in biological systems that produce peroxynitrite. At least four different mechanistic classes of reactions that have been ascribed to peroxynitrite can be envisioned to involve 2: (a) hydrolysis to nitrate, (b) one-electron or (c) two-electron oxidations, and (d) electrophilic nitration. Given the fast reaction of peroxynitrite with carbon dioxide and the ubiquitous presence of the latter, the role of CO_2 cannot be neglected in complex peroxynitrite reactions *in vitro* and *in vivo.*

Key Words: carbon dioxide; catalysis; electrophilic nitration; nitric oxide; nitrosoperoxycarbonate; nitrocarbonate; peroxynitrite, radical.

Peroxynitrite[2] ($ONOO^-$)[3] is a potent oxidant that can be formed in biological systems from the reaction of superoxide ($O_2^{\cdot-}$), a reducing agent, and the moderately oxidizing nitric oxide ($^{\cdot}NO$) (1–9). The reaction between $O_2^{\cdot-}$ and $^{\cdot}NO$ is a radical–radical combination and, therefore, is extremely rapid ($k = 4–7 \times 10^9$ M^{-1} s^{-1}) (10–12) and does not require enzymatic catalysis. Peroxynitrite is a versatile oxidant that is capable of reacting with all major classes of biomolecules, including antioxidants (such as ascorbate, glutathione, α-tocopherol, and uric acid) (13–19), carbohydrates (20), lipids (21, 22), nucleic acids (23–26), and proteins (27–34). The reactions of peroxynitrite that have been identified to date can be divided into at least five mechanistic classes: (a) isomerization to nitrate, (b) one-electron or (c) two-electron oxidations, (d) oxygen atom transfers, and (e) electrophilic nitrations (35–39).

It has been known for almost 3 decades that peroxynitrite is unstable in carbonate[2] buffers (40). Radi *et al.* (41) proposed that in carbonate buffers peroxynitrite forms an adduct, $O{=}N{-}OOCO_2^-$ (1), the nitrosoperoxycarbonate anion. Lymar and Hurst recently suggested that this adduct is formed from the reaction of peroxynitrite anion with CO_2 (Eq. [1]), rather than the reaction of ONOOH with HCO_3^- (42).

$$CO_2 + O{=}NOO^- \rightarrow \underset{1}{O{=}N{-}OOCO_2^-} \qquad [1]$$

The peroxynitrite anion/CO_2 reaction, with a rate constant of 3×10^4 M^{-1} s^{-1}, is one of the fastest reactions known for peroxynitrite (42), and the abundance of carbonate ($\geqslant$25 mM) in physiological fluids (43) suggests that this reaction may precede many of the reactions of peroxynitrite that occur *in vivo.* Beckman and his coworkers have shown that low concentrations of carbonate (1–5 mM) protect *Escherichia coli* from the toxic effects of peroxynitrite in *in vitro* bactericidal assays (43).

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[2] The IUPAC recommended name for peroxynitrite anion is oxoperoxonitrate(1−) and hydrogen oxoperoxonitrate for peroxynitrous acid (*Nomenclature of Inorganic Chemistry. Recommendations–1990,* Blackwell Sci, Oxford/London). We use the term "peroxynitrite" to indicate the total concentration of $ONOO^-$ + ONOOH. We use the term "carbonate" to refer collectively to all the carbonated species (CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}). When we refer to a particular carbonated species we use its chemical formula.

[3] Abbreviations used: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); $ABTS^{\cdot+}$, ABTS radical cation; CO_2, carbon dioxide; DTPA, diethylenetriaminepentaacetic acid; *I*, ionic strength; ONOOH, peroxynitrous acid; $ONOO^-$, peroxynitrite anion; $O{=}N{-}OOCO_2^-$ or 1, nitrosoperoxycarbonate anion; $O_2N{-}OCO_2^-$ or 2, nitrocarbonate anion; $^{\cdot}NO$, nitric oxide; $O_2^{\cdot-}$, superoxide; $^{\cdot}NO_2$, nitrogen dioxide; 4-HPA, 4-hydroxyphenylacetic acid.

0003-9861/96 $18.00

Understanding the kinetics of the peroxynitrite anion/CO_2 reaction and the chemistry of the nitrosoperoxycarbonate anion is absolutely essential in establishing the role of carbonate in peroxynitrite-mediated oxidations *in vivo*. We here report stopped-flow kinetic studies of the decay of peroxynitrite in carbonate-enriched buffers using pH jump and carbonic anhydrase as probes. The results confirm that $O{=}N{-}OOCO_2^-$ is formed from the reaction of CO_2 with $ONOO^-$ and not from the reaction of HCO_3^- with HOONO (42). Based on the reaction kinetics; the yields of nitrate, nitrite, and hydrogen peroxide; the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); and nitration of phenols by peroxynitrite in carbonate buffers, we propose that $O{=}N{-}OOCO_2^-$ rearranges to the nitrocarbonate anion, $O_2N{-}OCO_2^-$. The nitrocarbonate anion is proposed to serve as an oxidant and nitrating species in biologically relevant peroxynitrite-producing systems.[4]

MATERIALS AND METHODS

The following chemicals were purchased from Sigma: ABTS, diethylenetriaminepentaacetic acid (DTPA), FAD, NADPH, phenol, sodium azide, carbonic anhydrase II from human erythrocytes (EC 4.2.1.1, 3400 Wilbur–Anderson units/mg of solid), nitrate reductase from *Aspergillus* species (EC 1.6.6.2, 2 units/mg of protein), and peroxidase from horseradish (EC 1.11.1.7, 290 pupurogallin units/mg of solid). Sulfanilamide and 4-hydroxyphenylacetic acid (4-HPA) were purchased from Aldrich. All other chemicals were of the highest grade available.

Synthesis of Peroxynitrite

Peroxynitrite was synthesized by the ozonation of an aqueous solution of 0.1 M sodium azide at pH 12, as described earlier (44). To minimize contamination by the unreacted residual azide, ozonation was continued even after a maximal yield of peroxynitrite occurred (45). This procedure results in 20–30% loss of the peroxynitrite, but the final preparations contain only traces of unreacted azide (≤5 μM). Typical preparations contained peroxynitrite in the concentration range of 30–35 mM and were essentially free of hydrogen peroxide.

Stopped-Flow Kinetic Studies

Stopped-flow measurements of the disappearance of peroxynitrite were performed as described earlier (15, 35, 44), using a spectrophotometer manufactured by On-Line Instrument Systems (Bogart, GA) with a mixing time ≤1.5 ms. The stopped-flow instrument was equipped with an OLIS-RSM 1000 rapid scanning monochromator that was capable of collecting 1000 spectra per second. A stock solution of peroxynitrite was diluted to about 0.4 mM with 0.01 N NaOH containing 0 or 10 mM added $NaHCO_3$ and loaded into one of the two syringes of the stopped-flow apparatus. The other syringe was loaded with 0.2 M phosphate buffer, pH 7.0, containing 0.2 mM DTPA, 0 or 10 mM added $NaHCO_3$, and 0 or 1 μM native or heat-inactivated carbonic anhydrase. The cell and the individual reactants were thermostated at 25 ± 1°C. Equal volumes of phosphate buffer and the peroxynitrite solution were mixed in the cell (the pH of the final assay mixture was 7.2). Changes in absorbance were monitored over 260 to 364 nm for 1–15 s, by which time the decomposition of peroxynitrite was ≥99%.

The data obtained in the stopped-flow experiments were treated under two different conditions, those of near equilibrium and those of steady state in CO_2 (42, 46). Near-equilibrium conditions prevail when the added $NaHCO_3$ was allowed to equilibrate at pH 7.0 in phosphate buffers prior to allowing it to react with peroxynitrite. The concentration of CO_2 is relatively high and constant under these conditions (Table I). Steady-state conditions prevail when the added $NaHCO_3$, preequilibrated at pH 12, is rapidly switched to neutral pH. In this case, the CO_2 concentration remains at a very low level because the hydration/dehydration reaction is slow relative to the proton transfer reactions and the reaction of CO_2 with $ONOO^-$ (42, 46, 47). The rate laws for these experimental conditions are given in Table I.

Assay of Hydrogen Peroxide

Hydrogen peroxide was estimated by the horseradish peroxidase-coupled oxidation of ABTS to the ABTS radical cation ($ABTS^{\bullet+}$) (48, 49), as described earlier (44). Briefly, 0.1–1 μmol of peroxynitrite was decomposed by incubation with 1 ml of 0.1 M phosphate buffer, pH 7.0, containing 0.1 mM DTPA and 0 or 20 mM added $NaHCO_3$ for 5 min at room temperature. [Sodium bicarbonate was added 2 min before the addition of peroxynitrite. This allows the equilibration of the carbonate species prior to the reaction with peroxynitrite.] An aliquot (0.5 ml) of the decomposed peroxynitrite solution was mixed with 2.5 ml of 0.1 M phosphate buffer, pH 7.0, containing 6 μmol of ABTS and 30 units of horseradish peroxidase. After 2–5 min of incubation at room temperature, the $ABTS^{\bullet+}$ formed was measured spectrophotometrically at 404 nm (ϵ = 36,800 M^{-1} cm^{-1}) (50).

Estimation of Nitrate and Nitrite

Nitrate. Peroxynitrite (0.2–2 μmol) was decomposed by being incubated with 1 ml of 0.16 M phosphate buffer, pH 7.0, containing 0.16 mM DTPA and 20 mM added $NaHCO_3$. In one set of experiments, $NaHCO_3$ was added 2 min before the addition of peroxynitrite. In the other set, $NaHCO_3$ was added 2–5 min after the addition of peroxynitrite, when the peroxynitrite had already decomposed. Nitrate was estimated based on the nitrate reductase-coupled oxidation of NADPH as described by Bories and Bories (51). Briefly, an aliquot (0.1 ml) of the decomposed peroxynitrite solution was mixed with 3.4 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.24 mM NADPH, 20 μM FAD, and nitrate reductase (20 units/liter). The reaction mixtures were incubated for 1 h at room temperature. At the end of the incubation period, the change in absorbance at 340 nm was measured using a Hewlett Packard 8451A diode array spectrophotometer (ϵ = 6220 M^{-1} cm^{-1} for NADPH at 340 nm).

Nitrite. Nitrite was estimated according to the method of Bratton and Marshall (52), as described earlier (44, 53). In these assays, peroxynitrite (0.1–1 μmol) was decomposed by incubation with 1 ml of 0.1 M phosphate buffer, pH 7.0, or 0.1 M carbonate buffer, pH 7.0. Both buffers contained DTPA at a final concentration of 0.1 mM.

Nitration of Phenolic Compounds

Phenol. Peroxynitrite (2 μmol) was allowed to react with 30 μmol of phenol in 2 ml of 0.1 M phosphate buffer, pH 7.0, containing 0.1 mM DTPA and up to 20 mM added $NaHCO_3$ at room temperature for 10 min. As described above, $NaHCO_3$ was added 2 min before the addition of peroxynitrite to allow the equilibration of the carbonate species prior to the reaction with peroxynitrite. The nitrophenols formed were measured spectrophotometrically at 400 nm.

[4] For simplicity, we do not consider the protonated forms of nitrosoperoxycarbonate 1 and nitrocarbonate 2 because their pK_A values are likely to be lower than that of carbonic acid [pK_A = 3.5; I = 0.25 M (46)]. Therefore, at physiological pH and in the pH range studied here, the ionized forms predominate.

TABLE I

Approximate Concentrations of Various Carbonated Species Immediately Following Mixing in the Stopped-Flow Apparatus and the Applicable Rate Laws for the Decay of Peroxynitrite (0.2 mM after Mixing) under the Prevailing Conditions

Experimental conditions	Concentration (M)	Integrated rate law
Case I[a] Near equilibrium	$CO_2 = 5 \times 10^{-4}$ $H_2CO_3 = 8 \times 10^{-7}$ $HCO_3^- = 4 \times 10^{-3}$ $CO_3^{2-} = 1 \times 10^{-5}$	$\frac{[ONOO]_t}{[ONOO]_0} = \exp(-at)$
Case II[b] Steady state	$CO_2 = 3 \times 10^{-10}$ $H_2CO_3 = 1 \times 10^{-6}$ $HCO_3^- = 5 \times 10^{-3}$ $CO_3^{2-} = 1 \times 10^{-5}$	$\frac{[ONOO]_t}{[ONOO]_0} = \exp(-bt) - \frac{R}{b[ONOO]_0} \times [1 - \exp(-bt)]$

[a] The species concentrations were calculated assuming that a 10 mM $NaHCO_3$ solution equilibrated at pH 7.0 in 0.2 M phosphate buffer [ionic strength (I) ≈ 0.5 M] was rapidly changed (by a two-fold dilution with pH 12 water containing 0.4 mM peroxynitrite) to give a final carbonate concentration of 5 mM at pH 7.2 in 0.1 M phosphate buffer (I ≈ 0.25 M). Notice also that these conditions are of pseudo-first order. In the integrated rate law expression, the constant a is the observed rate constant, that is, the sum of the spontaneous decomposition rate constant (0.4 s^{-1}) and the pseudo-first-order rate constant for reaction with 5 mM carbonate (12.1 s^{-1}) at pH 7.2 and 25°C.

[b] These values were calculated assuming that a 10 mM $NaHCO_3$ solution equilibrated pH 12 (I ≈ 0 M) was rapidly changed (by a twofold dilution with 0.2 M phosphate buffer, pH 7.0) to give a final carbonate concentration of 5 mM at pH 7.2 in 0.1 M phosphate buffer (I ≈ 0.25 M). The thermodynamic properties reported by Alberty were used for these calculations (46). In the integrated rate law expression, b is the spontaneous rate constant for decomposition of peroxynitrite (0.4 s^{-1}) and R is the rate of dehydration of H_2CO_3 under the prevailing conditions.

4-Hydroxyphenylacetic acid. Nitration of 4-HPA was studied at pH 7.0, as described earlier (31, 53). Briefly, 0.2–2 μmol of peroxynitrite was allowed to react with 4 μmol of 4-HPA in 2 ml of 0.1 M carbonate buffer, pH 7.0, or 0.1 M phosphate buffer, pH 7.0, containing 0 or 5 mM added $NaHCO_3$ at room temperature for 10 min. Diethylenetriaminepentaacetic acid was included in all assays at a final concentration of 0.1 mM. 3-Nitro-4-hydroxyphenylacetic acid formed was estimated spectrophotometrically at 430 nm following the addition of 0.5 ml of 0.1 N NaOH (ϵ = 4400 M^{-1} cm^{-1}) (31).

Oxidation of ABTS

Peroxynitrite (0.01–0.2 μmol) was allowed to react with 4 μmol of ABTS in 2.0 ml of 0.1 M phosphate buffer, pH 7.0, containing 0.1 mM DTPA and 0 or 20 mM added $NaHCO_3$ at room temperature for 2 min. As described above, $NaHCO_3$ was added 2 min before the addition of peroxynitrite to allow the equilibration of the carbonate species prior to the reaction with peroxynitrite. The formation of $ABTS^{\bullet+}$ was estimated spectrophotometrically at 404 nm as described above.

RESULTS AND DISCUSSION

Formation of the Carbon Dioxide–Peroxynitrite Anion Adduct

We used two methods to determine the nature of the reactant pair that allows the formation of the $O{=}N{-}OOCO_2^-$ adduct in carbonate/peroxynitrite reactions. The first is a pH jump method that is essentially identical to that used by Lymar and Hurst (42). This method is based on the fact that the hydration/dehydration reaction of CO_2/H_2CO_3 (Eq. [2]) is slower than protonation reactions of CO_3^{2-} and HCO_3^- (Eqs. [3] and [4]) (46, 47). Our second approach is based on the catalytic action of the enzyme carbonic anhydrase on the dehydration of H_2CO_3 (Eq. [2]) (54). In both cases, the technique of stopped flow was employed to determine the kinetics of peroxynitrite decomposition.

$$H_2CO_3 \rightleftharpoons CO_2 + H_2O \quad [2]$$

$$CO_3^{2-} + H^+ \rightleftharpoons HCO_3^- \quad [3]$$

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \quad [4]$$

pH jump experiments. Figure 1 shows the time course of peroxynitrite decay at 25°C in 0.1 M phosphate buffer, pH 7.2, containing 0.1 mM DTPA and 0 or 5 mM added $NaHCO_3$. In the absence of added $NaHCO_3$ (trace A), the decay of peroxynitrite is slow and follows first-order kinetics with an observed rate constant, k_{obs}, equal to 0.4 s^{-1} (Table I). This compares well with our previously reported values of 0.34–0.40 s^{-1} (36, 44, 53). The decay of peroxynitrite was about threefold faster when $NaHCO_3$ (10 mM) was added to alkaline peroxynitrite and this solution was then mixed with an equal volume of phosphate buffer, pH 7.0 (trace B). The catalytic effect of $NaHCO_3$ was more pronounced if $NaHCO_3$ was preincubated with phosphate buffer (pH 7.0) and this solution was then mixed with an equal volume of alkaline peroxynitrite (trace C; k_{obs} = 12.5 s^{-1}). In both cases, the final concentration of carbonate was 5 mM and the pH of the reaction mixture measured at the outlet was 7.2. However, as shown in Table I, the striking difference between these two cases is the availability of CO_2 for reaction with peroxynitrite. This occurs because equilibria involving proton transfers (see Reactions [3] and [4]) are reestablished immediately following pH shock to 7.2, but the concentration of CO_2 remains at the previous equilibrium level. Therefore, it

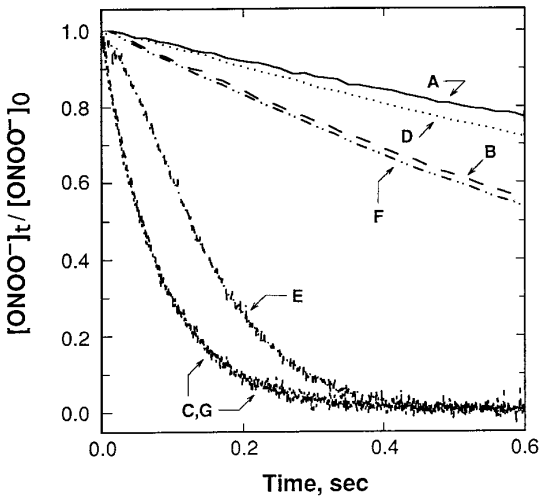


FIG. 1. Stopped-flow kinetic traces of the isomerization of peroxynitrite to form nitrate at pH 7.2 and 25°C. Trace A, peroxynitrite in 0.01 N NaOH was mixed with 0.2 M phosphate buffer, pH 7.0. Trace B, a mixture of peroxynitrite and 10 mM $NaHCO_3$ in 0.01 N NaOH was mixed with 0.2 M phosphate buffer, pH 7.0. Trace C, peroxynitrite in 0.01 N NaOH was mixed with 0.2 M phosphate buffer, pH 7.0, which also contained 10 mM $NaHCO_3$. Trace D, peroxynitrite in 0.01 N NaOH was mixed with 0.2 M phosphate buffer, pH 7.0, containing 1 μM carbonic anhydrase. Trace E, peroxynitrite and 10 mM $NaHCO_3$ in 0.01 N NaOH mixed with 0.2 M phosphate buffer, pH 7.0, containing 1 μM carbonic anhydrase. Trace F, a mixture of peroxynitrite and 10 mM $NaHCO_3$ in 0.01 N NaOH was mixed with 0.2 M phosphate buffer, pH 7.0, containing 1 μM heat-inactivated carbonic anhydrase. Trace G, peroxynitrite in 0.01 N NaOH was mixed with 0.2 M phosphate buffer, pH 7.0, containing 10 mM $NaHCO_3$ and 1 μM carbonic anhydrase. For all experiments, equal volumes of the phosphate buffer and the peroxynitrite solution were mixed in the cell. The cell and the individual reactants were thermostated at 25 ± 1°C. The pH of the final assay mixture was 7.2 and the concentration of peroxynitrite (after mixing) was about 0.2 mM. All assays contained DTPA at a final concentration of 0.1 mM. The decomposition of peroxynitrite was followed at 302 nm.

is likely that CO_2 and $ONOO^-$ constitute the reactant pair in the formation of 1 (Reaction [1]), in agreement with the conclusions of Lymar and Hurst (42).

Studies using carbonic anhydrase. Additional evidence that nitrosoperoxycarbonate anion is formed from the reaction of $ONOO^-$ with CO_2 and not ONOOH with HCO_3^- comes from the studies of carbonic anhydrase (Fig. 1). Carbonic anhydrase is a zinc-containing metalloenzyme that catalyzes the reversible hydration of CO_2, Reaction [2] (54).

As shown in Fig. 1, trace D, addition of carbonic anhydrase in the absence of added $NaHCO_3$ results in a slight increase in the rate of isomerization of peroxynitrite. When $NaHCO_3$ was added to alkaline peroxynitrite and this solution was then mixed with an equal volume of phosphate buffer containing 1 μM carbonic anhydrase, there is marked increase in the rate of decomposition of peroxynitrite (Fig. 1, trace E). The rate of peroxynitrite decay under these conditions is about 20 times faster than the spontaneous decomposition of peroxynitrite in the absence of added carbonate (Fig. 1, trace A) and about 6 times faster than the $NaHCO_3$-induced decomposition of peroxynitrite (trace B). Heat-inactivated carbonic anhydrase in the presence of added $NaHCO_3$ caused no increase in the rate of decay of peroxynitrite (Fig. 1, trace F).

These observations suggest a catalytic role for carbonic anhydrase in these peroxynitrite/carbonate reactions. Two possibilities exist: (i) carbonic anhydrase produces higher transient levels of CO_2 via the catalytic dehydration of H_2CO_3 (see Reaction [2]) or (ii) the enzyme may catalyze a direct reaction between CO_2 (or other carbonate isomers) and $ONOO^-$ (or ONOOH).

To test these possibilities, $NaHCO_3$ was preincubated with phosphate buffer (pH 7.0) containing 1 μM carbonic anhydrase and this solution was then mixed with an equal volume of alkaline peroxynitrite (Fig. 1, trace G). The rate of decomposition of peroxynitrite under these conditions (k_{obs} = 12.0 s^{-1}) is essentially the same as that observed in the corresponding control assay that did not contain carbonic anhydrase (Fig. 1, trace C). This rules out the possibility of direct catalysis of the peroxynitrite/carbonate reaction by carbonic anhydrase. Together with the results obtained in pH-jump experiments, these observations suggest that the catalytic action of carbonic anhydrase is a consequence of its speeding up the dehydration of H_2CO_3, resulting in higher steady-state levels of CO_2.

Products of Peroxynitrite Decay in Carbonate Buffers

Hydrogen peroxide. We find no evidence for the occurrence of H_2O_2 in preparations of peroxynitrite (0.1–1 μmol) that were decomposed at pH 7.0 in the presence or absence of added $NaHCO_3$ (20 mM). In assays where $NaHCO_3$ was added, the reactions were under near-equilibrium conditions with regard to the availability of CO_2 (~2 mM) (46). Using a second-order rate constant of 3×10^4 M^{-1} s^{-1} for the reaction of $ONOO^-$ with CO_2 (42), one can calculate that ≥99% of peroxynitrite in these solutions should have decomposed via the formation of $O{=}N{-}OOCO_2^-$.

The coupled enzyme assay used for the estimation of H_2O_2 in the above reactions has a detection limit ≤1 nmol, and the recovery of H_2O_2 added either before or after decomposition of peroxynitrite was ≥70% (44). Given these detection limits, the yields of H_2O_2 must be less than 1 mol% of the peroxynitrite originally employed. The failure to detect H_2O_2 in these peroxynitrite-decomposed solutions also rules out the formation of other hydroperoxides such as peroxycarbonate.[5]

Nitrite. We analyzed for NO_2^- in peroxynitrite solutions that were decomposed at pH 7.0 in 0.1 M phos-

[5] Peroxycarbonate, if formed, would be expected to hydrolyze to give H_2O_2 [Flanagan, J., Jones, D. P., Griffith, W. P., Skapski, A. C., and West, A. P. (1986) *J. Chem. Soc. Chem. Commun.* p. 20].

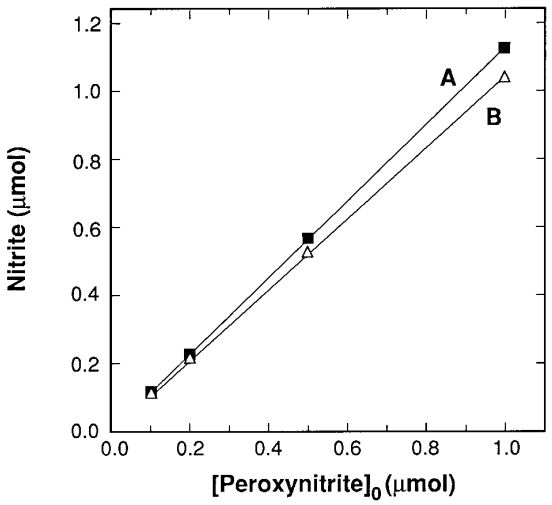


FIG. 2. Estimation of nitrite in preparations of peroxynitrite decomposed in (A) 0.1 M phosphate buffer, pH 7.0, or (B) 0.1 M carbonate buffer, pH 7.0. Both buffers contained DTPA at a final concentration of 0.1 mM. Other details are given under Materials and Methods.

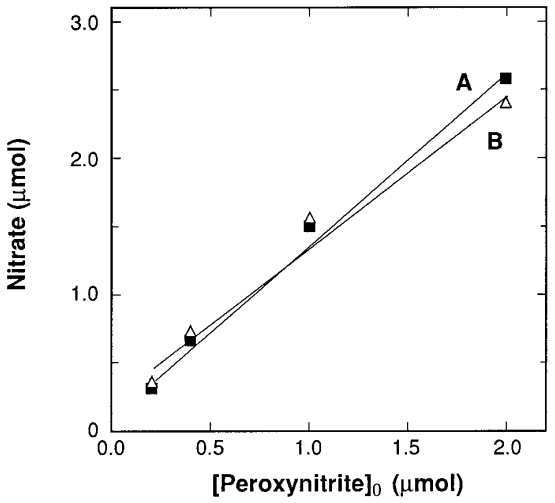


FIG. 3. Assay of nitrate in peroxynitrite preparations decomposed in 0.1 M phosphate buffer, pH 7.0, containing 0.1 mM DTPA and 20 mM added $NaHCO_3$. (A) $NaHCO_3$ was added before the addition of peroxynitrite and (B) $NaHCO_3$ was added after the decomposition of peroxynitrite. Nitrate was estimated according to the method of Bories and Bories (51). Other details are as given under Materials and Methods.

phate or 0.1 M carbonate buffer (Fig. 2). Both buffers contained DTPA at a final concentration of 0.1 mM. The decomposition of peroxynitrite in phosphate buffers proceeds via the isomerization of peroxynitrous acid, resulting in the formation of NO_3^- (36). Therefore, the concentration of NO_2^- in peroxynitrite preparations decomposed in phosphate buffers represents the contamination of NO_2^- in the original peroxynitrite preparation (Fig. 2, curve A). These contaminating nitrite levels are nearly the same as those found in peroxynitrite preparations that were decomposed in 0.1 M carbonate buffer, pH 7.0 (Fig. 2, curve B). This shows that NO_2^- is not formed in the CO_2-catalyzed decomposition of peroxynitrite. Assuming that the equilibrium concentration of CO_2 was about 10 mM, the decomposition of peroxynitrite in the carbonate buffer should have proceeded exclusively via the formation of $O{=}N{-}OOCO_2^-$ (Fig. 2, curve B).

Nitrate. Figure 3 shows the concentration of NO_3^- in peroxynitrite solutions that were decomposed at pH 7.0 in phosphate buffer containing 0.1 mM DTPA and 0 or 20 mM added $NaHCO_3$. Any NO_3^- present in these solutions results from the contamination of NO_3^- in the original peroxynitrite preparation plus any NO_3^- formed from the isomerization of peroxynitrite. The total nitrate is almost the same whether or not $NaHCO_3$ was present at the time of decomposition of peroxynitrite (Fig. 3), suggesting that nitrate is the major product in both the spontaneous (curve A) and the CO_2-catalyzed decomposition of peroxynitrite (curve B).

Reactions of Peroxynitrite in Carbonate Buffers

Nitration of phenols. Nitration of tyrosine residues in proteins has been used as an indicator of the production and reaction of peroxynitrite *in vivo* (39, 55–57). Because of this, it is particularly important to probe the catalysis of nitrations by CO_2. We have examined the effect of exogenously added $NaHCO_3$ on the peroxynitrite-mediated nitration of a substituted phenol, 4-HPA, in phosphate buffers (Fig. 4). We chose 4-HPA because it gives a single major nitration product, 3-

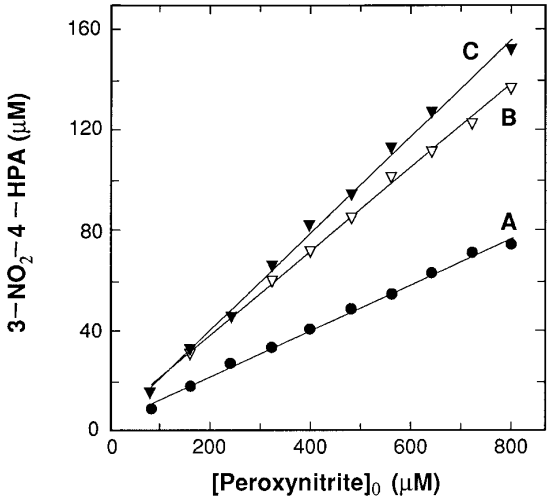


FIG. 4. Yields of 3-nitro-4-hydroxyphenylacetic acid from the reaction of peroxynitrite with 4-hydroxyphenylacetic acid at pH 7.0. Curves A and B refer to the reactions performed in 0.1 M phosphate buffer, pH 7.0, containing 0 and 5 mM added $NaHCO_3$, respectively, whereas curve C shows the nitration of 4-HPA in 0.1 M carbonate buffer, pH 7.0. The experimental details are given under Materials and Methods.

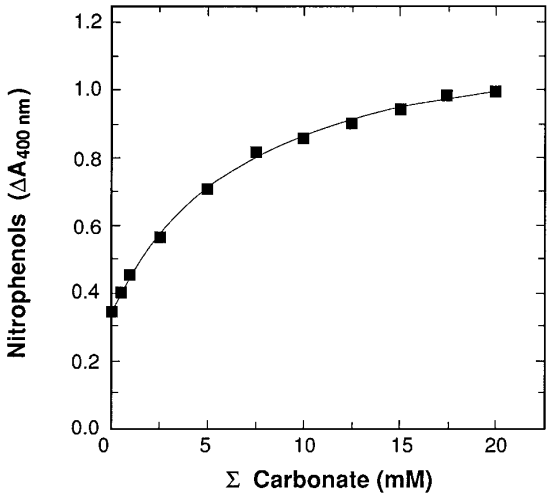


FIG. 5. Nitration of phenol by peroxynitrite in 0.1 M phosphate buffer, pH 7.0, containing 0.1 mM DTPA and up to 20 mM added $NaHCO_3$. Other details are given under Materials and Methods.

nitro-4-HPA (31). Diethylenetriaminepentaacetic acid was included in these reactions at a final concentration of 0.1 mM to minimize catalysis by transition metal ion impurities. As shown in Fig. 4, curve A, in the absence of added $NaHCO_3$, the yield of 3-nitro-4-HPA was 9 mol% based on the starting concentration of peroxynitrite. Addition of $NaHCO_3$ (5 mM) to the phosphate buffer increases the yield of 3-nitro-4-HPA to about 16 mol% (Fig. 4, curve B), and the yield increases only slightly further, to 19 mol%, when 100 mM carbonate buffer at pH 7.0 was used (Fig. 4, curve C) (without phosphate buffer).

We have also studied the effect on peroxynitrite-mediated nitrations of phenol by adding $NaHCO_3$ (0–20 mM) to phosphate buffers, pH 7.0 (Fig. 5). Unlike 4-HPA, the nitration of phenol gives several products (58), and the product distribution can be used to probe the underlying mechanism(s). As shown in Fig. 5, peroxynitrite gives a small yield of nitrophenols even in the absence of added $NaHCO_3$. The yield of nitrophenols increases by about threefold when $NaHCO_3$ is added to phosphate buffers at pH 7.0 to give a final concentration of 20 mM (Fig. 5). The ortho/para (o/p) ratio of the nitrophenols formed is almost the same in the presence and absence of added $NaHCO_3$ (58). Since some adventitious carbonate is present in peroxynitrite solutions and in the buffers used, these observations suggest that nitration of phenols in general could be mediated via the formation of $O{=}N{-}OOCO_2^-$ even when $NaHCO_3$ was not added (see below).

Oxidation of ABTS. Peroxynitrite is known to oxidize ABTS to the ABTS radical cation (53, 59, 60). We have allowed peroxynitrite to oxidize ABTS at pH 7.0 in phosphate buffers containing 0.1 mM DTPA and 0 or 20 mM added $NaHCO_3$. Even in the absence of added $NaHCO_3$, the yield of $ABTS^{\bullet+}$ is 0.24 mol/mol of peroxynitrite employed in the reaction (Fig. 6, curve A). Addition of $NaHCO_3$ to a final concentration of 20 mM increases this yield only marginally to 0.27 mol/mol of peroxynitrite (Fig. 6, curve B). As discussed earlier, the rearrangement of peroxynitrite to give nitrate in the latter case occurs exclusively via the formation of $O{=}N{-}OOCO_2^-$. Since the control solutions always contain some adventitious carbonate (see below), it is likely that some of the oxidation of ABTS proceeds via the formation of $O{=}N{-}OOCO_2^-$ even when $NaHCO_3$ was not added.

Possible Mechanisms

We suggest that nitrosoperoxycarbonate anion (1) and its rearranged product nitrocarbonate anion (2) are involved in reactions of peroxynitrite in carbonate-rich physiological fluids[4] (Scheme 1). The observations that must be considered in postulating such a mechanism are as follows: (i) An adduct formed from the reaction of CO_2 and $ONOO^-$, 1, reacts rapidly with water to give NO_3^- and HCO_3^- (Fig. 3), resulting in a marked catalysis of the isomerization of peroxynitrite to NO_3^- by CO_2 (Fig. 1). (ii) The decay of peroxynitrite in carbonate buffers gives little or no NO_2^- (Fig. 2), H_2O_2, or other hydroperoxidic material. (iii) There is always a higher yield of nitration of phenols by peroxynitrite in carbonate-enriched buffers (Figs. 4 and 5). (iv) The o/p ratio of nitrophenols formed is essentially the same in both control and carbonate-enriched buffers (58), suggesting either that nitration in the control assays is also mediated by adventitious CO_2 or that 2 and whatever nitrating species may be present in the absence of CO_2 have the same selectivity. (v) Lymar and Hurst report that

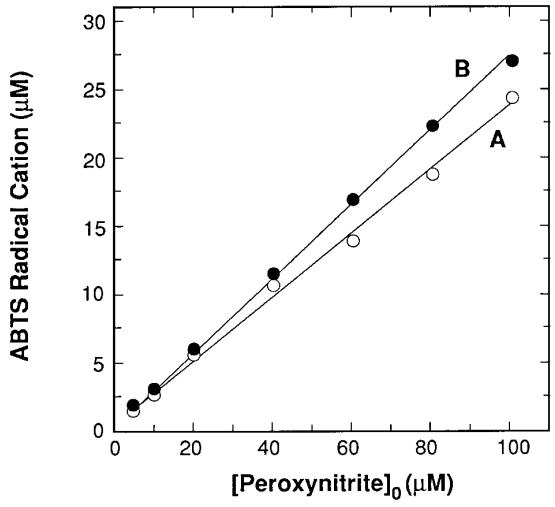
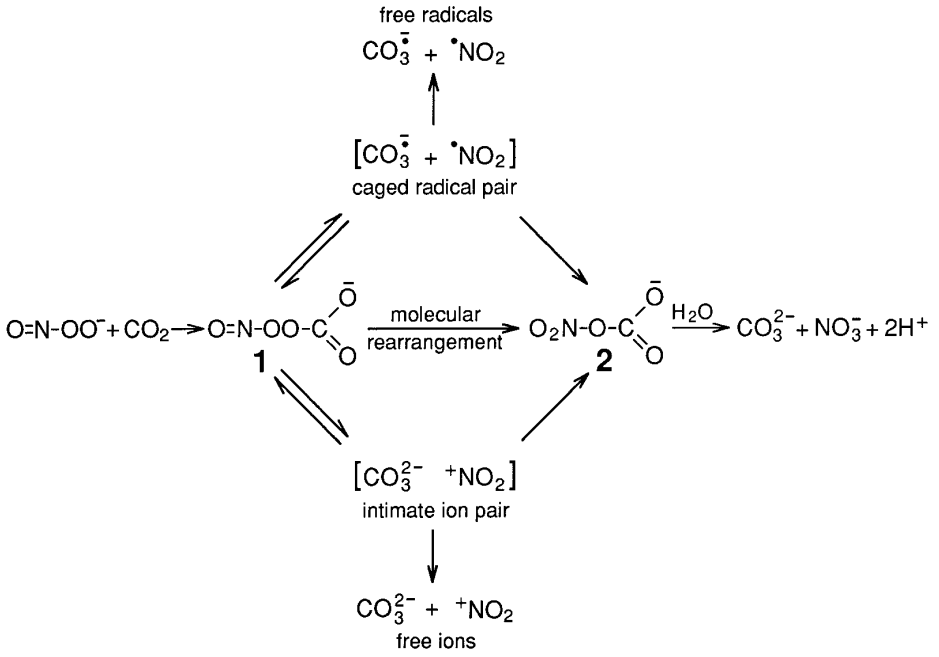


FIG. 6. Oxidation of ABTS by peroxynitrite in 0.1 M phosphate buffer, pH 7.0, containing 0.1 mM DTPA and (A) 0 or (B) 20 mM added $NaHCO_3$. The experimental details are given under Materials and Methods.



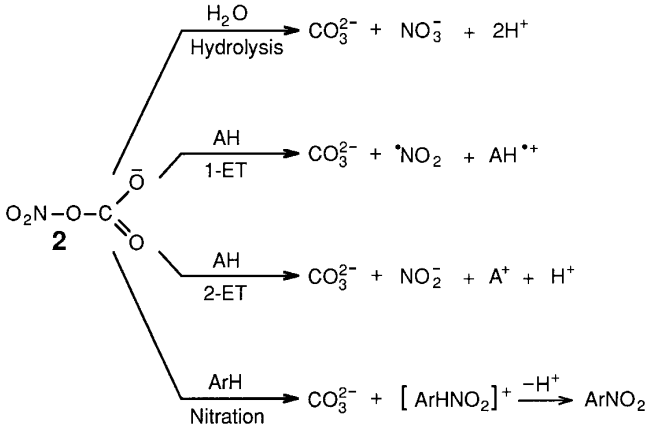
SCHEME 1. Schematic representation of the isomerization of peroxynitrite to nitrate in carbonate-enriched buffers. The scheme consists of three important reactions that occur in succession. The first in the sequence is the formation nitrosoperoxycarbonate anion (1) from the reaction of peroxynitrite anion with CO_2. This reaction is followed by the rearrangement of 1 to the nitrocarbonate anion (2), which, in turn, undergoes hydrolysis by water to give the final products nitrate and carbonate. The rearrangement of 1 to 2 can occur via a radical, an ionic, or a cyclic molecular group-transfer reaction.

the lifetime of the nitrating species formed in peroxynitrite/carbonate reactions is at least 2 orders of magnitude longer than that of the nitronium ion in water (42). (vi) The nitrosoperoxycarbonate anion and/or its subsequent isomerization product bring about the oxidation of ABTS to $ABTS^{\bullet+}$ even in situations where no carbonate was added (Fig. 6).

Based on the above observations, we propose that 1 rearranges to give 2 (Scheme 1). This rearrangement could occur via a radical, an ionic, or a cyclic molecular group-transfer reaction, as shown in Scheme 1. The radical and ionic paths involve homolysis or heterolysis (respectively) of 1 at the O–O bond resulting in the formation of caged radicals or intimate ion pairs. These caged pairs can diffuse apart to give free radicals or ions, or combine to form 2 or reform 1. The yields of NO_3^- (Fig. 3), NO_2^- (Fig. 2), and the nitration of phenols (Figs. 4 and 5) suggest that the caged ion or radical pairs may not diffuse out of the solvent cage to any significant extent. For instance, diffusion of the caged radical pair apart would result in the formation of free $^{\bullet}NO_2$, which would dimerize and disproportionate to give equimolar amounts of NO_3^- and NO_2^-. Similarly, diffusion of the caged ion pair apart would result in the formation of the free nitronium ion, which would rapidly react with water to give NO_3^- ($k = 5 \times 10^8\ s^{-1}$) (61) and, therefore, would not be an effective nitrating species.

Nitrocarbonate as the Active Species in Peroxynitrite Reactions

The nitrocarbonate anion may serve as the proximal oxidant in biologically relevant peroxynitrite-producing systems (Scheme 2). It is a mixed anhydride of nitric and carbonic acids and, in analogy with acylnit-



SCHEME 2. Schematic representation of four different mechanistic classes of reactions that are typical of peroxynitrite-producing systems which in actuality may involve the nitrocarbonate anion in carbonate-enriched physiological fluids: (from top to bottom) hydrolysis to nitrate, one-electron transfer (1-ET), two-electron transfer (2-ET), and electrophilic nitration.

rates (62), 2 would be expected to be a good nitrating agent. However, unlike acylnitrates that are easily hydrolyzed (63), 2 appears to be more stable to nucleophilic attack at the carbonyl carbon atom. The negative charge of 2 may hinder nucleophilic attack by water and render 2 a relatively long-lived species capable of carrying out nitrations in water at physiological pH.[4] Apart from nitration, at least three other types of reactions can be envisioned for 2: (i) hydrolysis to nitrate, (ii) one-electron, or (iii) two-electron oxidations (Scheme 2). Hydrolysis is the predominant reaction pathway for 2 in the absence of reactive substrates. In the presence of appropriate electron donors, a competitive one- or two-electron reduction of 2 may occur. One-electron reduction would give HCO_3^- and $^{\bullet}NO_2$, whereas a two-electron reduction would give HCO_3^- and NO_2^-.

Catalysis by Ubiquitous Carbon Dioxide

The catalytic effects of CO_2 on the reactions of peroxynitrite that are described here raise concerns about the true values of rate constants for peroxynitrite reactions that have been reported in the literature, since carbonate (and therefore CO_2) could be present in peroxynitrite preparations even if it is not purposefully added. Peroxynitrite solutions are often prepared in mild or moderate alkali (pH $\geqslant$ 12), and these alkaline solutions would inevitably be contaminated with some carbonate due to absorption of CO_2 from the air. Furthermore, the carbonate contamination would be expected to increase during the storage of stock peroxynitrite solutions. A small amount of carbonate also may be present even in freshly prepared phosphate or other buffers used to carry out peroxynitrite reactions. Even if precautions are taken to protect solutions from the air, the pellets of NaOH or KOH that are used to prepare the solutions often are covered with an opaque film of absorbed carbonate.

Despite these concerns about contamination of peroxynitrite studies *in vitro* with adventitious carbonate, there is striking agreement among values for the rate constant for the isomerization of peroxynitrite to give nitrate as reported by different groups, often using different preparations of peroxynitrite and quite different apparatus. This agreement undoubtedly results from the fact that the rate of the isomerization of peroxynitrite is not sensitive to the presence of small concentrations of adventitious CO_2. For example, if the concentration of CO_2 remains at a steady state value of 5 μM, only about 25% of the peroxynitrite would decay through the formation of nitrosoperoxycarbonate and/or nitrocarbonate, and this would lead to small increases in the rates for peroxynitrite disappearance that are comparable to the variations due to random errors.

However, many of the reactions of peroxynitrite with biological substrates probably occur exclusively through intermediates 1 and/or 2, and these reactions will be dramatically influenced by the presence of CO_2. These reactions cannot be studied without knowledge of the concentration of CO_2. For example, the yields of the nitration of phenols vary significantly in the presence of CO_2, as we report here.

Thus, in studies of complex peroxynitrite reactions systems *in vitro,* in the study of peroxynitrite in cell culture studies (that are done under 5% CO_2 and in carbonate buffers), and in studies of peroxynitrite-mediated transformations *in vivo,* the role of CO_2 cannot be neglected.

ACKNOWLEDGMENTS

This work was supported in part by a grant to W.A.P. from the Environmental Health Sciences Institute of the National Institutes of Health, Grant ES-06754. We also thank Professors Gary Winston and Andrew Maverick of Louisiana State University and Kendall Houk of the University of California at Los Angeles for helpful discussions.

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