

# Nitration and Nitrosation by Peroxynitrite: Role of CO<sub>2</sub> and Evidence for Common Intermediates

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**Abstract:** Peroxynitrite reacts with morpholine (MorH) to give both *N*-nitromorpholine (Mor-NO<sub>2</sub>) and *N*-nitrosomorpholine (Mor-N=O). The pH profile of Mor-NO<sub>2</sub> formation shows a bell-shaped curve with a maximum yield of 1.6 mol % (relative to peroxynitrite) centered around pH 9.0. Yields of Mor-N=O, on the other hand, increase until the pH reaches 10.0 and then reach a plateau; the maximum yield of Mor-N=O is 12 mol % relative to peroxynitrite. Both the nitration and nitrosation of MorH are catalyzed by low levels of CO<sub>2</sub>; however, excess CO<sub>2</sub> dramatically reduces the yields of Mor-N=O but not Mor-NO<sub>2</sub>, and the combined yields of Mor-N=O and Mor-NO<sub>2</sub> are about the same under conditions of high and low concentrations of CO<sub>2</sub>. These data indicate that both nitration and nitrosation by peroxynitrite are free radical processes. The morpholine radical (Mor<sup>•</sup>), formed from the reactions of carbonate and/or hydroxyl radicals with MorH, reacts with either •NO or •NO<sub>2</sub> and serves as a common precursor for Mor-N=O and Mor-NO<sub>2</sub>.

Peroxynitrite is a versatile oxidant<sup>1,2</sup> which is thought to mediate the toxic effects of nitric oxide (•NO).<sup>3</sup> Nitric oxide itself cannot cause either nitration or nitrosation,<sup>4</sup> and both of these reactions are thought to involve the intermediacy of peroxynitrite;<sup>3,5</sup> in fact, nitration is widely considered to be the footprint of peroxynitrite formation in vivo.<sup>6,7</sup> Nitration of tyrosine residues can alter phosphorylation/dephosphorylation of proteins, an important regulatory aspect of signal transduction.<sup>8</sup> Nitrosation of biological nucleophiles also can have pathophysiological consequences; e.g., nitrosation of purine and pyrimidine bases in DNA can produce deamination.<sup>9</sup> *S*-Nitrosothiols, which

are present in blood plasma in concentrations up to 0.5–1.3 μM,<sup>10a</sup> have long been known to have •NO-like activity.<sup>10b–c</sup>

Peroxynitrite and the products derived from its reaction<sup>11</sup> with CO<sub>2</sub> react with phenol to give substantial yields of 4-nitroso-phenol<sup>5c,12</sup> in addition to 2- and 4-nitrophenols and several other hydroxylated and quinone products.<sup>12,13</sup> Maximum yields of nitrosation are obtained at pH > 10,<sup>5c</sup> where little or no nitration of phenolic compounds generally is observed.<sup>13,14</sup> At first, the

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(1) The IUPAC-recommended name for peroxynitrite anion (ONOO<sup>-</sup>) is oxoperoxonitrate(1-), and hydrogen oxoperoxonitrate for peroxynitrous acid (ONOOH; pK<sub>a</sub> = 6.2–6.8). We use the term peroxynitrite to refer to the sum of ONOO<sup>-</sup> and ONOOH.

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(11) Carbonate is present in concentrations up to 25 mM in all physiological fluids, and about 7% of carbonate exists as free CO<sub>2</sub> at pH 7.2. Peroxynitrite anion reacts with CO<sub>2</sub> to form ONOOCO<sub>2</sub><sup>-</sup> (k<sub>2</sub> = 6 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup> at 37 °C). In the absence of peroxidases, virtually all of the peroxynitrite generated in biological environments will react with CO<sub>2</sub> before it can react with any other molecules. Therefore, it has become imperative to study the reactions of peroxynitrite with biomolecules in the presence of purposefully added carbonate; it also is wise to do experiments that do not have added carbonate but contain only adventitious carbonate. See: (a) Lyman, S. V.; Hurst, J. K. *J. Am. Chem. Soc.* **1995**, *117*, 8867. (b) Uppu, R. M.; Squadrito, G. L.; Pryor, W. A. *Arch. Biochem. Biophys.* **1996**, *327*, 335. (c) Denicola, A.; Freeman, B. A.; Trujillo, M.; Radi, R. *Arch. Biochem. Biophys.* **1996**, *333*, 49. (d) Floris, R.; Piersma, S. R.; Yang, G.; Jones, P.; Wever, R. *Eur. J. Biochem.* **1993**, *215*, 767. (e) Sies, H.; Sharov, V. S.; Klotz, L.-O.; Briviba, K. *J. Biol. Chem.* **1997**, *272*, 27812. (f) Lyman, S. V.; Hurst, J. K. *Chem. Res. Toxicol.* **1996**, *9*, 845.

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requirement for high pH was thought to arise from the limited availability of the phenolate ion at lower pH values (the  $pK_a$  of phenol is 9.9). However, a similar pH requirement was observed for nitrosation of aromatic primary amines<sup>15</sup> whose conjugate acids (protonated amines) have  $pK_a$  values of ca. 4.5.<sup>16</sup> The present study of the nitration and nitrosation reactions of peroxyntirite using morpholine (MorH) as a probe was undertaken since MorH gives fewer side reactions than do phenols and forms well-characterized nitration and nitrosation products. Our results suggest that both nitration and nitrosation are free radical processes requiring the simultaneous presence of a strong one-electron oxidant (such as  $HO^\bullet$  or  $CO_3^{\bullet-}$ ) and  $\bullet NO$  or  $\bullet NO_2$ .

## Experimental Section

**Chemicals and Reagents.** 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid, ammonium salt) (ABTS), diethylenetriaminepentaacetic acid (DTPA), MorH, and *N*-nitrosomorpholine (Mor-N=O) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade, and all solutions were prepared using deionized water of high resistivity ( $\geq 18 M\Omega cm^{-1}$ ). Peroxyntirite was synthesized by the ozonation of 0.1 M  $NaN_3$  (pH  $\sim 12$ ) as described earlier.<sup>17</sup> *N*-Nitrosomorpholine (Mor-NO<sub>2</sub>) was synthesized by the oxidation of Mor-N=O according to the method of Emmons<sup>18</sup> using 30% H<sub>2</sub>O<sub>2</sub> and trifluoroacetic acid. The product Mor-NO<sub>2</sub> was purified by reversed-phase (RP) HPLC (described below), and the GC/MS/EI analysis gave a molecular ion at  $m/z$  132.

**Reaction of Peroxyntirite with Morpholine.** Peroxyntirite<sup>19</sup> (2 mM) was allowed to react with 5 mM MorH in 2 mL of 0.1 M phosphate buffer (PB), pH 6.2–10.6, that also contained 0.1 mM DTPA and 0–50 mM added carbonate. The reactions were performed by adding aliquots (130–160  $\mu L$ ) of stock peroxyntirite solution (25–30 mM) to the MorH/PB solutions over a period of 10 s, with the contents being constantly stirred and the stirring continued for an additional 10 s. To minimize the adsorption of CO<sub>2</sub> from the air, the reaction mixtures were kept in sealed vials until further analysis (see below).

To study the effects of high and low concentrations of CO<sub>2</sub> on the peroxyntirite/MorH reaction, we used a pH-jump technique,<sup>5c,14c</sup> allowing rapid mixing of 0.1 M  $NaHCO_3/Na_2CO_3$  (preequilibrated at pH 7.0 or 10.0 in 0.2 M PB; also contained 10 mM MorH) with solutions of 4 mM peroxyntirite that also contained 0 or 0.14 N added NaOH. Addition of NaOH was necessary to change the pH of carbonate solutions from 7.0 to 10.2. In assays where carbonate was preequilibrated at pH 10.0, no NaOH was added, and yet there was a slight jump in the pH from 10.0 to 10.2 upon mixing of individual solutions. Mixing of the reactant solutions was performed using a vortex mixer, and the final concentrations of peroxyntirite, MorH, and carbonate were 2, 5, and 50 mM, respectively. In assays where the carbonate species were preequilibrated at pH 7.0 and 10.0, the initial concentrations of CO<sub>2</sub> after mixing of all reagents were 5 mM and ca. 1  $\mu M$ , respectively (Table 1).

**Reversed-Phase HPLC and GC/MS/EI Analysis.** Upon complete decay of peroxyntirite (assessed by the absence of ABTS oxidation<sup>5c</sup>), the reaction mixtures were filtered (pore size, 0.2  $\mu m$ ) and then analyzed

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(19) We have examined the yields of nitrosation of MorH at various low and high initial concentrations of peroxyntirite (0–2 mM) at pH 10.2. Nitrosation of MorH occurs at all concentrations of peroxyntirite, much in the same way as described for phenol<sup>5c</sup> and 1,2-phenylenediamine.<sup>15</sup> In these systems, the dependence of nitrosation on peroxyntirite concentration is somewhat curvilinear (for example, see Figure 3A in ref 5c), particularly in reactions performed without added carbonate but supposedly containing some adventitious carbonate.<sup>11b,13a</sup>

**Table 1.** Formation of Mor-N=O and Mor-NO<sub>2</sub> in the Reaction of 2 mM Peroxyntirite with 5 mM MorH in the Presence of High and Low Concentrations of CO<sub>2</sub> at pH 10.2

assay conditions	yields of Mor-N=O ( $\mu M$ )	yields of Mor-NO <sub>2</sub> ( $\mu M$ )	combined yields of Mor-N=O and Mor-NO <sub>2</sub> ( $\mu M$ )
high CO <sub>2</sub> (5 mM) <sup>a</sup>	27 $\pm$ 3 ( <i>n</i> = 7)	236 $\pm$ 12 ( <i>n</i> = 7)	263 $\pm$ 13 ( <i>n</i> = 7)
low CO <sub>2</sub> ( $\sim 1 \mu M$ ) <sup>b</sup>	195 $\pm$ 3 ( <i>n</i> = 4)	32 $\pm$ 6 ( <i>n</i> = 4)	227 $\pm$ 8 ( <i>n</i> = 4)

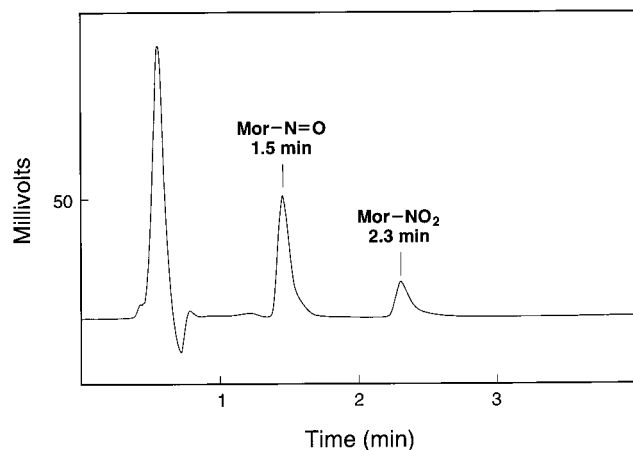
<sup>a</sup> Peroxyntirite (2 mM) was allowed to react with ca. 2.5-fold excess CO<sub>2</sub> (5 mM) at a final pH of 10.2. For this, a solution of 10 mM MorH plus 0.1 M carbonate was preequilibrated for 2 min at pH 7.0 in 0.2 M PB (*T* = 25 °C) and then mixed with an equal volume of 4 mM peroxyntirite in 0.14 N NaOH to reach the desired pH of 10.2. <sup>b</sup> The reaction was performed similarly to that described in footnote a but using solutions of 10 mM Mor plus 0.1 M carbonate (preequilibrated at pH 10.0 in 0.2 M PB, *T* = 25 °C) and 4 mM peroxyntirite (pH  $\sim 12$ , contains no added NaOH). The concentration of CO<sub>2</sub> in the final reaction mixture was ca. 1  $\mu M$  and the pH was 10.2. Other assay conditions were as described in the Experimental Section, and the products Mor-N=O and Mor-NO<sub>2</sub> were estimated by RP-HPLC. Values represent mean  $\pm$  SD of 4–7 determinations.

by RP-HPLC using a Perkin-Elmer series 410 liquid chromatograph (Perkin-Elmer, Norwalk, CT) provided with a Perkin-Elmer LC-95 UV/vis spectrophotometer and a Waters Spherisorb S3 ODS2 column (2.0  $\times$  100 mm; Waters Corp., Taunton, MA). The separation of products was achieved using an isocratic solvent system that consisted of 5% (v/v) acetonitrile in 27 mM acetate/30 mM citrate buffer (pH 3.2) at a flow of 0.5 mL/min (sample volume was 20  $\mu L$ , and the eluent was monitored at 254 nm). The products were identified by matching their retention times with those of authentic compounds, and Labcalc software (Galactic Industries, Salem, NH) was used to integrate the peak areas.

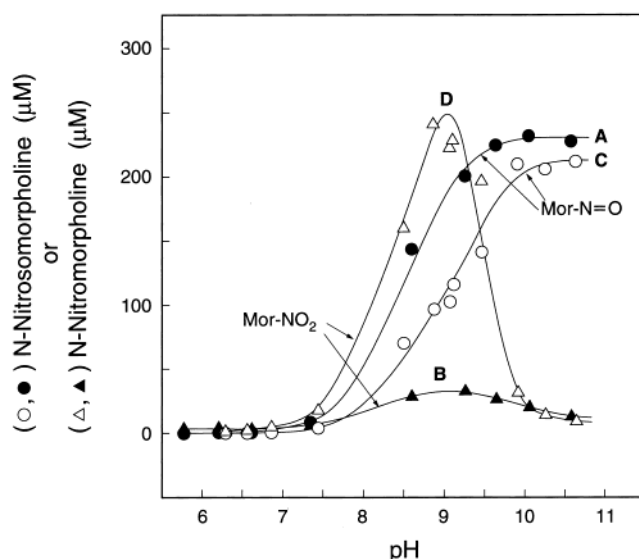
In some cases, the products of the peroxyntirite/MorH reaction, separated previously by RP-HPLC, were extracted into dichloromethane and then analyzed using a Hewlett-Packard 5890 gas chromatograph equipped with a mass-selective detector 5970 and a DB-5MS column (12.5 m length  $\times$  0.20 mm internal diameter  $\times$  0.33  $\mu m$  film thickness). Helium gas was used as the carrier at a flow of 0.85 mL/min. The conditions for chromatography were as follows: injection port, 250 °C; detector, 280 °C; oven, 40 °C for 3 min, 20 °C/min up to 280 °C, and 280 °C for 10 min; split, 25:1; and solvent delay, 3 min. Retention times of Mor-N=O and Mor-NO<sub>2</sub> were 8.4 and 9.2 min, respectively. The ion fragmentation patterns were compared with those of authentic Mor-N=O and Mor-NO<sub>2</sub>.

## Results

**Reaction of Peroxyntirite with Morpholine in the Absence of Purposefully Added Carbonate.** Figure 1 shows the RP-HPLC analysis of the products of peroxyntirite/MorH reaction at pH 7.4 in the absence of purposefully added carbonate. As can be seen, the reaction at this pH mainly produces two products. The first product elutes with a retention time (1.5 min) identical to that of Mor-N=O. The second product, which was formed in somewhat lower yield compared to that of Mor-N=O, has a retention time identical to that of Mor-NO<sub>2</sub> (2.3 min). The nitrated and nitrosated products from several RP-HPLC runs were extracted into dichloromethane, and the organic layer was subjected to GC/MS/EI analysis to conclusively establish their structures. We find that the product that elutes with a retention time of 1.5 min has fragmentation identical to that of Mor-N=O with ions at  $m/z$  116, 86, and 56, corresponding to the molecular ion ( $M^+$ ) (C<sub>4</sub>H<sub>8</sub>N<sub>2</sub>O<sub>2</sub><sup>+</sup>), and fragments with successive loss of  $\bullet NO$  (C<sub>4</sub>H<sub>8</sub>NO<sup>+</sup>) and HCHO (C<sub>3</sub>H<sub>6</sub>N<sup>+</sup>), respectively. The GC/MS/EI analysis of the product that elutes with a retention time of 2.3 min is the same as that of Mor-NO<sub>2</sub> with ions at  $m/z$  132, 86, and 56, corresponding to



**Figure 1.** RP-HPLC analysis of the products of peroxynitrite reaction with MorH at pH 7.4. The reactions were carried out in a final volume of 2 mL of 0.1 M PB that also contained 0.1 mM DTPA, 2 mM peroxynitrite, and 5 mM MorH. The reaction was initiated with the addition of peroxynitrite, and all other conditions were as described in the Experimental Section.



**Figure 2.** Yields of Mor-N=O and Mor-NO<sub>2</sub> in the peroxynitrite/MorH reaction in the presence and in the absence of purposefully added carbonate at pH 6.2–10.6. Peroxynitrite (2 mM) was allowed to react with 5 mM MorH in 2 mL of 0.1 M PB and 0 (●, ▲) or 20 mM added carbonate (○, Δ). The solutions also contained 0.1 mM DTPA. The reaction in all cases was initiated with the addition of peroxynitrite, and the products Mor-N=O (●, ○) and Mor-NO<sub>2</sub> (▲, Δ) were analyzed by RP-HPLC as described in the Experimental Section.

M<sup>+</sup> (C<sub>4</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub><sup>+</sup>), and fragments with successive loss of •NO<sub>2</sub> (C<sub>4</sub>H<sub>8</sub>NO<sup>+</sup>) and HCHO (C<sub>3</sub>H<sub>6</sub>N<sup>+</sup>).

The pH dependence of Mor-N=O formation in the peroxynitrite/MorH reaction is different from that of Mor-NO<sub>2</sub> (Figure 2). We detect Mor-N=O at pH values as low as 7.0, and the yields reach a maximum of about 12 mol % (relative to peroxynitrite) at pH ≥ 10 (curve A). The formation of Mor-N=O parallels the availability of unprotonated MorH<sup>20</sup> (MorH<sub>2</sub><sup>+</sup> ⇌ MorH + H<sup>+</sup>; pK<sub>a</sub> = 8.5). The formation of Mor-NO<sub>2</sub>, on the other hand, follows a bell-shaped profile with a maximum yield of about 1.6 mol % (relative to peroxynitrite) occurring at pH 9.0 (curve B).

Although the yields of both nitration and nitrosation products are small, they are significant and typical of peroxynitrite-mediated reactions that are zero-order in substrate and involve either the activated peroxynitrous acid (ONOOH\*)<sup>21–23</sup> or the

products derived from the reaction of peroxynitrite with adventitious and purposefully added carbonate.<sup>2,5b–d,12–15,24</sup> The yields of products in all these reactions range from 0.1 to 30 mol % relative to peroxynitrite. These low yields suggest that the reactive intermediates from peroxynitrite primarily decompose to the innocuous products NO<sub>3</sub><sup>−</sup> and NO<sub>2</sub><sup>−</sup> in competition with nitration, nitrosation, and other oxidation reactions.

**Catalytic Role of CO<sub>2</sub> in the Nitrosation of Morpholine by Peroxynitrite.** Although the reactions in Figure 2 (curves A and B) were performed in the absence of added carbonate, these reaction mixtures do contain adventitious carbonate<sup>11b</sup> (ca. 0.1 mM).<sup>13a</sup> Two possible sources for carbonate contamination are the stock solutions of peroxynitrite (maintained at pH ≥ 12 for reasons of stability) and the buffers used in these reactions. In the pH range of 6.2–10.6 used in these reactions (Figure 2, curves A and B), at equilibrium, concentration of CO<sub>2</sub> ranges from 50 μM to 4 nM. (The molar fractions of CO<sub>2</sub> relative to total carbonate are 0.5 and 4 × 10<sup>−6</sup> at pH 6.2 and 10.6, respectively.<sup>25</sup>) Carbon dioxide is a catalyst for the decomposition of peroxynitrite<sup>26</sup> and mostly is regenerated at the end of the catalytic cycle.<sup>27</sup> Therefore, these low concentrations of CO<sub>2</sub> may play a major role in nitration as well as nitrosation.

Indeed, low levels of CO<sub>2</sub> catalyze the nitrosation of MorH by peroxynitrite. As shown in Figure 3, the accumulation of Mor-N=O is much faster in reactions containing 50 mM added carbonate (curve A) than in those that contain small amounts of adventitious carbonate<sup>13a</sup> (ca. 0.1 mM) (curve B). Assuming a molar fraction of 2 × 10<sup>−5</sup> for CO<sub>2</sub> at pH 10.2,<sup>25</sup> the concentration of CO<sub>2</sub> in assays without any added carbonate and with 50 mM added carbonate can be calculated to be 0.002 and 1 μM, respectively.

**Modulation of Nitrosation and Nitration Potentials of Peroxynitrite by CO<sub>2</sub>.** To investigate the nature of reactive species in the peroxynitrite/CO<sub>2</sub> system that cause nitration and nitrosation, we designed pH-jump experiments along the lines suggested by Lyman et al.<sup>14c</sup> and Uppu et al.<sup>5c</sup> We chose two sets of experimental conditions. In the first set of conditions, to obtain high initial concentrations of CO<sub>2</sub> at alkaline pH, we used a rapid pH jump of 0.1 M carbonate from pH 7.0 to 10.2. Under these conditions, [CO<sub>2</sub>]<sub>0</sub> > [peroxynitrite]<sub>0</sub> (Table 1), and this allows a rapid and nearly total reaction of peroxynitrite with CO<sub>2</sub>, forming ONOOCO<sub>2</sub><sup>−</sup>. (The decay of CO<sub>2</sub> by competing reactions with water ( $k = 0.05 \text{ s}^{-1}$ ) and HO<sup>−</sup> ( $k = 8500 \text{ M}^{-1} \text{ s}^{-1}$ ) is a slow process at pH 10.2.<sup>25a</sup>) Under these conditions, we find significant formation of Mor-NO<sub>2</sub> with small yields of Mor-N=O (Table 1), suggesting that the products from homolysis of ONOOCO<sub>2</sub><sup>−</sup> (CO<sub>3</sub><sup>•−</sup> and •NO<sub>2</sub>) can cause nitration but not nitrosation. We find significant yields of Mor-N=O but not Mor-NO<sub>2</sub> in the second set of conditions in which peroxy-

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(22) For the sake of simplicity, we show HO• in Scheme 1. However, the mechanism does not necessarily depend on HO•; it only requires an activated species from ONOOH.

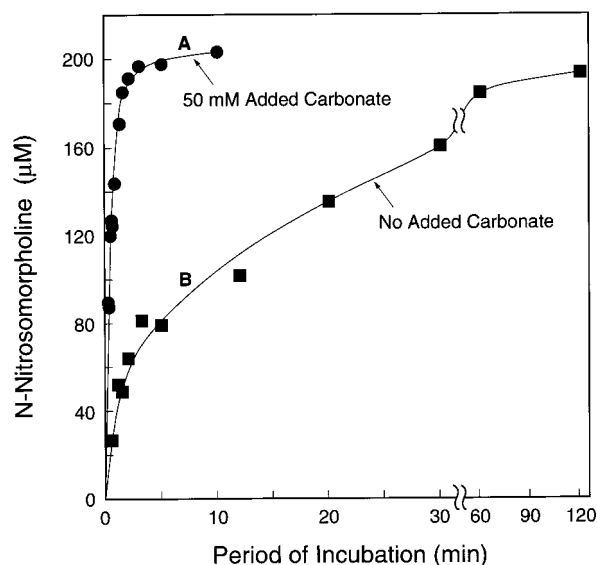
(23) There is recent evidence in favor of ONOOH homolysis, giving HO• and •NO<sub>2</sub> free radicals in yields up to 30 mol %: (a) Merényi, G.; Lind, J. *Chem. Res. Toxicol.* **1998**, *11*, 243. (b) Coddington, J. W.; Hurst, J. K.; Lyman, S. V. *J. Am. Chem. Soc.* **1999**, *121*, 2438. (c) Goldstein, S.; Meyerstein, D.; van Eldik, R.; Czapski, G. *J. Phys. Chem. A* **1999**, *103*, 6587.

(24) Moro, M. A.; Darley-Usmar, V. M.; Lizasoain, I.; Su, Y.; Knowles, R. G.; Radomski, M. W.; Moncada, S. *Br. J. Pharmacol.* **1995**, *116*, 1999.

(25) (a) Kern, D. M. *J. Chem. Educ.* **1960**, *37*, 14. (b) Alberty, R. A. *J. Phys. Chem.* **1995**, *99*, 11028.

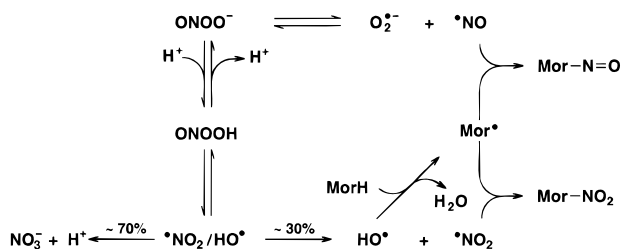
(26) Pryor, W. A.; Lemercier, J.-N.; Zhang, H.; Uppu, R. M.; Squadrito, G. L. *Free Radical Biol. Med.* **1997**, *23*, 331.

(27) Zhang, H.; Squadrito, G. L.; Pryor, W. A. *Nitric Oxide: Biol. Chem.* **1997**, *1*, 301.



**Figure 3.** Time course of the formation of Mor-N=O in the peroxyntirite/MorH reaction at pH 10.2 in the (A) presence and (B) absence of 50 mM purposefully added carbonate. Peroxyntirite (2 mM) was allowed to react with 5 mM MorH in 2 mL of 0.1 M PB containing 0.1 mM DTPA and 0 or 50 mM added carbonate. All reactions were initiated with the addition of peroxyntirite, and the product Mor-N=O was estimated at various intervals of time by RP-HPLC. Other assay conditions were as described in the Experimental Section.

**Scheme 1.** Postulated Reactions of Peroxyntirite with MorH Leading to the Formation of Mor-N=O and Mor-NO<sub>2</sub> in the Absence of Carbonate<sup>a</sup>



<sup>a</sup> Another possible source of  $\bullet\text{NO}$  involves the 1-e<sup>-</sup> oxidation of ONOO<sup>-</sup> by HO<sup>•</sup> (eq 3) followed by dissociation of the product ONOO<sup>•</sup> to  $\bullet\text{NO}$  plus O<sub>2</sub> (eq 4) (see text).

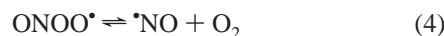
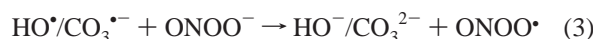
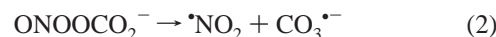
nitrite (2 mM) was allowed to react with MorH (5 mM) in the presence of low, catalytic concentrations of CO<sub>2</sub> (ca. 1  $\mu\text{M}$ ) at pH 10.2 (Table 1), similar to the nitrosation of phenol<sup>5c</sup> and 1,2-phenylenediamine<sup>15</sup> by peroxyntirite/CO<sub>2</sub>. Unlike the reactions with high [CO<sub>2</sub>]<sub>0</sub>, the reactions with low [CO<sub>2</sub>]<sub>0</sub> take several minutes to complete (see Figure 3), allowing for maximal interaction between the products of homolysis of ONOOCO<sub>2</sub><sup>-</sup> and ONOO<sup>-</sup>.

**Discussion**

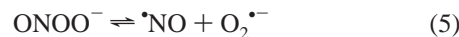
During the past decade, following seminal work of Beckman et al.,<sup>28</sup> numerous reports have been published on the oxidative and free radical reactions of peroxyntirite.<sup>2,3,5,11–15,21–24,26–29</sup> A vast majority of these reports center on the nitration of tyrosine

and model compounds (e.g., phenol), since nitration both marks the presence of peroxyntirite and is a new type of posttranslational modification that affects phosphorylation/dephosphorylation of tyrosine residues in proteins.<sup>8</sup>

In contrast to nitration, only a few examples of nitrosation by peroxyntirite have been reported,<sup>5b–d,12,15</sup> and these reactions occur in alkaline solutions where little or no nitration is observed.<sup>13,14</sup> We have postulated<sup>15</sup> that nitrosation is a free radical process requiring the simultaneous presence of a strong one-electron oxidant (such as HO<sup>•</sup> or CO<sub>3</sub><sup>•-</sup>) and  $\bullet\text{NO}$ . The higher yields of nitrosation under alkaline conditions are consistent with the formation of the free radicals HO<sup>•</sup> (eq 1)<sup>23</sup> and CO<sub>3</sub><sup>•-</sup> (eq 2)<sup>30</sup> and their secondary reactions with ONOO<sup>-</sup> (eq 3),<sup>5c,23,31,32</sup> giving ONOO<sup>•</sup> and its dissociation products  $\bullet\text{NO}$  and O<sub>2</sub> (eq 4).<sup>33</sup>



In alkaline solutions, in addition to the reactions of HO<sup>•</sup> and CO<sub>3</sub><sup>•-</sup> (eq 3), some  $\bullet\text{NO}$  can be formed from the dissociation of ONOO<sup>-</sup> (eq 5).<sup>23a,34</sup>



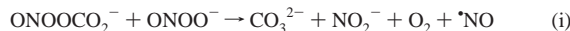
In model systems such as ours, HO<sup>•</sup> (eq 1) and  $\bullet\text{NO}$  (eqs 3 and 5) come from slow reactions of peroxyntirite<sup>11</sup> that would not be expected to occur in vivo. In contrast, in biological systems,  $\bullet\text{NO}$  is continuously produced by nitric oxide synthase, NOS,<sup>35</sup> and so the formation of  $\bullet\text{NO}$  from peroxyntirite (eqs 3–5) is not required. Nevertheless, in vitro systems such as ours have the advantage of providing a conceptual framework for understanding the mechanisms of nitration and nitrosation in  $\bullet\text{NO}$ -producing biological systems (see below).

**Possible Mechanisms: (A) At Very Low Concentrations of Carbonate.** Scheme 1 exemplifies the reactions that can

(30) (a) Goldstein, S.; Czapski, G. *J. Am. Chem. Soc.* **1998**, *120*, 3458. (b) Lymar, S. V.; Hurst, J. K. *Inorg. Chem.* **1998**, *37*, 294. (c) Bonini, M. G.; Radi, R.; Ferrer-Sueta, G.; Ferreira, A. M. D. C.; Augusto, O. *J. Biol. Chem.* **1999**, *274*, 10802

(31) (a) Pfeiffer, S.; Gorren, A. C. F.; Schmidt, K.; Werner, E. R.; Hansert, B.; Bohle, D. S.; Mayer, B. *J. Biol. Chem.* **1997**, *272*, 3465. (b) Goldstein, S.; Saha, A.; Lymar, A. V.; Czapski, G. *J. Am. Chem. Soc.* **1998**, *120*, 5549.

(32) A reviewer asked if ONOOCO<sub>2</sub><sup>-</sup> could react with ONOO<sup>-</sup>, forming  $\bullet\text{NO}$  and other products as shown in eq i



The adduct ONOOCO<sub>2</sub><sup>-</sup> has a very short half-life<sup>11a–c,14c</sup> and a similar adduct, RCH(O<sup>-</sup>)ONO, formed in reactions of ONOO<sup>-</sup> with aldehydes, does not have significant oxidative capability.<sup>29b</sup> Therefore, we believe that ONOOCO<sub>2</sub><sup>-</sup> does not oxidize ONOO<sup>-</sup>. Even if ONOOCO<sub>2</sub><sup>-</sup> would react with peroxyntirite directly, the reaction is indistinguishable from those of HO<sup>•</sup> and CO<sub>3</sub><sup>•-</sup> with ONOO<sup>-</sup> (eq 3).

(33) Frears, E.; Nazhat, N.; Blake, D.; Symons, M. *Free Radical Res.* **1997**, *27*, 31.

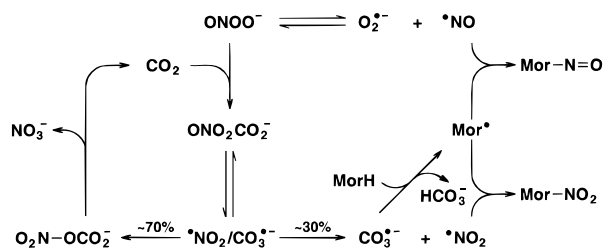
(34) The dissociation constant for ONOO<sup>-</sup> giving  $\bullet\text{NO}$  plus O<sub>2</sub><sup>•-</sup> is 0.017 s<sup>-1,23a</sup> whereas the rate constants for reactions of HO<sup>•</sup> and CO<sub>3</sub><sup>•-</sup> with ONOO<sup>-</sup> are (4–5) × 10<sup>9</sup> s<sup>-1,23b,c</sup> and 8 × 10<sup>7</sup> M<sup>-1</sup> s<sup>-1,23c</sup> respectively. Therefore, the relative yields of  $\bullet\text{NO}$  from reactions 3 and 4 depend on pH, carbonate and buffer concentrations, and impurities present in the system.

(35) (a) Vallance, P.; Patton, S.; Bhagat, K.; MacAllister, R.; Radomski, M.; Moncada, S.; Malinski, T. *Lancet* **1995**, *346*, 153. (b) Pinsky, D. J.; Patton, S.; Mesaros, S.; Brovkovich, V.; Kubaszewski, E.; Grunfeld, S.; Malinski, T. *Circ. Res.* **1997**, *81*, 372.

(28) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1620.

(29) (a) Vasquez-Vivar, J.; Santos, A. M.; Junqueira, V. B. C.; Augusto, O. *Biochem. J.* **1996**, *314*, 869. (b) Uppu, R. M.; Winston, W. W.; Pryor, W. A. *Chem. Res. Toxicol.* **1997**, *10*, 1331. (c) Kirsch, M.; Lomonosova, E. E.; Korsh, H.; Sustmann, R.; De Groot, H. *J. Biol. Chem.* **1998**, *273*, 12716. (d) Hodges, G. R.; Ingold, K. U. *J. Am. Chem. Soc.* **1999**, *121*, 10695. (e) Yang, D.; Tang, Y.-C.; Chen, J.; Wang, X.-C.; Barberger, M. D.; Houk, K. N.; Olson, L. *J. Am. Chem. Soc.* **1999**, *121*, 11976.

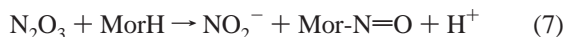
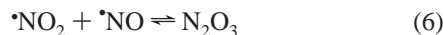
**Scheme 2.** Schematic Representation of the Formation of Mor-N=O and Mor-NO<sub>2</sub> in the Peroxynitrite/MorH Reaction in the Presence of Purposefully Added Carbonate<sup>a</sup>



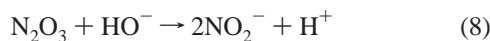
<sup>a</sup> Another possible source of \*NO involves the 1-e<sup>-</sup> oxidation of ONOO<sup>-</sup> by CO<sub>3</sub><sup>•-</sup> (eq 3) followed by the dissociation of the product ONOO<sup>•</sup> to \*NO plus O<sub>2</sub> (eq 4) (see text).

occur in the absence of purposefully added carbonate. Preparations of peroxynitrite contain ONOOH and ONOO<sup>-</sup> and low levels of their respective homolysis products \*NO<sub>2</sub> plus HO<sup>•</sup> (eq 1) and \*NO plus O<sub>2</sub><sup>•-</sup> (eq 5).<sup>23</sup> Considering the highly reactive and unselective nature<sup>36</sup> of HO<sup>•</sup> and the abundance of MorH (5 mM), the principal target for the reaction of HO<sup>•</sup> in our system is MorH. The radical Mor<sup>•</sup> then reacts with \*NO or \*NO<sub>2</sub>, forming Mor-N=O or Mor-NO<sub>2</sub> (Scheme 1).

The direct reaction of \*NO<sub>2</sub> with \*NO results in the formation of N<sub>2</sub>O<sub>3</sub> (eq 6),<sup>23,31a,37</sup> and this could account for some nitrosation of MorH by peroxynitrite (eq 7).



However, in systems such as ours which use alkaline buffers and high concentrations of carbonate and phosphate, the hydrolysis of N<sub>2</sub>O<sub>3</sub> (eq 8) is markedly accelerated.<sup>38</sup>



This raises doubts regarding the contribution of N<sub>2</sub>O<sub>3</sub> or other nitrosonium ion (NO<sup>+</sup>) donors<sup>5c</sup> (such as N<sub>2</sub>O<sub>4</sub> or a peroxy isomer of N<sub>2</sub>O<sub>5</sub>) to the overall yields of nitrosation of MorH by peroxynitrite under alkaline conditions.<sup>39</sup>

**(B) In the Presence of Purposefully Added Carbonate.** Scheme 2 shows the reactions that can occur in the peroxynitrite/

(36) Ross, A. B.; Mallard, W. G.; Helman, W. P.; Buxton, G. V.; Huie, R. E.; Neta, P. *NDRL-NIST Solution Kinetics Database*, Ver. 2.0; NIST: Gaithersburg, MD, 1994.

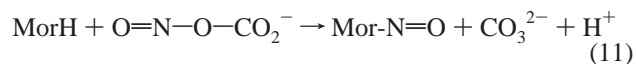
(37) Pfeiffer et al.<sup>31a</sup> postulated the formation of N<sub>2</sub>O<sub>3</sub> to explain the high yields of NO<sub>2</sub><sup>-</sup> and O<sub>2</sub> during the alkaline decomposition of peroxynitrite in the absence of added reactants, RH (in our system RH = MorH). The direct reaction of HO<sup>•</sup> with ONOO<sup>-</sup>, as postulated by Pfeiffer et al., is less likely in the presence of RH and other reactive impurities (such as buffers and NO<sub>2</sub><sup>-</sup>) because of competing reactions that allow for faster removal of HO<sup>•</sup> (Scheme 1). However, considering the recent evidence that \*NO<sub>2</sub> and \*NO can be formed (respectively) from the homolysis of ONOOH (eq 1) and ONOO<sup>-</sup> (eq 5)<sup>23</sup> and that the rates of R<sup>•</sup> reaction with \*NO and \*NO<sub>2</sub> are comparable to that of the \*NO/\*NO<sub>2</sub> reaction,<sup>36</sup> some N<sub>2</sub>O<sub>3</sub> may be formed (eq 6) in reactions of peroxynitrite in the absence of purposefully added carbonate.

(38) (a) Caulfield, J. L.; Singh, S. P.; Wishnok, J. S.; Deen, W. M.; Tannenbaum, S. R. *J. Biol. Chem.* **1996**, *271*, 25859. (b) Lewis, R. S.; Tannenbaum, S. R.; Deen, W. M. *J. Am. Chem. Soc.* **1995**, *117*, 3933.

(39) One of the reviewers suggested using N<sub>3</sub><sup>-</sup> to abate nitrosation by N<sub>2</sub>O<sub>3</sub> and related NO<sup>+</sup> donors. We studied the effect of N<sub>3</sub><sup>-</sup> (0–66 mM) on the yields of nitration and nitrosation in the peroxynitrite/CO<sub>2</sub>/MorH system at pH 10.2. Our results show that N<sub>3</sub><sup>-</sup> inhibits both nitration and nitrosation to a similar extent (0–70%), suggesting that N<sub>3</sub><sup>-</sup> acts as a general free-radical quencher rather than as a specific scavenger of NO<sup>+</sup>-carrier(s) in the peroxynitrite/CO<sub>2</sub>/MorH system. This is consistent with our hypothesis that peroxynitrite-mediated oxidations are predominantly free radical processes.

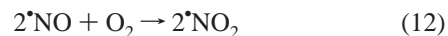
MorH system in the presence of purposefully added carbonate. We suggest that nitration and nitrosation occur as a result of the complex interplay of the free radical species CO<sub>3</sub><sup>•-</sup>, \*NO<sub>2</sub>, \*NO, and O<sub>2</sub><sup>•-</sup>, species which are formed in reactions 2–5.<sup>5c,23,30–34</sup> Nitration occurs when the radicals CO<sub>3</sub><sup>•-</sup> and \*NO<sub>2</sub> react with MorH. Since CO<sub>3</sub><sup>•-</sup> is a more potent oxidant [*E*<sup>o</sup>(CO<sub>3</sub><sup>•-</sup>/CO<sub>3</sub><sup>2-</sup>) = 1.5 V] than is \*NO<sub>2</sub> [*E*<sup>o</sup>(\*NO<sub>2</sub>/NO<sub>2</sub><sup>-</sup>) = 1.04 V],<sup>40</sup> nitration can involve an initial H-atom abstraction (or one-electron oxidation) from MorH by CO<sub>3</sub><sup>•-</sup>,<sup>41</sup> followed by the combination of Mor<sup>•</sup> with \*NO<sub>2</sub> (Scheme 2).<sup>42</sup> Nitrosation occurs from the trapping of Mor<sup>•</sup> by \*NO in competition with \*NO<sub>2</sub> (Scheme 2).

Another possible mechanism for nitrosation by peroxynitrite/CO<sub>2</sub> involves the reaction of CO<sub>3</sub><sup>•-</sup> with \*NO (eqs 9 and 10) and the subsequent NO<sup>+</sup> transfer<sup>5c</sup> from O=N–O–CO<sub>2</sub><sup>-</sup> to MorH (eq 11).



The reaction of \*NO with CO<sub>3</sub><sup>•-</sup> was studied in detail by Czapski et al.,<sup>43</sup> who find that the overall rate constant for reactions 9 and 10 is about 3.5 × 10<sup>9</sup> M<sup>-1</sup> s<sup>-1</sup>. This shows that the intermediate O=N–O–CO<sub>2</sub><sup>-</sup>, if formed, would be extremely short-lived and incapable of nitrosating MorH (or any substrate) at the concentrations used in our experiments.<sup>39</sup>

**Predictions Based on Free Radical Mechanisms and Their Experimental Validation.** At pH 10.2, the yields of nitration are about 10-fold higher in reactions performed using high initial concentrations of CO<sub>2</sub> than in reactions performed with very low concentrations of CO<sub>2</sub> (Table 1). The yields of nitrosation, on the other hand, are much smaller in reactions performed using high CO<sub>2</sub> than in reactions performed with low CO<sub>2</sub> (Table 1). In both conditions, the H<sup>+</sup>-catalyzed decomposition of peroxynitrite<sup>21</sup> (eq 1) and the autoxidation<sup>3</sup> of \*NO (eq 12) are extremely slow; therefore, peroxynitrite reacts primarily with CO<sub>2</sub> and decomposes via the intermediate formation of ONOOCO<sub>2</sub><sup>-</sup> (eq 2).



This means peroxynitrite gives comparable yields of CO<sub>3</sub><sup>•-</sup> under both high and low concentrations of CO<sub>2</sub>. If nitration and nitrosation of MorH by peroxynitrite/CO<sub>2</sub> proceed via a common radical intermediate Mor<sup>•</sup> (Scheme 2), the combined yields of nitration and nitrosation should be about the same under conditions of high and low concentrations of CO<sub>2</sub>, as is observed (see Table 1).

The free radical mechanism in Scheme 2 is consistent with the high yields of Mor-N=O and low yields of Mor-NO<sub>2</sub> that we observe under alkaline conditions at low concentrations of

(40) Stanbury, D. M. *Adv. Inorg. Chem.* **1989**, *33*, 69.

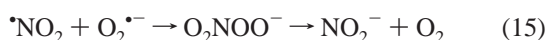
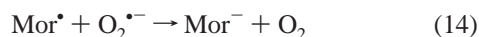
(41) The second-order rate constant for the reaction of CO<sub>3</sub><sup>•-</sup> with MorH is not known. However, based on the reaction of CO<sub>3</sub><sup>•-</sup> with piperidine, *N*-methylpiperidine, and 1,4-diazabicyclo[2, 2, 2]octane (Elango, T. P.; Ramakrishnan, V.; Vancheesan, S.; Kuricose, J. C. *Tetrahedron* **1985**, *41*, 3837), we presume that this rate could be in the order of (3–10) × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> under the conditions employed in our assays.

(42) There also might be minor products (arising from β-scission or other processes) from the reaction of Mor<sup>•</sup> with O<sub>2</sub> that we could not observe in our RP-HPLC analysis of the products.

(43) Czapski, G.; Holcman, J.; Bielski, B. H. J. *J. Am. Chem. Soc.* **1994**, *116*, 11465.

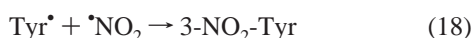
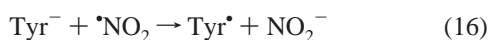
CO<sub>2</sub> (Table 1). As a first approximation, we calculate that the formation of •NO<sub>2</sub> at low concentrations of CO<sub>2</sub> is limited by the rate at which CO<sub>2</sub> is replenished from HCO<sub>3</sub><sup>-</sup> ( $k_{\text{HCO}_3^-} [\text{HCO}_3^-] = (2 \times 10^{-4} \text{ s}^{-1}) \times (0.016 \text{ M}) = 3.2 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$ ). In these reactions, •NO<sub>2</sub> and CO<sub>3</sub><sup>•-</sup> are formed predominantly from the homolysis of ONOOCO<sub>2</sub><sup>-</sup> (eq 2), and, therefore, the rate of •NO formation from the reaction of CO<sub>3</sub><sup>•-</sup> with ONOO<sup>-</sup> (eqs 3 and 4)<sup>23c,33</sup> is about the same as or somewhat smaller than the rate of •NO<sub>2</sub> formation. A major source of •NO under these alkaline conditions is the dissociation of ONOO<sup>-</sup> (eq 5); for example, at early stages of these reactions, the rate of •NO production from the spontaneous dissociation of 2 mM peroxyntirite ( $k_f[\text{ONOO}^-] = (0.017 \text{ s}^{-1}) \times (0.002 \text{ M}) = 3.4 \times 10^{-5} \text{ M s}^{-1}$ ) is about 10 times larger than the rate of •NO<sub>2</sub> formation. This explains why nitrosation but not nitration is favored under alkaline conditions at low concentrations of CO<sub>2</sub> (Table 1).<sup>44</sup>

The pH profiles for nitration of MorH by peroxyntirite are bell-shaped both in the presence and in the absence of purposefully added carbonate (Figure 2, curves B and D). This pattern also is typical in several other reactions of peroxyntirite forming stable nitration products.<sup>12–14</sup> According to these free radical mechanisms (Schemes 1 and 2), the lower yields of nitration, in part, can be explained as due to scavenging of key intermediates (in the present case, Mor• and •NO<sub>2</sub>; eqs 13–15) by •NO and O<sub>2</sub><sup>•-</sup> formed from the homolysis of ONOO<sup>-</sup> (eq 5) and/or the reaction of CO<sub>3</sub><sup>•-</sup> with ONOO<sup>-</sup> (eqs 3 and 4).



In fact, with increases in pH, both the homolysis of HOONO (eq 1) and the CO<sub>2</sub>-catalyzed decomposition of peroxyntirite (eq 2), which are the major sources of HO•, CO<sub>3</sub><sup>•-</sup>, and •NO<sub>2</sub>, become rate limiting, while the homolysis of ONOO<sup>-</sup>, giving •NO plus O<sub>2</sub><sup>•-</sup> (eq 5), and the reaction of CO<sub>3</sub><sup>•-</sup> with ONOO<sup>-</sup> (eq 3) become more favorable.<sup>23,34</sup> This, together with the fact that both •NO and •NO<sub>2</sub> react with R• at about the same rates,<sup>36</sup> explains why the yields of nitration but not nitrosation decrease at alkaline pH, giving a bell-shaped profile (Figure 2, curves B and D).

At high initial concentrations of CO<sub>2</sub>, in contrast to MorH (Table 1), phenol<sup>5c</sup> and tyrosine (TyrH)<sup>14c</sup> give lower yields of nitration at pH > 10. These reduced yields probably result from scavenging of •NO<sub>2</sub> by the phenolate ion (eq 16), resulting in poor coupling of the intermediate phenoxy<sup>5c</sup> or tyrosinyl<sup>14c</sup> radicals (eq 17) with •NO<sub>2</sub> (eq 18). For example, a change in pH from 7.5 to 11.3 increases the rate of •NO<sub>2</sub>/tyrosine reaction by about 100 times<sup>45</sup> without causing a major change in the reactivity of CO<sub>3</sub><sup>•-</sup> toward the phenol substrate.<sup>46</sup> (The pK<sub>a</sub> values of the hydroxyl in phenol and tyrosine are 9.9 and 10.1, respectively.)



Nitrogen dioxide does not react with either MorH<sub>2</sub><sup>+</sup> or MorH,<sup>47</sup> and this explains why the yields of Mor-NO<sub>2</sub> remain high under alkaline conditions at high initial concentrations of CO<sub>2</sub> (Table 1).

**General Conclusions and Implications.** Peroxyntirite and the products derived from its reaction with CO<sub>2</sub> can nitrate as well as nitrosate MorH over a wide range of pH in vitro. Both nitration and nitrosation can be explained by free radical pathways involving an initial H-atom abstraction (or one-electron oxidation) from MorH by HO• or CO<sub>3</sub><sup>•-</sup>. Depending on the relative availability of O<sub>2</sub><sup>•-</sup> (eq 5), •NO (eqs 4 and 5) and •NO<sub>2</sub> (eqs 1 and 2), the Mor• produced in these reactions can react further to give regenerated MorH (eq 14), Mor-N=O (eq 13), or Mor-NO<sub>2</sub> (Schemes 1 and 2). In addition, some MorH may be nitrosated by N<sub>2</sub>O<sub>3</sub> (eq 7) formed from the reaction of •NO<sub>2</sub> with •NO (eq 6).

These in vitro free radical mechanisms for peroxyntirite suggest how nitration and nitrosation may occur in vivo. In biological systems, •NO produced from NOS is down-regulated by the reactions of O<sub>2</sub><sup>•-</sup> and oxygenated heme proteins, giving peroxyntirite.<sup>28,48</sup> Peroxyntirite then isomerizes to NO<sub>3</sub><sup>-</sup> (~70%) via the reaction<sup>11a–c</sup> with CO<sub>2</sub> with small but significant yields of free radicals CO<sub>3</sub><sup>•-</sup> and •NO<sub>2</sub> (~30%) from the peroxyntirite/CO<sub>2</sub> reaction.<sup>2c,27,30</sup> (Since autoxidation of •NO to •NO<sub>2</sub> is slow in biological systems,<sup>3</sup> the peroxyntirite/CO<sub>2</sub> reaction could be a source of •NO<sub>2</sub> in vivo.) Therefore, in vivo, the free radicals CO<sub>3</sub><sup>•-</sup> and •NO<sub>2</sub> in the presence of •NO (from NOS) mediate nitration and nitrosation.

Nitrogen dioxide is a less potent oxidant than CO<sub>3</sub><sup>•-</sup>,<sup>40</sup> and most of the in vitro studies of peroxyntirite/CO<sub>2</sub> are performed using substrates (e.g., phenol and tyrosine) that CO<sub>3</sub><sup>•-</sup> and •NO<sub>2</sub> react with at different rates.<sup>36</sup> Generally, at pH 7.0–7.5, CO<sub>3</sub><sup>•-</sup> reacts with RH at rates that are 10<sup>2</sup>–10<sup>3</sup> times higher than those of RH with •NO<sub>2</sub>;<sup>36</sup> however, the product R• formed in either of these reactions reacts with •NO<sub>2</sub> at rates close to the diffusion limit.<sup>45,49</sup> Therefore, in vitro reactions by peroxyntirite/CO<sub>2</sub> at and around the neutral pH give mainly nitration products via the one-electron oxidation of RH by CO<sub>3</sub><sup>•-</sup> followed by the reaction of R• with •NO<sub>2</sub>. However, in biological systems, where there is a continuous formation<sup>35</sup> of •NO from NOS, •NO and •NO<sub>2</sub> compete for reaction with R•, which can arise from autoxidation of RH as well as from the reaction of RH with CO<sub>3</sub><sup>•-</sup>. In vivo, in contrast with our in vitro system, sequential reactions of CO<sub>3</sub><sup>•-</sup> and •NO<sub>2</sub> are not necessary to form nitration products. In vivo, the yields of nitration and nitrosation products critically depend on the relative concentrations of •NO<sub>2</sub> and •NO.

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(44) A reviewer suggested the addition of •NO to alkaline peroxyntirite solutions to test if nitrosation is enhanced. We are not sure to what extent an experiment of this kind can lend support to our hypothesis that a mixture of •NO and ONOO<sup>-</sup> (or, more correctly, •NO plus CO<sub>3</sub><sup>•-</sup>) causes nitrosation in vivo. •NO autoxidation generates nitrosating intermediates (like N<sub>2</sub>O<sub>3</sub>), and these can react directly with ONOO<sup>-</sup> (Goldstein, G.; Czapski, G.; Lind, J.; Merényi, G. *Chem. Res. Toxicol.* **1999**, *12*, 132). Thus, by adding •NO, one would introduce a peroxyntirite-independent pathway for nitrosation as well as potentially interfering cross reactions. The system the reviewer proposes is even more complex than our system, and finding increased nitrosation, as one would expect, could not be easily interpreted.

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