Peroxynitrite Mediated Oxidations: Spin Trap Studies; Nitration and Hydroxylation of Phenol Result From Different Intermediates; CO(2) Catalyzes the Decomposition of Peroxynitrite.

Jean-noel Lemercier
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PEROXYNITRITE MEDIATED OXIDATIONS: SPIN TRAP STUDIES; NITRATION AND HYDROXYLATION OF PHENOL RESULT FROM DIFFERENT INTERMEDIATES; CO$_2$ CATALYZES THE DECOMPOSITION OF PEROXYNITRITE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Chemistry

by

Jean-Noël Lemercier
B.S., Université d'Angers, 1990
December, 1997
To My Parents,

My Brothers and Sister,

To Vinita.
ACKNOWLEDGMENTS

I would like to thank my advisor and mentor, Professor William A. Pryor for his support and guidance throughout my graduate career.

Thanks are also due to the members of my committee, Drs. Cartledge, Fischer, Kestner, Larkin, and Winston for their scientific insights, concern, and help.

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FOREWORD

This dissertation is composed of three chapters that investigate the mechanisms of reaction of peroxynitrite, the mediator of nitric oxide toxicity in vivo.

The first chapter, “SPIN TRAP STUDIES ON THE DECOMPOSITION OF PEROXYNITRITE” was authored by Jean-Noël Lemercier, Giuseppe L. Squadrito, and William A. Pryor and was published in Archives of Biochemistry and Biophysics in August 1995. This chapter reports on the elucidation of the mechanism of decomposition of peroxynitrite using ESR spectroscopy, and more precisely spin-trapping.

The second chapter, “CARBON DIOXIDE MODULATION OF HYDROXYLATION AND NITRATION OF PHENOL BY PEROXYNITRITE”, authored by Jean-Noël Lemercier, Sarojini Padmaja, Rafael Cueto, Giuseppe L. Squadrito, Rao M. Uppu, and William A. Pryor, has been submitted for publication to Archives of Biochemistry and Biophysics in March 1997. This chapter investigates the mechanisms of nitration and hydroxylation of phenol mediated by peroxynitrite.

Lastly, the third chapter, “The catalytic role of carbon dioxide in the decomposition of peroxynitrite” was authored by Jean-Noël Lemercier, Houwen Zhang, Giuseppe L. Squadrito, Rao M. Uppu, and
William A. Pryor. The manuscript has been submitted for publication in *Free Radical Biology and Medicine* in April 1997. This chapter reports on the regeneration of CO₂ in the CO₂-catalyzed decomposition of peroxynitrite.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDRF</td>
<td>endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>O$_2$</td>
<td>molecular oxygen</td>
</tr>
<tr>
<td>RONO</td>
<td>alkyl nitrite</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>nitrite</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>nitrate</td>
</tr>
<tr>
<td>N$_3^-$</td>
<td>azide</td>
</tr>
<tr>
<td>SIN-1</td>
<td>3-morpholinosydnonimine</td>
</tr>
<tr>
<td>DMPO</td>
<td>5,5-dimethyl-1-pyrroline-$N$-oxide</td>
</tr>
<tr>
<td>DMPOx</td>
<td>5,5-dimethyl-1-pyrrolidin-2-one-1-oxy</td>
</tr>
<tr>
<td>*DMPO-OH</td>
<td>DMPO-hydroxyl radical spin adduct</td>
</tr>
<tr>
<td>*DMPO-OOH</td>
<td>DMPO-superoxide spin adduct</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>hfsc</td>
<td>hyperfine splitting constant</td>
</tr>
<tr>
<td>MAH</td>
<td>molecule-assisted homolysis</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>dtpa</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Chemical Symbol</td>
<td>Chemical Name</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>H₂CO₃</td>
<td>carbonic acid</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>bicarbonate</td>
</tr>
<tr>
<td>CO₃²⁻</td>
<td>carbonate</td>
</tr>
<tr>
<td>CO₃⁻</td>
<td>carbonate radical</td>
</tr>
<tr>
<td>HOONO</td>
<td>peroxynitrous acid</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>peroxynitrite anion</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>NO₂⁺</td>
<td>nitronium ion</td>
</tr>
<tr>
<td>NO₂</td>
<td>nitrogen dioxide</td>
</tr>
<tr>
<td>HOONO⁺</td>
<td>activated intermediate of HOONO</td>
</tr>
<tr>
<td>O=N-OOCO₂⁻</td>
<td>nitrosperoxycarbonate anion</td>
</tr>
<tr>
<td>O₂N-OCO₂⁻</td>
<td>nitrocarbonate anion</td>
</tr>
</tbody>
</table>
ABSTRACT

In the first project the spin trap DMPO was used to probe for the production of hydroxyl radicals from peroxynitrite. Peroxynitrite reacts with DMPO to give \( \cdot \text{DMPO-OH} \), suggesting that \( \cdot \text{DMPO-OH} \) results from a reaction between DMPO and \( \text{HOONO} \) or between DMPO and an activated intermediate of \( \text{HOONO} \). Thiols produce a large increase in the \( \cdot \text{DMPO-OH} \) signal intensity. Experiments employing SOD suggest that this increase results from the decomposition of \( \cdot \text{DMPO-OOH} \). These results are incompatible with the decomposition of peroxynitrite to form \( \cdot \text{OH} \) but suggest that an intermediate of \( \text{HOONO} \) is produced that is less reactive and more selective than the hydroxyl radical.

In the second project, we have examined the formation of hydroxyphenols, nitrophenols, and the minor products 4-nitrosophenol, benzoquinone, 2,2'-biphenol, and 4,4'-biphenol from the reaction of peroxynitrite with phenol with and without added carbonate. Without added carbonate, both the yields and rates of formation of nitrophenols and hydroxyphenols have different pH profiles. The sum of the rate constants for nitration and hydroxylation is identical to the rate constant for the spontaneous decomposition of peroxynitrite. When carbonate is added, hydroxylation is inhibited, whereas the rates of formation and
yields of nitrophenols increase. The maximum yield of nitrophenols is 20 mol%, about 4-fold higher than without added carbonate. The \(a/p\) ratio of nitrophenols is independent of carbonate. These results suggest that hydroxylation and nitration occur via two different intermediates. We suggest that ONOOH\(^*\) is the hydroxylating species, and that \(O = N - OO - CO_2\), the product of the peroxynitrite/CO\(_2\) reaction, or secondary products derived from it, is the nitrating agent.

The third project is a study of the reaction of peroxynitrite with limiting concentrations of CO\(_2\). Peroxynitrite adds to CO\(_2\) to give \(O = N - OO - CO_2\) (1). The reformation of CO\(_2\) (not CO\(_3^+\) or HCO\(_3^-\)) from \(O = N - OO - CO_2\) is demonstrated. When the concentration of CO\(_2\) is limiting, the reformation of CO\(_2\) amplifies the fraction of peroxynitrite that reacts with CO\(_2\). Even low CO\(_2\) concentrations dissolved from ambient air can cause deviations from predicted kinetic behavior. The reactions attributed to peroxynitrite depend on the availability of CO\(_2\), since \(O = N - OO - CO_2\), and intermediates derived from it, oxidize and nitrate.
INTRODUCTION

Nitric Oxide

Early studies suggested that nitric oxide (*NO), a simple hydrophobic gaseous molecule, was noxious. In fact, *NO was known as an environmental toxin present in smog and cigarette smoke, and a suspected carcinogen. Surprisingly, in 1987, *NO was identified as the endothelium-derived relaxing factor (EDRF), an endogenous vasodilator mimicked by nitroglycerin and nitroprusside (1-5). Nitric oxide was subsequently shown to be an ubiquitous intercellular messenger in all vertebrates and also to be produced by the cells of the immune and nervous systems. Many biological processes including respiration, digestion, platelet aggregation, blood flow modulation, muscle tone, neurotransmission, phagocyte activity, and neuronal synaptic plasticity (6-11) involve nitric oxide. The biosynthesis of *NO involves the oxidation of L-arginine at the guanidino nitrogen by the enzyme nitric oxide synthase, yielding citrulline and *NO (12) (Fig. I). Nitric oxide undergoes three types of reactions (13): reaction with heme compounds [e.g., activation of guanylate cyclase which is responsible for signal transduction and reaction with oxyhemoglobin which yields nitrate (13-15)]; reaction with iron sulfur proteins; and reaction with reactive oxygen species such as molecular oxygen or superoxide.
Figure 1. Biosynthesis of Nitric Oxide

\[
\begin{align*}
\text{L-arginine} & \xrightarrow{\text{NADPH}} \text{NG-hydroxy-L-arginine} \\
\text{NG-hydroxy-L-arginine} & \xrightarrow{1/2 \text{NADPH}} \text{O}_2 \rightarrow \cdot \text{NO} \\
\cdot \text{NO} & \xrightarrow{\text{O}_2} \text{L-citrulline} \\
\text{L-citrulline} & \xrightarrow{\text{O}_2} \text{Nitric Oxide}
\end{align*}
\]
Nitric Oxide Toxicity. Nitric oxide has been linked to oxidative stress related pathological conditions such as multiple sclerosis (16), amyotrophic lateral sclerosis (17), acute lung injury (18), endotoxemia (19), ischemia-reperfusion injury (20-23), rheumatoid arthritis (24), and chronic inflammation (24,25). However, 'NO itself may not be directly involved in those conditions since it is a weak oxidant (26). The oxidative reactions of NO have traditionally been attributed to NOx species formed from its reaction with oxygen. However, the concentrations in vivo of nitric oxide (5-4000 nM) and oxygen (20-200 μM) are generally too low for their termolecular reaction to occur. Biological membranes may be an exception since both nitric oxide and oxygen are about 10 times more soluble in lipid media than in aqueous media, and the reaction of 'NO with O2 may be more likely.

In 1968, McCord and Fridovich showed that superoxide (O2−) is produced by virtually all aerobically metabolizing cells (27). Beckman et al. (28) have postulated that peroxynitrite, the product of the reaction of 'NO with superoxide, is the mediator of the toxicity of nitric oxide in vivo (Equation [I]).

\[ \text{Equation [I]} \]

\[ \text{O}_2^+ + \text{'NO} \rightarrow \text{O} = \text{N-O-O}^- \]
The formation of peroxynitrite is likely in vivo. A variety of cells such as neutrophils, macrophages, leukocytes, nerve cells, brain cells, and endothelial cells are able to produce nitric oxide and superoxide simultaneously (28-37). The rate of reaction between \( \cdot \)NO and superoxide is near diffusion controlled (38-40); therefore the formation of peroxynitrite can compete with the scavenging of superoxide by superoxide dismutase and the reaction of \( \cdot \)NO with heme compounds when sufficient levels of both nitric oxide and superoxide are present (41). Typical concentrations of nitric oxide produced for signal transduction (10 to 400 nM) are much lower than the concentration of SOD. (The intracellular concentration of superoxide dismutase is 4-10 \( \mu \)M in the brain and the liver.) However, concentrations of \( \cdot \)NO high enough for formation of peroxynitrite may be attained near the sites of synthesis of both \( \cdot \)NO and \( \cdot \)O\(_2\). For instance, Malinski et al. (42) have estimated the concentration of nitric oxide during cerebral ischemia to be 2-4 \( \mu \)M.

A number of experimental observations show that the formation of peroxynitrite from the reaction of \( \cdot \)NO with superoxide in vivo is probable (43-48). Superoxide scavengers such as superoxide dismutase and thiols have been shown to enhance the biological activity of nitric oxide (29,49,50). On the other hand, superoxide generating compounds inactivate nitric oxide by diverting nitric oxide to peroxynitrite (2,51,52).
Also, by reacting with superoxide, ^\textsuperscript{\cdot}\textit{NO} can even act as an antioxidant (34,53,54). Human, pig and \textit{E. coli} aconitases are inactivated by peroxynitrite but not by nitric oxide in the absence of superoxide (55,56). Nitrotyrosine residues were detected in atherosclerotic lesions of human coronary arteries (57), in lung sections of patients with acute lung injury (58), and in the synovial fluids from rheumatoid patients (24). The nitration of phenolic residues is generally considered to be the hallmark of peroxynitrite formation \textit{in vivo}, since unlike ^\textsuperscript{\cdot}\textit{NO} and ^\textsuperscript{\cdot}\textit{NO}_2^-, peroxynitrite is a nitrating agent at physiological concentrations (59,60).

**Reactions of Peroxynitrite.** The diffusion-controlled reaction between the two free radicals ^\textsuperscript{\cdot}\textit{NO} and \textit{O}_2^- results in the release of 22 kcal/mol and is irreversible (38-40,61). The chemistry of peroxynitrite as an inorganic acid has been reported previously (62-68). Peroxynitrite is a weak acid, with a pK\textsubscript{a} = 6.8 (69,70). It is stable in alkaline solution, but its conjugate acid, peroxynitrous acid decomposes to form nitrate in a reaction with first order kinetics and an Arrhenius activation energy of 17.3 Kcal/mol (Equation [II]) (61).

\[
\text{ONOO}^- + \text{H}^+ \rightleftharpoons \text{ONOOH} \rightarrow \text{NO}_3^- + \text{H}^+ \quad \text{[II]}
\]
In addition to nitrating tyrosine, peroxynitrite has been shown to react with a variety of biological compounds, including thioethers (70,71), thiols (61,72), lipids (73,74), ascorbic acid (75,76), deoxyribose (28), and myeloperoxidase (77) (Table I). It has also been shown to trigger apoptosis (78,79) and to react with DNA (80,81). Early studies proposed that peroxynitrite homolyzed to nitrogen dioxide and the hydroxyl radical (28), which was suggested to be responsible for peroxynitrite-mediated biological oxidative damage (28,63,82,83). However, Moreno et al. (71) showed that hydroxyl radical scavengers do not quench the oxidative reactions of peroxynitrite. Subsequently, Koppenol et al. (61), used thermokinetic data to argue against homolysis and proposed that an activated form of peroxynitrous acid, HOONO*, formed on the pathway leading from peroxynitrous acid to nitrate, is the oxidant. Spin trap studies, including ours (see Chapter 1), have shown that the hydroxyl radical is not formed as a major product from homolytic fission of peroxynitrous acid upon decomposition (84-87).

Recently, peroxynitrite was shown to react with CO₂ (88,89) (Equation [III]).

\[
\text{ONOO}^- + \text{CO}_2 \rightarrow \text{O=N-O-O-CO}_2^- \\
\text{[III]}
\]
Table I. Reaction of Peroxynitrite with Plasma Constituents at pH 7.4.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration C (M)</th>
<th>$k_{app}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{app} \times C$ (s$^{-1}$)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.3 (69)</td>
<td></td>
<td>0.3</td>
<td>(69)</td>
</tr>
<tr>
<td>Cysteine</td>
<td>$4 \times 10^{-5}$</td>
<td>$5 \times 10^3$</td>
<td>0.2</td>
<td>(72)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>$1 \times 10^{-4}$</td>
<td>$2 \times 10^3$</td>
<td>0.2</td>
<td>(61,90)</td>
</tr>
<tr>
<td>Methionine</td>
<td>$6 \times 10^{-8}$</td>
<td>250</td>
<td>$1.5 \times 10^{-3}$</td>
<td>(70)</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>$1 \times 10^{-4}$</td>
<td>40</td>
<td>$4 \times 10^{-3}$</td>
<td>(75,76,90)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>$1 \times 10^{-5}$</td>
<td>50</td>
<td>$5 \times 10^{-4}$</td>
<td>(91)</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>$2.5 \times 10^{-2}$</td>
<td>$2 \times 10^3$</td>
<td>50</td>
<td>(88)</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>$1 \times 10^{-4}$</td>
<td>$3 \times 10^6$</td>
<td>300</td>
<td>(77)</td>
</tr>
</tbody>
</table>

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Carbon dioxide is ubiquitous in vivo and its physiological concentration ranges from 1 to 2 mM. A comparison of the rate constant for the reaction of peroxynitrite with CO$_2$ ($3 \times 10^4$ M$^{-1}$s$^{-1}$) (88), and the rate of isomerization of peroxynitrous acid (1.38 s$^{-1}$) (69) clearly shows that the reaction shown in Equation [III] will predominate over that shown in Equation [II] in most biological fluids (Table I). Myeloperoxidase, found in leukocyte phagosomes, is the only biomolecule that reacts sufficiently fast to compete with CO$_2$ for peroxynitrite (92) (Table I). Consequently, oxidative injury involving peroxynitrite is expected to occur primarily via the intermediacy of $\cdot$ONOOCO$_2$. Therefore, the role of peroxynitrite in the toxicity of $\cdot$NO needs to be reconsidered in terms of the reactivity of the adduct formed in the reaction between peroxynitrite and CO$_2$. Carbon dioxide has been shown to inhibit the toxicity of peroxynitrite to *Escherichia Coli* (92,93), to inhibit the oxidation of thiols and to enhance peroxynitrite mediated nitration (94,95). The nitration of tyrosine residues remains therefore, a good marker for the toxicity of $\cdot$NO in vivo.

**Preparations of Peroxynitrite.** Experimentally, peroxynitrite can be introduced in a reaction system in two ways. Aliquots from a stock solution may be added to a vigorously stirred solution in bolus amounts, or peroxynitrite can be generated continuously in order to mimic physiological conditions. Stock solutions of peroxynitrite can be prepared
Table II. Syntheses of Peroxynitrite.\(^a\)

<table>
<thead>
<tr>
<th>Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preparative Syntheses</strong></td>
<td></td>
</tr>
<tr>
<td>Reaction of Ozone with Azide</td>
<td>(96, 97)</td>
</tr>
<tr>
<td>Autooxidation of Hydroxylamine</td>
<td>(98-100)</td>
</tr>
<tr>
<td>Reaction of H(_2)O(_2) with HNO(_2)</td>
<td>(64,100-102)</td>
</tr>
<tr>
<td>Reaction of H(_2)O(_2) with Alkyl Nitrite</td>
<td>(103-105)</td>
</tr>
<tr>
<td>Reaction of (^\circ\text{NO}) with H(_2)O(_2)</td>
<td>(106)</td>
</tr>
<tr>
<td>Reaction of (^\circ\text{NO}) with KO(_2)</td>
<td>(100)</td>
</tr>
<tr>
<td>Reaction of (^\circ\text{NO}) with Me(_2)N(^+)O(_2)(^-)</td>
<td>(107)</td>
</tr>
<tr>
<td>Photolysis of KNO(_3)</td>
<td>(67,108)</td>
</tr>
<tr>
<td><strong>In Situ Syntheses</strong></td>
<td></td>
</tr>
<tr>
<td>(^\circ\text{NO}) and O(_2)(^-) Generated Independently (E.g., from S-Nitrosoglutathione and Xanthine/Xanthine oxidase)</td>
<td>(54,109,110)</td>
</tr>
<tr>
<td>(^\circ\text{NO}) and O(_2)(^-) Generated Simultaneously (E.g., from autoxidation of SIN-1)</td>
<td>(111-114)</td>
</tr>
</tbody>
</table>

\(^a\) From reference (115).
using one of the many published syntheses (Table II). Each of these preparations contains contaminants. For example, nitrite and nitrate are almost invariably present. Hydrogen peroxide, H$_2$O$_2$, may be present in high concentrations, specially in preparations involving the reactions of H$_2$O$_2$ with NO$_2$, H$_2$O$_2$ with $^\cdot$NO, and H$_2$O$_2$ with RONO. Peroxynitrite preparations involving the ozonation of azide, N$_3^-$, will contain sodium azide as it is a starting material. The level of azide impurity may be diminished by extending the ozonation after the maximum yield of peroxynitrite has been attained. Peroxynitrite can also be generated continuously in lower amounts and in situ over an extended period of time. One can either use compounds that can generate nitric oxide and superoxide simultaneously (such as 3-morpholinosydnonimine, HCl, SIN-1), or use two separate generating systems for the production of nitric oxide and superoxide. Nitric oxide may be produced from one of the many nitric oxide donors available, and superoxide may be generated enzymatically by a xanthine/xanthine oxidase system for example.

**Purpose of this Investigation.** The role and mechanisms of action of peroxynitrite as the mediator of the toxicity of nitric oxide *in vivo* have been under scrutiny for a few years in Dr. W.A. Pryor's laboratory. Two mechanisms had been proposed to explain the peroxynitrite mediated reactions. The earlier mechanism suggested that peroxynitrite homolyzed
to form the hydroxyl radical (\(\cdot \text{OH}\)) which was thought to be responsible for peroxynitrite oxidations (Equation [IV]).

\[
\text{HOONO} \rightarrow \text{HO}^* + \cdot \text{NO}_2 \quad \text{[IV]}
\]

However, since scavengers of \(\cdot \text{OH}\) could not completely quench the formation of oxidized products (71), a second mechanism was proposed, postulating the formation of an activated intermediate. We have studied the mechanism of decomposition of peroxynitrite using spin trapping in order to confirm the hypothesis that peroxynitrite does not homolyze to hydroxyl radicals upon decomposition.

Further, since nitrotyrosine has been proposed as an \textit{in vivo} marker of the presence of peroxynitrite (57), we have studied the mechanisms of nitration and hydroxylation by peroxynitrite using phenol as a model. In light of the recent finding that peroxynitrite rapidly reacts with CO\(_2\) (88), we have also investigated the effect of CO\(_2\) on the kinetics and formation of products of the reaction of peroxynitrite with phenol.

Finally, we have studied the effect of limiting amounts of CO\(_2\) on the kinetics of the decomposition of peroxynitrite in order to explore the mechanism of reaction of the peroxynitrite/CO\(_2\) adduct.
CHAPTER 1. SPIN TRAP STUDIES ON THE DECOMPOSITION OF PEROXYNITRITE*

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Introduction

Peroxynitrite\textsuperscript{1} is a potent oxidant that oxidizes biomolecules such as thiols (72), lipids (54,73), deoxyribose (28) and alpha-1-proteinase inhibitor (71). Peroxynitrite is formed from the near diffusion-controlled reaction (k = 6.7 ± 0.9 x 10\textsuperscript{9} M\textsuperscript{-1}s\textsuperscript{-1}) of nitric oxide and superoxide (39). Peroxynitrite formation \textit{in vivo} likely occurs near cells such as macrophages, neutrophils and endothelial cells that produce both nitric oxide and superoxide (4,7,28,33,116,117).

The toxicity of both nitric oxide and superoxide can be attributed at least in part to oxidations by peroxynitrite, but the mechanisms by which peroxynitrite oxidizes biomolecules are a matter of controversy. The homolytic scission of peroxynitrite to the free radicals HO\textsuperscript{•} and \textsuperscript{•}NO\textsubscript{2} has been suggested as one possibility (28,62). However, it also has been argued that the formation of \textsuperscript{•}NO\textsubscript{2} and HO\textsuperscript{•} is not thermodynamically feasible (54,72), and a mechanism involving an excited intermediate that can afford reaction products similar to those observed in reactions of the hydroxyl radical has been proposed (41,61,70). The excited intermediate of peroxynitrite is proposed to be less reactive and more selective than

\textsuperscript{1}Peroxynitrite refers to the sum of 'OONO and its conjugate acid, HOONO, i.e., the stoichiometric concentration of peroxynitrite. When a discussion is specifically limited to either the anion or the free acid, either its name or its structure will be given.
the hydroxyl radical (41,70), which reacts at near diffusion-controlled 
rates with many biomolecules (118,119).

The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) is often 
used to determine the intermediacy of hydroxyl radicals in a variety of 
systems. In the past two years, three research groups (84,85,120) have 
reported somewhat divergent observations using this spin trap to study 
the decomposition of peroxynitrite. Augusto et al. (84) and Carmichael et 
al. (120), under certain conditions, observed the hydroxyl radical spin 
adduct of DMPO (*DMPO-OH), whereas Shi et al. (85) instead observed 
the spin trap oxidation product 5,5-dimethyl-1-pyrrolidin-2-one-1-oxy 
(DMPOx).

In the system Augusto et al. used, glutathione strongly enhances 
the intensity of the *DMPO-OH spin adduct ESR signal; this was 
rationalized by Augusto et al. by proposing that GSH scavenges oxidants 
that would otherwise react with the spin adduct (84). However, as also 
suggested by Augusto et al., it is possible that peroxynitrite reacts with 
GSH faster than it decomposes to nitrate and/or reacts with DMPO (84).
The concept of using a spin trap to test for the production of hydroxyl 
radicals from peroxynitrite was novel and extremely useful. However, 
although the observation of the ESR signal for *DMPO-OH is striking, it is 
recognized that the detection of the hydroxyl radical spin adduct is not,
by itself, conclusive evidence that peroxynitrite decomposes to form the hydroxyl radical. For example, a bimolecular reaction between HOONO and DMPO to give a spin adduct is also possible. This type of interaction, termed molecule-assisted homolysis, is known for a variety of peroxides (121-125). Furthermore, the reaction of an intermediate formed in the decomposition of peroxynitrite to nitrate with DMPO could also give a spin adduct.

We here report a reexamination of the reaction kinetics of peroxynitrite and DMPO, with and without glutathione or cysteine, and the effects of superoxide dismutase (SOD) on the formation of ESR spin adducts.

Experimental procedures

Materials. Superoxide dismutase (from bovine erythrocytes; EC 1.15.1.1), cysteine, glutathione, sodium nitrite, horse heart cytochrome c type VI, xanthine, EDTA and grade I buttermilk xanthine oxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Chelex-100 was obtained from Bio-Rad Laboratories (Richmond, CA). DMPO was obtained from OMRF (Oklahoma City, OK). Peroxynitrite was synthesized by ozonation of sodium azide (96). The peroxynitrite solution obtained this way was kept at -20°C until two layers separated and the top bright
yellow layer was collected. Peroxynitrite concentration was determined spectrophotometrically ($\lambda = 302 \text{ nm}, \epsilon = 1670 \text{ M}^{-1}\text{cm}^{-1}$) (64).

**Stopped-Flow Studies.** Kinetic experiments were conducted on a stopped-flow spectrophotometer (Kinetic Instruments, Inc.; Ann Arbor, MI and On-Line Instrument Services, Jefferson, GA) with a mixing time of less than 1.5 ms at 25 ± 0.1 °C. Stock solutions of peroxynitrite were diluted to 0.8 mM with deionized water. A stock solution of DMPO (2.8 M; $\lambda = 227 \text{ nm}, \epsilon = 8000 \text{ M}^{-1}\text{cm}^{-1}$ (126)) was diluted with buffer (0.2 M phosphate buffer pH 7.20-7.30 treated with Chelex-100 to remove any adventitious transition metal ions) to the desired concentrations. Equal volumes of peroxynitrite and DMPO solutions were mixed in the cell of the stopped-flow apparatus. Peroxynitrite (0.4 mM after mixing) decomposition in the presence of 0 to 75 mM of DMPO was followed at 302 nm. Each experiment was repeated 10 times to determine an average rate constant. The final pH, measured at the outlet, ranged from 7.27 to 7.31. The decomposition of peroxynitrite followed a first order decay (except in the experiments in the presence of thiol). The data were fitted using OLIS Stopped-Flow software (OLIS Inc., Jefferson, GA).

**Kinetic treatment.** We propose a mechanism, given by Equations [1.1-1.5], for which the general rate law is expressed in Equation [1.6].

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\[ \text{HOONO} \xrightarrow{k_3} \text{H}^- + \text{OONO} \quad [1.1] \]
\[ \text{HOONO} \xrightarrow{k_i} \text{HOONO}^- \quad [1.2] \]
\[ \text{HOONO}^- \xrightarrow{k_2} \text{H}^- + \text{NO}_2^- \quad [1.3] \]
\[ \text{HOONO}^- + \text{DMPO} \xrightarrow{k_4} \text{Products} \quad [1.4] \]
\[ \text{HOONO} + \text{DMPO} \xrightarrow{k_3} \text{Products} \quad [1.5] \]

\[ -\frac{d(P)}{dt} = -k_1[\text{HOONO}^\ast] + k_i[\text{HOONO}] + k_2[\text{DMPO}][\text{HOONO}] \quad [1.6] \]

where \([P]\) represents the sum of the concentrations of \(\text{HOONO}\) and \(^{-}\text{OONO}\). We assume that \(\text{HOONO}^\ast\) is formed in a steady state:

\[ k_i[\text{HOONO}] = k_N[\text{HOONO}^\ast] + k_i[\text{HOONO}^\ast] + k_2[\text{DMPO}][\text{HOONO}^\ast] \quad [1.7] \]

\[ [\text{HOONO}^\ast] = \frac{k_i[\text{HOONO}]}{k_N + k_1 + k_2[\text{DMPO}]} \quad [1.8] \]

Equation [1.6] can then be rewritten as

\[ -\frac{d(P)}{dt} = -k_1 \frac{k_i[\text{HOONO}]}{k_N + k_1 + k_2[\text{DMPO}]} + k_i[\text{HOONO}] + k_2[\text{DMPO}][\text{HOONO}] \quad [1.9] \]
Considering the dissociation constant of peroxynitrous acid given by:

\[ K_a = \frac{[H^+][OONO]}{[HOONO]} \quad [1.10] \]

the rate law can be written as a function of just [P] as:

\[
\frac{-d[P]}{dt} = \frac{[H^+]}{K_a + [H^+]} \left[ \frac{k_4k_1 + k_2k_3[DMPO]}{k_4 + k_1 + k_2[DMPO]} + k_3[DMPO] \right] P \quad [1.11]
\]

Thus, a general expression for \( k_{obs} \), the observed rate constant for the decomposition of peroxynitrite, as a function of the concentration of DMPO and pH can be derived (Equations [1.12-1.16]). [We have previously described a similar treatment of peroxynitrite reactions (70).]

\[
k_{obs} = \frac{a(1 + b[DMPO])}{1 + \frac{ab}{c}[DMPO]} + d[DMPO] \quad [1.12]
\]

\[
a = \frac{[H^+]}{K_a + [H^+] \times \frac{k_4k_1}{k_4 + k_1}} \quad [1.13]
\]

\[
b = \frac{k_3}{k_4} \quad [1.14]
\]
Two cases are considered. For the first case, DMPO is assumed to undergo a bimolecular reaction with ground-state HOONO and $k_2^*$ is set to zero. To examine this case, $k_{obs}$ was fitted to Equation [1.17] using TableCurve 2D (Jandel Scientific, San Rafael, CA).

$$k_{obs} = a - d[DMPO]$$  \[1.17\]

For the second case, DMPO is assumed to react exclusively with HOONO$^*$ and $k_2$ is set to zero (70). To examine this case, $k_{obs}$ was fitted to Equation [1.18].

$$k_{obs} = \frac{a(1 + b[DMPO])}{1 - \frac{ab}{c} [DMPO]}$$  \[1.18\]

In this treatment, it is assumed that spin adducts derived from DMPO do not react further with peroxynitrite. Reaction of the DMPO-spin adducts with peroxynitrite appears unlikely since further oxidation would
yield DMPOx which we do not observe. Also, there was no delay before the single exponential decay was observed and we did not notice any significant deviation from the first-order kinetics in our time-course plots for concentrations of DMPO up to 75 mM. Thus, we do not have any indication of a reaction of peroxynitrite with the product of the reaction of DMPO and peroxynitrite.

ESR Studies. ESR spectra were obtained at room temperature on a Bruker-100 spectrometer provided with a TM110 cavity. The data collection employed a software written using Asyst from Keithley/Metrabyte (Taunton, MA). Rapid mixing was attained by vortexing the DMPO solutions (80-100 mM (10-20 μl) in 0.1 M phosphate buffer pH 7.2-7.3 treated with Chelex-100) during the addition of peroxynitrite in order to ensure complete mixing before any significant decomposition of the peroxynitrite occurred. The reaction mixtures (final volume 1.5 ml) were then transferred immediately to a flat cell and the spectra recorded. Experiments in the presence of glutathione or cysteine and/or SOD were conducted as above by adding the specific amounts of thiol and/or SOD to the buffer solution containing DMPO prior to the addition of peroxynitrite.

SOD Activity Assay. The assay developed by McCord and Fridovich (127) was used to determine the activity of SOD; it is based on

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the rate of reduction of ferricytochrome c at 550 nm by superoxide produced by xanthine/xanthine oxidase. The spectra were recorded on a Lambda 6 UV/Vis spectrophotometer manufactured by Perkin Elmer (Norwalk, CT) at 25°C (λ = 550 nm). A 0.05 M phosphate buffer pH 7.8 containing 1 x 10^-4 M EDTA was used; the activity of SOD was measured before and after reaction with 0.32 mM peroxynitrite.

Results

Stopped-Flow Study of the Reaction of Peroxynitrite and DMPO.
The observed rate constant (k_{obs}) increases linearly with increasing concentration of DMPO as shown in Fig. 1.1. The rate increases by 46% when DMPO goes from 0 to 75 mM; we were forced to limit our study to concentrations of DMPO below 75 mM, since DMPO interferes with the measurement of absorbance at higher concentrations.

Effect of Glutathione and Cysteine on the Lifetime of Peroxynitrite.
As shown in Figure 1.2, the half-life of 0.8 mM peroxynitrite, determined from the decay of peroxynitrite followed at 302 nm, decreases from 1.1 s in the absence of thiol to 0.6 s in the presence of 1 mM glutathione and 0.1 s in the presence of 5 mM cysteine. (For each thiol, the concentration used was the one that gave the strongest ESR signal).

Effect of SOD on the ESR Signal from Decomposition of Peroxynitrite in the Presence of DMPO. As shown in Figure 1.3, SOD has no effect on the intensity of the •DMPO-OH spin adduct signal, identified
Figure 1.1. Stopped-Flow Study of the Reaction between HOONO and DMPO. The rates of reaction were obtained by following the disappearance of HOONO at 302 nm. The concentration of DMPO varied from 0 to 75 mM. The concentration of peroxynitrite was 0.4 mM. The buffer used was 0.1 M phosphate, pH 7.3.
Figure 1.2. Decay of Peroxynitrite at pH 7.3 without any Thiol, with Glutathione, and with Cysteine. Effect of 1 mM glutathione and 5 mM cysteine on the rate of disappearance of peroxynitrite was followed at 302 nm in 0.1 M phosphate buffer at 25°C, pH 7.3. The initial concentration of peroxynitrite was 0.4 mM.
Figure 1.3. Effect of SOD on the ESR Signal from Decomposition of Peroxynitrite in the Presence of DMPO. Spectra obtained from samples in which 0.8 mM peroxynitrite had been decomposed in a 0.1 M phosphate buffer at pH 7.2 which contained 80 mM DMPO and SOD. The ESR acquisition parameters used were power, 20 mW; amplitude modulation, 0.63 G; time constant, 0.2 s; receiver gain, $6.3 \times 10^8$; scanning time, 100 s; scan width, 100 G. (A) Control (no peroxynitrite); (B) with peroxynitrite but no SOD; (C) with 10.2 U/ml SOD; (D) with 20.5 U/ml SOD; (E) with 30.7 U/ml SOD.
from its hyperfine splitting constant (hfsc) of \( a_N = 1.49 \text{ mT} \); \( a_H = 1.49 \text{ mT} \) (128). As shown in Figure 1.4, superoxide dismutase (from bovine erythrocyte) is not inactivated by peroxynitrite or its decomposition products under the conditions of our experiments where SOD is in the order of \( 10^{-8} \text{ M} \). Curve A in Figure 1.4 shows the time course of the reduction of ferricytochrome c by superoxide (produced from a xanthine/xanthine oxidase system). The rate of reaction obtained from the linear part of the curve (time < 300 sec) is \( 0.0264 \pm 0.0013 \) absorbance units/min. Curve B represents the time course of the same reaction when 3.7 units of SOD have been added to the system. SOD inhibits the reduction of ferricytochrome c by superoxide (by destroying the superoxide) and therefore the rate of reaction is reduced to \( 0.0061 \pm 0.0011 \) absorbance units/min. Curve C was obtained when the same amount of SOD had been previously reacted with 0.32 mM of peroxynitrite. The rate in this case is unchanged, with a value of \( 0.0077 \pm 0.0021 \) absorbance units/min. Curve D was obtained when the peroxynitrite (0.32 mM) was allowed to decompose in the buffer prior to the addition of 3.7 units of SOD. The observed rate again is unchanged, with a value of \( 0.0087 \pm 0.0016 \) absorbance units/min. These findings are in agreement with the results of Beckman and coworkers (59) who reported no loss of activity when 1 mg/ml of bovine erythrocyte SOD
Figure 1.4. Effect of HOONO on the Activity of SOD. Spectra obtained from the reduction of 1 x 10^-5 M ferricytochrome c by superoxide (from 5 x 10^-6 M xanthine/6 x 10^-8 M xanthine oxidase) followed at 550 nm in a 0.05 M phosphate buffer, pH 7.8, containing 1 x 10^-4 M EDTA. (A) Without SOD; (B) with one unit of unreacted SOD; (C) with one unit of SOD reacted with 0.32 mM HOONO; (D) reverse addition.

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(Cu, Zn SOD) was exposed to up to 10 mM peroxynitrite. A slight deactivation of SOD seems to occur in the presence of peroxynitrite at the beginning of the time runs (Curves C and D), however the order of addition of peroxynitrite and SOD to the reaction mixture did not affect the time run, therefore the small deactivation is most likely due to reaction of the products of the decomposition of peroxynitrite with SOD.

Effect of SOD on the ESR Signal from Decomposition of Peroxynitrite in the Presence of DMPO and Glutathione. Figure 1.5 shows the ESR spectra obtained when peroxynitrite is allowed to decompose in the presence of DMPO (80 mM) and glutathione (1 mM) and various concentrations of SOD (0 to 28.8 U/ml). Clearly the ESR signal intensity decreases as the activity of SOD increases; it is barely detectable at 28.8 U/ml of SOD, as shown in curve E.

Effect of SOD on the ESR Signal from the Decomposition of Peroxynitrite in the Presence of DMPO and Cysteine. Figure 1.6 shows the ESR spectra obtained with cysteine (5 mM); increasing amounts of SOD (0 to 24 U/ml) cause a decrease in the ESR signal intensity. Figure 1.7 summarizes the data presented in Figs. 1.3, 1.5, and 1.6 in terms of relative intensities.

Effect of Oxygen on the Formation of the *DMPO-OH ESR Spin Adduct. As shown in Figure 1.8, the ESR signal for the spin adduct decreased sharply when the reaction system was purged with argon. For
Figure 1.5. Effect of SOD on the ESR Signal from Decomposition of Peroxynitrite in the Presence of DMPO and GSH. Spectra obtained from samples in which 0.8 mM peroxynitrite had been decomposed in a 0.1 M phosphate buffer, pH 7.2, which contained 80 mM DMPO, SOD and 1 mM GSH. The ESR acquisition parameters used were power, 20 mW; amplitude modulation, 0.63 G; time constant, 0.2 s; receiver gain, $2 \times 10^5$; scanning time, 100 s; scan width, 100 G. (A) Control (no SOD); (B) with 7.2 U/ml SOD; (C) with 14.4 U/ml SOD; (D) with 21.6 U/ml SOD; (E) with 28.8 U/ml SOD.
Figure 1.6. Effect of SOD on the ESR Signal from Decomposition of Peroxynitrite in the Presence of DMPO and Cysteine. Spectra obtained from samples in which 0.8 mM peroxynitrite had been decomposed in a 0.1 M phosphate buffer, pH 7.2, which contained 80 mM DMPO, SOD and 5 mM cysteine. The ESR acquisition parameters used were power, 20 mW; amplitude modulation, 0.63 G; time constant, 0.2 s; receiver gain, 2 x 10^5; scanning time, 100 s; scan width, 100 G. (A) Control (no SOD); (B) with 12 U/ml SOD; (C) with 24 U/ml SOD.
Figure 1.7. Comparison of the ESR Signal Intensities as a Function of the Concentration of SOD without any Thiol, with GSH, and with Cysteine. The signal intensities were obtained from the amplitude of the tallest line for each signal in arbitrary units.
Figure 1.8. Effect of Oxygen on the Formation of the DMPO-OH Spin Adduct. Spectra obtained from samples in which 0.8 mM peroxynitrite had been decomposed in a 0.1 M phosphate buffer at pH 7.2 which contained 80 mM DMPO and either 5 mM cysteine (A, B, and C) or 1 mM GSH (D and E). The solutions were purged with oxygen (A and D) or argon (B, C, and D) for 10 min prior to the addition of 0.8 mM peroxynitrite. Spectrum (B) was obtained 1 min after addition of peroxynitrite. Spectrum (C) was obtained 10 min after addition of peroxynitrite. The ESR acquisition parameters used were power, 20 mW; amplitude modulation, 0.63 G; time constant, 0.2 s; receiver gain, 2 x 20⁵; scanning time, 100 s; scan width, 100 G.
5 mM cysteine, Figs. 1.8A and 1.8C show the signal decreased by 71% in the Ar-purged sample; for GSH (Figs. 8D and 8E), the signal decreased by 48%, which is consistent with the findings of Augusto et al (84). The DMPO-cysteine spin-adduct was observed when the incubation mixture had been purged with argon for 10 min before the addition of peroxynitrite and the spectrum was taken 1 min thereafter.

Discussion

The Rate of Decomposition of Peroxynitrite Increases with the Concentration of DMPO. If free hydroxyl radicals were spin trapped by DMPO, the HOONO-DMPO reaction would be first-order in peroxynitrite and zero-order in DMPO, since the reaction of DMPO with the hydroxyl radical occurs at a near diffusion-controlled rate (118,119). We observe that the decomposition of peroxynitrite occurs at a faster rate as the concentration of DMPO increases (Fig. 1.1); thus, considering the high reactivity of HO*, it is unlikely that DMPO reacts directly with the hydroxyl radical. Rather, it appears that DMPO reacts in a rate-determining step with HOONO or a reactive intermediate formed from HOONO.

Studies of the relative rates at which different hydroxyl radical scavengers trap the HO* proposed to be formed from peroxynitrite give relative rate constants that do not agree with the known rate constants
for the hydroxyl radical (41,70,71,118,129). The ability of substrates like DMPO to increase the observed rate constant of peroxynitrite decomposition and the failure to reconcile relative rate constants obtained from competition experiments with those for the hydroxyl radical constitute the strongest evidence against the participation of the hydroxyl radical (41,61,70,71).

**A General Mechanism for Peroxynitrite Oxidations.** A mechanism has been proposed to explain the complex kinetics observed for the reaction of peroxynitrite with methionine and 2-keto-4-thiomethylbutanoic acid (70). The reaction of peroxynitrite with these two thiomethyl compounds gives plots of $k_{obs}$ versus the concentration of the thiomethyl compound that can be approximated by straight lines, but the plots have a $y$-intercept that is larger than the value of $k_{obs}$ obtained in the absence of substrate (41,70). To explain the downward curvature in these plots at low substrate concentrations, we suggested that the thiomethyl compounds could react either with ground state HOONO or with HOONO*. For this mechanism, an equation like Eq. [1.13] fits the data obtained for the thiomethyl compounds. In this mechanism, the curvature in the plot of $k_{obs}$ versus the concentration of thiomethyl compound arises because the thiomethyl compound reacts with first-order kinetics with HOONO* when the substrate concentration is low and
with ground state HOONO with second-order kinetics when the substrate concentration is higher. At high substrate concentrations, the plot tends towards a straight line typical of a second-order reaction (but extrapolates to a misleadingly large y-intercept as discussed above).

**A General Mechanism for the Reaction of Peroxynitrite with DMPO.**

Since the rate of the reaction of peroxynitrite with DMPO depends on the concentration of DMPO, we suggest that the spin adduct is formed from a direct reaction of peroxynitrite or its activated intermediate with DMPO. Thus, we propose a mechanism for the production of spin adduct similar to the one elaborated for the reactions of peroxynitrite with thiomethyl compounds (70). However, with DMPO it is not possible to reach high enough DMPO concentrations to test whether Figure 1.1 shows the expected downward curvature, since DMPO interferes with our stopped-flow measurements when present at high concentrations. This limitation precludes us from estimating the relative reactivities of HOONO* and HOONO with DMPO. Nevertheless, we here interpret the data in terms of two limiting cases, namely those where either HOONO* or HOONO react with DMPO. It is important, however, to bear in mind that these are limiting cases and that intermediate situations may occur. The approximate approach presented here gives upper limits for \( k_2 \) and \( k_2^*/k_N \) for DMPO.
Our reaction kinetic modeling considers two limiting situations, where the main reaction pathway involves either \( \text{HOONO}^{*} \) \((k_2 = 0)\) or \( \text{HOONO} \) \((k_2^{*}/k_N = 0)\). A computer fit of the data according to the first case (Eq. [1.17]) affords \( k_2^{*}/k_N = 7.6 \text{ M}^{-1} \) \((r^2 = 0.99)\) (see the Experimental Procedures; note \( k_N \) is the rate constant of Eq. [1.3]). For the second case (Eq. [1.18]), a computer fit of the data affords a second order rate constant, \( k_2 \), equal to \( 8.7 \text{ M}^{-1}\text{s}^{-1} \) \((r^2 = 0.99)\) (see Experimental Procedures).

In either of these two limiting cases, DMPO is consumed in a rate-limiting step and, therefore, we suggest that the observed ESR signals arise from reaction of DMPO with \( \text{HOONO}, \text{ HOONO}^{*} \), or with both species, and not from the participation of free hydroxyl radicals. Indeed, free hydroxyl radicals react with DMPO at a near diffusion-controlled rate \((4.3 \times 10^9 \text{ M}^{-1}\text{s}^{-1})\) \((118,119)\) and cannot account for the substrate concentration dependence of \( k_{\text{obs}} \) that we observe.

**The Thiomethyl Compounds Are More Reactive than DMPO**

towards Peroxynitrite. Table 1.1 gives the upper limits of \( k_2 \) and \( k_2^{*}/k_N \) for DMPO, as well as the values obtained for \( k_2 \) and \( k_2^{*}/k_N \) using methionine and 2-keto-4-thiomethylbutanoic acid as substrates. Peroxynitrite is more reactive toward thiomethyl compounds than DMPO. Nevertheless, DMPO is consumed in a rate-limiting step to produce the
Table 1.1. Rate Constants $k_2$ and $k_2^*$ for DMPO, Methionine and KTBA.

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>$k_2$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_2^*/k_N$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case I</td>
<td>0</td>
<td>7.6</td>
</tr>
<tr>
<td>Case II</td>
<td>8.7</td>
<td>0</td>
</tr>
<tr>
<td>Methionine</td>
<td>181</td>
<td>1250</td>
</tr>
<tr>
<td>KTBA</td>
<td>277</td>
<td>6230</td>
</tr>
</tbody>
</table>

Note: The values obtained for DMPO are limits obtained considering reaction with either HOONO ($k_2^* = 0$) or HOONO* ($k_2 = 0$).
•DMPO-OH ESR spin adduct. It is this more sluggish reaction of DMPO toward peroxynitrite (relative to the more reactive thiomethyl compounds) and its absorbance at 302 nm that precludes estimating relative reactivities of HOONO* or HOONO toward DMPO, since high concentrations of DMPO would be required to reach the linear phase of the kinetics.

**The Decomposition of Peroxynitrite Does Not Produce Superoxide.**

The •DMPO-OH ESR signal also could arise from the participation of superoxide, since the superoxide adduct of DMPO (•DMPO-OOH) rapidly decomposes to form the hydroxyl adduct, •DMPO-OH. However, the •DMPO-OH ESR signal intensity is not affected by superoxide dismutase (0 to 30.7 U/ml), suggesting the spin adduct signal arises from a superoxide-independent reaction pathway (Figure 1.3). Superoxide dismutase (bovine erythrocyte) is not inactivated by peroxynitrite or its decomposition products under our conditions (Figure 1.4), in agreement with previous observations by Ischiropoulos et al. (59).

**Peroxynitrite Accelerates the Autoxidation of Thiols.** Augusto et al. investigated the effects of glutathione and cysteine on the DMPO-peroxynitrite reaction system and observed the •DMPO-OH ESR signal intensity greatly increased in the presence of these thiols (84). We have confirmed this observation; addition of 1 mM GSH or 5 mM cysteine (a
strong ESR signal was observed under these conditions) results in an enhancement of the •DMPO-OH ESR signal intensity, 30-fold in the case of GSH and 6-fold for cysteine. Augusto et al. concluded thiols scavenge excess peroxynitrite and nitrogen dioxide, thereby preventing their reacting with •DMPO-OH to form ESR-silent products. However, we find the half-life of peroxynitrite (0.8 mM) varies from about 1.1 s in the absence of GSH to about 0.6 s in the presence of 1 mM GSH and to about 0.1 s in the presence of 5 mM cysteine (Figure 1.2). Under our conditions, GSH and cysteine react with peroxynitrite competing with the spontaneous decomposition to nitrate, indicating that peroxynitrite reacts faster with the two thiols than with DMPO. Moreover, the intensity of the •DMPO-OH ESR signal in the presence of glutathione (Figure 1.5) or cysteine (Figure 1.6) decreases in the presence of SOD in a concentration-dependent fashion (Figure 1.7). The intensity of the •DMPO-OH ESR signal in the presence of glutathione or cysteine also decreases when the incubation mixtures were flushed with argon for 10 min before addition of peroxynitrite (Figure 1.8). These observations suggest that most of the •DMPO-OH observed in the presence of thiols arises from the reaction of DMPO with superoxide to form the short-lived •DMPO-OOH adduct, which then decomposes to the •DMPO-OH adduct. [Superoxide is known to be implicated during the aerobic oxidation of
thiols (130-132). These results contrast sharply with our data on the peroxynitrite-DMPO reaction in the absence of thiol, where SOD has no effect on the ESR signal strength and superoxide is not involved.

We suggest that peroxynitrite initiates the autoxidation of the thiols, dramatically increasing the concentration of *DMPO-OOH, and hence *DMPO-OH. A possible mechanism for this initiation is an initial MAH reaction between thiols and peroxynitrite (Eq. [1.19]), followed by the propagation steps shown in Eqs. [1.20] and [1.21], and finally reaction of DMPO with superoxide to form *DMPO-OH in Equation [1.22] (84).

\[
\begin{align*}
\text{MAH} & \\
\text{RS}^- + ^\cdot\text{OONO} + \text{H}^+ & \rightarrow \text{RS}^* + \text{OH}^- + \text{NO}_2^- \quad [1.19] \\
\text{RS}^* + \text{RS}^- & \rightarrow \text{RSSR}^- \quad [1.20] \\
\text{RSSR}^- + \text{O}_2 & \rightarrow \text{RSSR} + \text{O}_2^- \quad [1.21] \\
\text{DMPO} + \text{O}_2^- & \rightarrow [*\text{DMPO-OOH}] \rightarrow *\text{DMPO-OH} \quad [1.22]
\end{align*}
\]

As shown in Equations [1.19-1.22], the autoxidation of thiols (and therefore the production of superoxide and *DMPO-OH) is predicted to depend on the amount of oxygen present in solution. The solubility of oxygen from air in water at 25°C is 0.25 mM (133). Thus, under conditions in which oxygen is limiting, thiyi radicals arising from glutathione or cysteine will react primarily with DMPO to form the thiyl...
radical spin adduct rather than propagate thiol autoxidation to form superoxide. This could explain why Shi et al. (85), who used high concentrations of peroxynitrite (10 mM) and glutathione (10 mM), report the formation of the thyl radical spin adduct and not $^*$DMPO-OH from the decomposition of peroxynitrite in the presence of GSH and DMPO (100 mM). We observe the thyl radical spin adduct in the spectrum obtained one minute after addition of peroxynitrite when the incubation mixture had been purged with argon. However, the thyl radical spin adduct rapidly decayed and was not observable after 10 minutes (Fig. 1.8).

Thus, considering the limiting amount of oxygen, it is likely that under the experimental conditions of Shi et al. most of the ESR signal arises from the direct reaction of thyl radicals with DMPO.

Shi and coworkers (85) also reported the detection of 5,5-dimethyl-1-pyrrolidin-2-one-1-oxy (DMPOx) by ESR during the decomposition of 10 mM peroxynitrite, a concentration 12.5 times higher than that used in our ESR experiments. It is possible that under these conditions of large peroxynitrite excess, the initial $^*$DMPO-OH spin adduct is over-oxidized to DMPOx by two equivalents of peroxynitrite according to Eq. [1.23].

$$\text{DMPO} \rightarrow \text{DMPO-OH}^- \rightarrow \text{DMPOx}$$ [1.23]
Conclusions

The spin trap DMPO has been shown to react with peroxynitrite in a bimolecular reaction (Fig. 1.1), and we propose this reaction leads to the production of the *DMPO-OH spin adduct. Thus, it is unlikely that the production of *DMPO-OH is entirely due to free hydroxyl radicals. Rather, we suggest a molecule-assisted homolytic reaction between DMPO and HOONO, HOONO*, or both, that produces the observed spin adduct (Eq. [1.24]).

\[
\text{DMPO} + \left\{ \begin{array}{c}
\text{HOONO} \\
\text{and/or} \\
\text{HOONC*}
\end{array} \right\} \overset{\text{MAH}}{\rightarrow} \begin{array}{c}
N \\
\text{CH} \\
\text{H}_3\text{C} \\
\text{H}_3\text{C} \\
\text{O}
\end{array} \rightarrow \text{·NO}_2
\]  

[1.24]

The reaction of glutathione (1 mM) or cysteine (5mM) with peroxynitrite (0.4 mM) occurs significantly faster than the first order decomposition of peroxynitrite (Fig. 1.2); thus, thiols react with peroxynitrite significantly faster than does DMPO. In the presence of DMPO, the reactions of glutathione or cysteine with peroxynitrite result in a dramatic increase on the *DMPO-OH ESR signal intensity; we suggest that this is due to the peroxynitrite-initiated autoxidation of thiols, leading to the *DMPO-OOH adduct, which decays to form the *DMPO-OH adduct according to Eq. [1.22]. When the amount of oxygen in solution was...
limited, the thiol spin adduct was observed, most likely arising from direct reaction of thiol radicals with DMPO.

Thus, the observation of the *(DMPO-OH spin adduct either in the presence or the absence of thiols does not constitute unequivocal proof for the spontaneous formation of hydroxyl radicals from peroxynitrite.
CHAPTER 2. CARBON DIOXIDE MODULATION OF HYDROXYLATION AND NITRATION OF PHENOL BY PEROXYNITRITE
Introduction

Peroxynitrite\(^2\) can be formed \textit{in vivo} from the diffusion-controlled reaction of nitric oxide and superoxide radicals (39,40). \textit{In vitro}, peroxynitrite has been shown to react with almost all classes of biomolecules including proteins, lipids, carbohydrates, antioxidants, and nucleic acids (54,70,71,73,76,81,134-137). The nitration of tyrosine residues in proteins is often taken as evidence for the formation of peroxynitrite \textit{in vivo} (57), since tyrosine and other phenolics are nitrated by peroxynitrite (57,138,139) but not by nitric oxide (140,141).

Peroxynitrite is known to nitrate and hydroxylate aromatic compounds (63,134,138,139,142). Halfpenny and Robinson (63), who first studied these reactions, suggested a radical mechanism. Hydroxyl and \(\cdot\text{NO}_2\) radicals have been suggested to be formed during the decomposition of peroxynitrite (28) and to be the species responsible for the peroxynitrite-mediated hydroxylation and nitration of phenylalanine and tyrosine (138). However, several lines of evidence, including the lack of effect of typical hydroxyl radical scavengers (71), experiments performed using spin traps (e.g., 5,5'-dimethylpyrroline-N-oxide) (84-87)

\[^2\]The term peroxynitrite is used to refer to the sum of both peroxynitrite anion (ONOO\(^-\)) and its conjugated acid, peroxynitrous acid (ONOOH). The term carbonate represents the sum of all carbonated species (CO\(_2\), H\(_2\)CO\(_3\), HCO\(_3^-\) and CO\(_3^{-}\)). If a particular carbonated species is referred to, it is represented by its chemical formula.
and a study on the effect of viscosity on the rate of decomposition of peroxynitrite (143), do not indicate the formation of free hydroxyl radical during the decomposition of peroxynitrous acid. The nitronium ion, \( \text{N}^+\text{O}_2^- \), formed from peroxynitrous acid at low pH (139) or from the reaction of peroxynitrite with transition metals (59,134), has also been proposed to be the active nitrating species.

Peroxynitrite is more unstable in carbonate than in phosphate buffers (66). Radi et al. (144) observed an increase in luminol chemiluminescence induced by peroxynitrite in the presence of bicarbonate and proposed that bicarbonate reacts with peroxynitrite to form an intermediate, \( \text{O} = \text{N}-\text{O}-\text{O}-\text{C(O)}\text{O}^- \) (1). Lymar and Hurst (88) reported that the peroxynitrite anion reacts with \( \text{CO}_2 \) with a rate constant of \( 3.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \) at 25 °C to form 1, and Uppu et al. (89), using carbonic anhydrase as a probe, confirmed that the peroxynitrite anion and \( \text{CO}_2 \) are the reactive species.

Both Lymar et al. (94) and Uppu et al. (89) have shown that peroxynitrite-mediated aromatic nitration is enhanced by \( \text{CO}_2 \). Yermilov et al. (145) also have shown that peroxynitrite serves as a potent nitrating agent in the presence of carbonate and suggested that \( \text{CO}_2 \) suppresses the hydroxyl-radical-like activity of peroxynitrous acid.

Recently, Denicola et al. (142) showed that the peroxynitrite-mediated
hydroxylation of benzoate, which they suggested is due to the activated intermediate ONOOH*(61,70), is inhibited in the presence of CO2.

We here report a study of the products and stopped-flow kinetics of the reaction of peroxynitrite with phenol in the presence and absence of purposefully added carbonate.3 We find that different intermediates are responsible for nitration and hydroxylation and suggest possible mechanisms.

Experimental Procedures

Materials. Sodium azide, phenol, o-, p-, m-nitropheno(s, o-hydroxyphenol and diethylenetriaminepentaacetic acid (dtpa) were purchased from Sigma Chemical Company (St. Louis, MO). Sodium bicarbonate and sodium chloride were from EM Science (Gibbstown, New Jersey). Benzoquinone, nitrosophenol, 2,2'- and 4,4'-biphenols, and p-hydroxyphenol were from Aldrich Chemical Company (Milwaukee, WI). All other chemicals were of the highest grade available. All solutions were prepared using deionized water.

Synthesis of Peroxynitrite. Peroxynitrite was synthesized according to the method of Pryor et al. (96), which involves the reaction

3Aqueous solutions typically absorb CO2 from ambient air. Therefore, our phosphate buffers contain carbonate. "Purposefully added carbonate" refers to the amount of carbonate that was added to a given reaction mixture and does not account for the inevitable carbonate contamination.
of azide with ozone. A Sander 200 ozonizer from Erwin Sander (Uetze-Eltze, Germany) was used to ozonize the azide solutions. To reduce the contamination by unreacted azide, ozonation was prolonged even after a maximal yield of peroxynitrite was obtained (97). This procedure helps to reduce the amount of unreacted azide (≤ 5 μM), although a 20-30% loss of peroxynitrite occurs. The peroxynitrite solutions thus obtained were quantified spectrophotometrically (ε302 = 1670 M⁻¹ cm⁻¹) (66) using a Hewlett Packard Model 8451A diode array spectrophotometer.

**GC-MS Analysis of Nitrophenols.** The products of the reaction between phenol and peroxynitrite were analyzed using gas chromatography-mass spectrometry (GC-MS). Typical experiments contained 1 mM phenol, 1 mM peroxynitrite in 0.1 M phosphate buffer, and various amounts of NaHCO₃ (0-20 mM). The final pH after the addition of peroxynitrite was measured in each case. The reaction mixture (5 mL) was subsequently loaded onto a conditioned Supelclean LC-18 (3 mL capacity) solid-phase extraction column (Supelco, Bellefonte, PA) and eluted with 3 mL of methanol. An aliquot (3 μL) of the methanolic extract was then injected into a Hewlett Packard (Palo Alto, CA) GC model 5890 connected to a mass-selective detector, model 5970B, operating in the selective ion mode (SIM) using ions with 94, 108, 110, and 139 amu. m-Nitrophenol was used as an internal standard.
since it was not detected as a product in the reactions studied. The GC conditions were: column 12 m x 0.2 mm x 0.33 μm, J&W DB-5MS; helium flow 0.7 mL per minute; split 20:1; injector 250°C; detector 280°C; column: 100°C, 2 min, 10°C/min to 280°C.

**HPLC Analysis of the Products of the Reaction of Peroxynitrite with Phenol.** The products of the reaction of peroxynitrite with phenol were analyzed using a Perkin-Elmer series 410 liquid chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a Perkin-Elmer model 235 diode array detector and a Spherisorb S5 ODS2 column (5 μm particle size, 250x4.6 mm; Phase Separations, Norwalk, CT). Typical samples for analysis contained 5 mM phenol, 0.8 mM peroxynitrite and 0 or 25 mM carbonate in 0.5 M phosphate (pH ≥ 6) or acetate (pH < 6) buffer. Since CO₂ rapidly reacts with peroxynitrite, the mixing of reactants in assays that contained added carbonate was performed using a stopped-flow apparatus. The final pH was measured in each case. The ionic strength was adjusted to 1 M using sodium chloride. Separation of the products was achieved using a mobile phase that contained a mixture of acetonitrile and 27 mM acetate/30 mM citrate buffer (pH 3.2). The elution utilized an isocratic 26/74 aqueous buffer/acetonitrile for 17 min, 26/74 to 30/70 buffer/acetonitrile for 2 min, isocratic 30/70 buffer/acetonitrile for 20 min, 30/70 to 26/74 buffer/acetonitrile for 2 min.
min, at a flow rate of 1 mL min$^{-1}$. The eluent was monitored at 245 nm for detection of benzoquinone and at 280 nm for detection of all of the other compounds. The peaks were identified by matching their UV-Vis spectra and retention times with authentic compounds. The peak areas were quantified using external standards. The following standards were used: hydroquinone, nitrosophenol, catechol, benzoquinone, phenol, o- and p-nitrophenols, 2,2'- and 4,4'-biphenols. The chromatographic separation was conducted at pH 3.2, and the presence of nitrate or nitrite in the sample injected could have led to nitration of phenol during the analytical procedure. Therefore, a control experiment was carried out in which phenol was added to a solution of phosphate buffer, pH 7.0, containing 0.8 mM of decomposed peroxynitrite. No products were detected and 100% phenol was recovered.

**Stopped-Flow Studies.** Kinetic experiments were conducted on a stopped-flow spectrophotometer manufactured by On-Line Instrument Systems, Inc., (Bogart, GA) with a mixing time of less than 1.5 ms at 25°C and equipped with a 2-cm mixing cell and an OLIS-RSM 1000 rapid scanning monochromator capable of collecting one thousand spectra per second. Stock solutions of peroxynitrite were diluted to the desired concentration with deionized water adjusted at pH 11.0. A stock solution of phenol was diluted with a buffer containing 0.2 mM dtpa [acetate (pH
3.5-5.5) and phosphate (pH 6.0-10.0)] to the required concentrations.

For experiments with carbonate, a fixed amount of NaHCO₃ was added to phenol and then diluted with the buffer. The ionic strength of the solutions was maintained at 0.5 M using sodium chloride. Equal volumes of peroxynitrite and buffered phenol solutions were mixed in the stopped-flow apparatus. The kinetics of the reaction between peroxynitrite and carbonate were monitored by the decay of peroxynitrite at 302 nm (pH ≥ 6.0) or peroxynitrous acid at 260 nm (pH < 6.0). For the reaction of peroxynitrite with phenol, the kinetics were followed by monitoring the build-up of hydroxyphenols at 290 nm and nitrophenols at 400 nm. The data were analyzed using the OLIS-RSM 1000 global fit software to extract pseudo-first-order rate constants. Results from six experiments were averaged. Temperatures were maintained to ± 0.2°C using a VWR Model 1167 circulating water bath.

**UV-Vis Analysis of the Maximum Yield of Nitration as a Function of the Concentration of Phenol.** The maximum yield of the sum of o- and p-nitrophenols was estimated by measuring the absorbance at 400 nm of solutions in which 1 mM of peroxynitrite had reacted with 0-30 mM phenol in the presence of 20 mM carbonate in a 0.5 M sodium phosphate buffer, pH 7.6.
Results

Kinetics of the Reaction of Phenol with Peroxynitrite. We studied the reaction of peroxynitrite with excess phenol in the pH range 4.0 to 8.5. The plot of the pseudo-first-order rate constant, \( k_{\text{obs}} \), vs pH for the formation of nitrophenols is different from that for the formation of hydroxyphenols (Fig. 2.1). The maximum rate constant for hydroxylation is obtained around pH 4.5, whereas the rate constant for nitration reaches a maximum around pH 6.5. Figure 2.2 shows the pH profiles of the sum of the observed rate constants for hydroxylation and nitration of phenol and the observed rate constant for the decomposition of peroxynitrite in the absence of any substrate. The two plots are nearly identical, suggesting that the decomposition of peroxynitrite yields two intermediates, one that is able to effect nitrations and the other that is able to cause hydroxylations. The data of Fig. 2.1 are not in doubt; Ramezanian et al. (139) also obtain a different pH dependence for hydroxylation and nitration of phenol. They proposed the mechanism shown in Scheme 2.1:

\[
\begin{align*}
\text{ONOO}^- + H^+ &\rightarrow \text{HOONO} \quad \text{Rate step} \\
\text{HOONO}^* &\rightarrow \text{ArH} \rightarrow \text{ArOH} \\
&\quad \rightarrow \text{ArNO}_2 \\
&\rightarrow \text{NO}_3^-
\end{align*}
\]

Scheme 2.1. Mechanism Proposed by Ramezanian et al. (139) for the Reaction of Phenol with Peroxynitrite involving a single intermediate, HOONO\(^*\).
Figure 2.1. Effect of pH on the Observed Rate Constants for Hydroxylation and Nitration of Phenol in its Reaction with Peroxynitrite at 25°C. Peroxynitrite (0.25 mM) was allowed to react with phenol (5 mM) in the pH range 4.0 to 10.0 in a 0.2 M phosphate buffer containing 0.1 mM dtpa with ionic strength adjusted to 0.5 M. The formation of hydroxyphenols (O) was followed at 290 nm and that of nitrophenols (●) at 400 nm. The formation of hydroxyphenols was not detected above pH 6.0.

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Figure 2.2. Effect of pH on the Observed Rate Constants for the Spontaneous Decomposition of Peroxynitrite in the Absence of Any Substrate and for the Sum of the Observed Rate Constants of Hydroxylation and Nitration. Peroxynitrite was allowed to decompose in 200 mM acetate (pH < 6.0) or phosphate buffers (pH ≥ 6.0) or allowed to react with phenol (5 mM) in the pH range 4.0 to 10.0. The buffers contained 0.1 mM dtpa with ionic strength adjusted to 0.5 M. (□) The sum of the observed rate constants of hydroxylation and nitration as a function of pH reported from Fig. 2.1. (■) Observed rate constant for the spontaneous decomposition of peroxynitrite in the absence of phenol as a function of pH.
However, this mechanism predicts that the observed rate constants for nitration, hydroxylation, nitrate formation, and peroxynitrite decomposition should all be equal to the rate of formation of the activated intermediate, HOONO\(^*\), which is formed in the rate limiting step. This is not what is found. Ramezanian et al. (139) were unaware of the reaction of peroxynitrite with CO\(_2\) at the time they published their study, and they correctly pointed out that their mechanism did not satisfactorily explain the different pH dependences for the rates of nitration and hydroxylation.

As stated in the text, the data of Fig. 2.2 require more than one intermediate, and we here suggest that they are HOONO\(^*\) and O = N\(=\)O\(=\)CO\(_2\)^\(-\), as shown in Scheme 2.2.

![Scheme 2.2. Mechanism for the Reaction of peroxynitrite with Phenol involving Two Intermediates, O = N\(=\)O\(=\)CO\(_2\)^\(-\) and HOONO\(^*\).](image)

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analyzed by HPLC. As shown in Figs. 2.3 and 2.4, the reaction of peroxynitrite with phenol yields mainly \( o^- \) and \( p^- \)-hydroxyphenols, and \( o^- \) and \( p^- \)-nitrophenols. Small yields of \( p^- \)-nitrosophenol are found at pH above 7.5, a mechanistically interesting reaction we are investigating further. Benzoquinone, \( 2,2' \)- and \( 4,4' \)-biphenols are formed in very low yields (less than 0.9% based on the initial concentration of peroxynitrite), both in the presence and absence of carbonate (Fig. 2.4).

In the absence of added carbonate, hydroxyphenols are formed mainly in the pH range 4.0-6.5, whereas the nitrophenols are produced in the pH range 6.0-8.0. In the presence of added carbonate, the yields of nitrophenols increase whereas hydroxylation is inhibited (compare Fig. 2.3 and Fig. 2.5). The yields of \( o^- \) and \( p^- \)-nitrophenols formed in the reaction of phenol (1 mM) with peroxynitrite (1 mM) at pH 7.6 were determined by GC-MS (Fig. 2.6). The combined yield of \( o^- \) and \( p^- \)-nitrophenols obtained in the absence of added carbonate is 3.6 mol% relative to peroxynitrite employed in the reaction (2.1). The yields of both \( o^- \) and \( p^- \)-nitrophenols increase with increasing concentrations of carbonate as shown in Fig. 2.6. The maximum combined yield of nitrophenols in the presence of 20 mM NaHCO\(_3\) is 142 ± 0.5 \( \mu \)M (14.2 mol% relative to peroxynitrite), which is approximately 4-fold higher than the combined yield of nitrophenols obtained without added carbonate (Table 2.1).
Table 2.1. Comparison of the Yields of ortho- and para-Nitrophenols and their Ratio in the Absence and Presence of Added Carbonate.

<table>
<thead>
<tr>
<th>Total added carbonate (mM)</th>
<th>% Yield of ortho-nitrophenol</th>
<th>% Yield of para-nitrophenol</th>
<th>ortho/para ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.0 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>1.25</td>
</tr>
<tr>
<td>20</td>
<td>7.9 ± 0.2</td>
<td>6.3 ± 0.4</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Typical experiments contained 1 mM phenol, 1 mM peroxynitrite in 100 mM phosphate buffer and 0 or 20 mM of added NaHCO₃. Nitrophenols were analyzed by GC-MS as described in the methods. The final pH was measured to be 7.6. The samples were thermostated at 25°C. Each value represents the mean of two different determinations.
Figure 2.3. Yields of the Products of the Reaction of Peroxynitrite with Phenol in the Absence of Added Carbonate as a Function of pH. Peroxynitrite (0.8 mM) was allowed to react with phenol (5 mM) in the pH range 4.0-8.5. Acetate buffers were used from pH 4.0 to pH 5.5 and phosphate buffers were used for pH 6.0 and above. All buffers contained 0.5 mM dtpa and their ionic strength was adjusted to 1 M with sodium chloride. (▼), Hydroquinone; (▼), catechol; (●), o-nitrophenol; (○), p-nitrophenol.
Figure 2.4. Yields of Minor Products from the Reaction of Peroxynitrite with Phenol in the Presence and Absence of 25 mM Carbonate as a Function of pH. Peroxynitrite (0.8 mM) was allowed to react with phenol (5 mM) in the presence of carbonate (25 mM) in the pH range 4.0-8.5. Acetate buffers were used from pH 4.0 to pH 5.5 and phosphate buffers were used for pH 6.0 and above. All buffers contained 0.5 mM dtpa and their ionic strength was adjusted to 1 M with sodium chloride. (♦), Benzoquinone; (■), 4,4'-biphenol; (□), 2,2'-biphenol; (○), p-nitrosophenol.
Figure 2.5. Yields of the Products of the Reaction of Peroxynitrite with Phenol in the Presence of 25 mM Added Carbonate as a Function of pH. Peroxynitrite (0.8 mM) was allowed to react with phenol (5.0 mM) in the presence of carbonate (25.0 mM) in the pH range 4.0-8.5. Acetate buffers were used from pH 4.0 to pH 5.5 and phosphate buffers were used for pH 6.0 and above. All buffers contained 0.5 mM dtpa and their ionic strength was adjusted to 1 M with sodium chloride. (▼), hydroquinone; (▲), catechol; (○), p-nitrophenol; (●), o-nitrophenol.
Figure 2.6. Yields of α- and ρ-Nitrophenols Obtained from the Carbonate-Catalyzed Nitration of Phenol as a Function of the Concentration of Carbonate at pH 7.6. Peroxynitrite (1 mM) was allowed to react with phenol (1 mM) in 0.1 M phosphate buffer containing varying concentrations of carbonate (0-20 mM). The yields were measured by GC-MS as described in the materials and methods. Each point represents the mean of two different determinations. (○), ρ-nitrophenol; (●), α-nitrophenol.
Figure 2.7 shows the yield of nitration products as a function of phenol concentration in the presence of 20 mM added carbonate and 1 mM peroxynitrite. The maximum yield of nitration products was 20 mol% based on the concentration of peroxynitrite employed in the reaction (Fig. 2.7). The \( o/p \) ratio of nitrophenols remains constant at 1.25 ± 0.09 over the range of 0-20 mM of added carbonate (Table 2.1).

**Kinetics of the Nitration of Phenol by Peroxynitrite in the Presence of Carbonate.** The kinetics of the peroxynitrite-mediated nitration of phenol were studied at pH 7.4 and 25°C in the presence of varying concentrations of added carbonate. As shown in Fig. 2.8, the observed pseudo-first-order rate constant, \( k_{obs} \), increases linearly with the concentration of carbonate. In general, in the presence of a given concentration of carbonate, the value of \( k_{obs} \) is unaffected by increasing the concentration of phenol (which was in excess over that of peroxynitrite), indicating the reaction is zero-order in phenol (data not shown). Overall, in the presence of added carbonate, the reaction is always first-order in peroxynitrite, first-order in carbonate, and zero-order in phenol. The apparent second-order rate constant for the carbonate-catalyzed nitration of phenol is 1.25 ± 0.02 \( \times 10^3 \) M\(^{-1}\)s\(^{-1}\) at pH 7.4 and 25°C (Fig. 2.8).
Figure 2.7. Influence of the Concentration of Phenol on the Total Yield of Nitration from the Reaction of Phenol with Peroxynitrite in the Presence of Added Carbonate. Phenol (0-30 mM) was reacted with peroxynitrite (1 mM) in the presence of 20 mM added carbonate. All reactions were carried out in 0.2 M phosphate buffer at pH 7.6. The yields were estimated from the absorbance of the reaction mixtures at 400 nm.
Figure 2.8. Stopped-flow Study of the Nitration of Phenol by Peroxynitrite in the Presence of Varying Amounts of Carbonate at 25°C and pH 7.4. Peroxynitrite (0.4 mM) was allowed to react with varying concentrations of added carbonate (5.0 to 30.0 mM) in a 0.2 M phosphate buffer containing 20.0 mM phenol and 0.1 mM dtpa. The ionic strength was maintained at 0.5 M using sodium chloride. The kinetics were followed by monitoring the build-up of nitrophenol at 400 nm. The straight line represents the plot of $k_{obs}$ for nitrophenol formation as a function of the concentration of carbonate. The apparent second-order rate constant for the carbonate-catalyzed nitration was determined from the slope.
Figure 2.9 shows the apparent second-order rate constant for the nitration of phenol by peroxynitrite in the presence of added carbonate at pH values ranging from 4.0 to 8.5. The pH profile of $k_{\text{app}}$ is bell-shaped with a maximum around pH 6.5, similar to that for the reaction of peroxynitrite with carbonate (38). This suggests that the peroxynitrite anion $\cdot CO_2$ reaction and phenolic nitrations have the same rate determining step, i.e., the formation of the $O = NOCO_2^-$ adduct (1). We obtained the rate constant for the $CO_2$-catalyzed nitration of phenol by peroxynitrite from a fit of Eq. (2.1) to the data in Fig. 2.9. (The derivation of Equation [2.1] is shown in the Appendix.)

$$k_{\text{app}} = \frac{k_2K_1[H^+]}{(K_1 + [H^+])(K_2 + [H^+])} \quad [2.1]$$

At 25 °C, the value of $k_{\text{app}}$ is $3.1 \pm 0.3 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$. This value is similar to the values reported by Lymar and Hurst (88) and Denicola et al. (142) for the reaction of ONOO$^-$ with CO$_2$.

Discussion

Nitration and Hydroxylation in the Absence of Purposefully Added Carbonate. The reaction of phenol with peroxynitrite is zero-order in phenol. In the absence of added carbonate, the plot of $k_{\text{obs}}$ vs pH for the formation of hydroxyphenols is different from that for the formation of
Figure 2.9. Apparent Second-Order Rate Constant for the Carbonate-Catalyzed Nitration of Phenol by Peroxynitrite as a Function of pH at 25°C. Peroxynitrite (0.25 mM) was allowed to react with phenol (5 mM) in presence of 20-50 mM added carbonate at different pH values and the formation of nitrophenol was followed at 400 nm. Apparent second-order rate constants were calculated from the slope of the plot of $k_{obs}$ vs [carbonate]. Each point represents the mean of six measurements. (■): experimental data and (−): fit to Eq. [2.1].
formation of hydroxyphenols is different from that for the formation of nitrophenols; the maximum rate of hydroxylation occurs around pH 5.5 whereas the rate of nitration reaches its maximum around pH 7.0 (Fig. 2.1). Figure 2.2 shows that the sum of the values of $k_{\text{obs}}$ for the formation of hydroxyphenols and nitrophenols approximately equals the value of $k_{\text{obs}}$ obtained for the spontaneous decay of peroxynitrite to nitrate, under identical conditions, in the absence of any substrate. The pH dependences of the yields of hydroxyphenols and nitrophenols also differ and match the trends observed in the plot of $k_{\text{obs}}$ vs pH; the yield of hydroxyphenols is maximum around pH 5.5 while nitration shows a maximum at approximately pH 7.0.

A mechanism involving two intermediates is necessary to explain the pH profiles of nitration and hydroxylation. If nitrophenols and hydroxyphenols came from a single intermediate (formed in a rate limiting process), then the $k_{\text{obs}}$ for hydroxylation, nitration, and for the spontaneous decomposition of peroxynitrite would be identical. Our interpretation involving two intermediates differs from that of Ramezanian et al. (139), who postulated that a single intermediate, formed during the isomerization of peroxynitrous acid to nitrate (61,70), is responsible for both nitration and hydroxylation of phenolic compounds (139). (Note, however, that these authors, in agreement with our data, report that hydroxylation and nitration have different pH dependences.)
Nitration and Hydroxylation in the Presence of Purposefully Added Carbonate. Addition of carbonate (25 mM) causes hydroxylation to decrease and nitration to increase over the pH range 4.0-8.5. The rate of nitration is a maximum where the concentrations of $\text{ONOO}^-$ and $\text{CO}_2$ are a maximum, since $\text{ONOO}^-$ and $\text{CO}_2$ react to form the nitrating species, adduct 1, as shown in Eq. [2.2 and 2.3] and Scheme 2.3. The ratio of HOONO/ONOO$^-$ reaches a maximum in acidic pH and ONOO$^-$ reaches a maximum at basic pH. [The pK of HOONO is 6.8 (41, 61) and the pK value for the hydration of carbonic acid, [Eq. 2.10] in the Appendix, has a value of 6.1-6.3 (88, 146).] Thus, nitration reaches a maximum rate at about pH 6.5 (Figure 2.9).

\[
\begin{align*}
\text{ONOO}^- + \text{H}^+ &\rightarrow \text{ONOOH} \rightarrow \text{ONOOH}^+ \rightarrow \text{H}^+ + \text{NO}_3^- & [2.2] \\
\text{O} = \text{NOO}^- + \text{CO}_2 &\rightarrow \text{O} = \text{N-O-O-CO}_2^- & [2.3]
\end{align*}
\]

Further Oxidation of Catechol and Hydroquinone. To ensure that hydroxyphenols were not formed and subsequently consumed, 50 $\mu$M catechol or hydroquinone was added before addition of peroxynitrite (Table 2.2). Hydroquinone and catechol were partially recovered (64% and 72%, respectively). This shows that the reaction of the hydroxylated products with the peroxynitrite/$\text{CO}_2$ adduct(s) causes a 28-36% decrease
Table 2.2. Effect of Spiking the Peroxynitrite/Phenol Reaction with Catechol or Hydroquinone.

<table>
<thead>
<tr>
<th>5 mM phenol + 0.8 mM peroxynitrite + 25 mM carbonate PLUS</th>
<th>Final Yields</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydroquinone (µM)</td>
</tr>
<tr>
<td>Nothing</td>
<td>13.7 ± 1.2</td>
</tr>
<tr>
<td>+ 50 µM catechol before reaction.</td>
<td>6.4 ± 4.3</td>
</tr>
<tr>
<td>+ 50 µM catechol added after reaction.</td>
<td>15.6 ± 1.7</td>
</tr>
<tr>
<td>+ 50 µM hydroquinone before reaction.</td>
<td>45.5 ± 2.1</td>
</tr>
<tr>
<td>+ 50 µM hydroquinone added after reaction.</td>
<td>57.6 ± 3.2</td>
</tr>
</tbody>
</table>

All reactions were carried out using fast flow mixing in a 0.25 M acetate buffer containing 0.1 mM dtpa. The final pH was measured to be 4.9. The yields of hydroquinone and catechol were measured by HPLC.
in their yield. However, in the peroxynitrite/phenol reactions, we observe decreases in the yields of hydroquinone of 73% and catechol of 93%, indicating that hydroxylation of phenol by peroxynitrite is inhibited in the presence of added carbonate.

**Nitration by Peroxynitrite.** The plots of the apparent rate constant vs pH for the formation of nitrophenols in the presence and absence of added carbonate show the same trend, a bell-shaped curve, with a maximum around pH 6.5 (Fig. 2.9 and ref. (88)). The o/p ratio of nitrophenols formed is also the same, both in the presence and absence of added carbonate (Table 2.1). Aqueous solutions in equilibrium with air are inevitably contaminated with carbonate (89,146). Therefore, we suggest that the same species or species with the same selectivity (i.e., intermediate 1 or the secondary products derived from it) are responsible for nitration, both in the presence and absence of added carbonate.

**Possible Mechanisms for the CO₂-Catalyzed Nitrations of Phenol.** Our results show that the rate constants for formation and yields of nitrophenols increase with increasing concentrations of added carbonate (Figs. 2.6 and 2.8). The reaction is first-order in both peroxynitrite and carbonate but remains zero-order in phenol, suggesting that the reaction between the peroxynitrite anion and CO₂ leading to the formation of adduct 1, \( O = N-OOCO₂^- \), is rate limiting. Intermediate 1 structurally
resembles classic nitrosating species such as $O = N - Cl$ and $O = N - O - N = O$ ($N_2O_3$) and, therefore, could nitrosate phenol to give nitrosophenol, which might be further oxidized to nitrophenol. However, Challis and Lawson (147) reported that nitrosation by $N_2O_3$ is rapid and yields almost exclusively the $p$-isomer, with an $o/p$ ratio of 0.03. In the present study, an $o/p$ ratio of 1.25 is obtained for nitrophenol formation, a value clearly inconsistent with nitrosation of phenol by a nitrosating species similar to $N_2O_3$ (followed by oxidation).

We observed a maximum yield of 20% of phenolic nitration by peroxynitrite in the presence of excess $CO_2$ (Fig. 2.7), identical to the maximum yield of nitration of tyrosine and 4-hydroxyphenylacetate by the peroxynitrite/$CO_2$ adduct(s) reported by Lymar et al. (94) and Uppu et al. (89). Lymar et al. (94) proposed a mechanism in which a reactive intermediate is derived from adduct 1 and reacts with tyrosine to yield the tyrosyl radical, $'NO_2$, and $HCO_3^-$; subsequent coupling of the tyrosyl radical with $'NO_2$ gives nitrotyrosine.

Thermodynamical calculations (88, 148) suggest that intermediate 1 can undergo both heterolysis and homolysis to produce the radical pair, $O_2N'/CO_3^-$, and the ionic pair, $O_2N^+/CO_3^-$, respectively (Scheme 2.3). Both the ionic and radical pairs may rearrange to form $O_2N-O-CO_2^-$, (2), as proposed by Uppu et al. (89). Adduct 2, which structurally resembles the
classic nitrating species acyl nitrate, RCOONO₂, is a plausible nitrating species (Eq [2.4]).

\[
\text{O}_2\text{N}^-\text{O}^-\text{C}^-\text{O}^- + \text{ArH} \rightarrow \text{H}_2\text{NO}_{\text{2}}\text{[CO}_3\text{]}^- \rightarrow \text{ArNO}_2 + \text{HCO}_3^- \quad [2.4]
\]

Heterolytic cleavage of 1 produces the nitronium ion, \(\text{NO}_2^+\) and carbonate, \(\text{CO}_3^{2-}\) with a calculated \(\Delta G^\circ = 1.5 \text{ Kcal/mol at pH 7.0}\) (Scheme 2.3). The nitronium ion is the active nitrating species in classic aromatic nitrations, and therefore is a possible nitrating species in our system (Eq. [2.5]).

\[
\text{O}_2\text{N}^-\text{CO}_3^{2-} + \text{ArH} \rightarrow \text{H}_2\text{NO}_{\text{2}}\text{[CO}_3\text{]}^- \rightarrow \text{ArNO}_2 + \text{HCO}_3^- \quad [2.5]
\]

The \(o/p\) ratio of 1.25 for nitrophenols obtained in the present study is the same as the \(o/p\) ratio of 1-1.3 reported for classic aromatic nitrations (149,150), suggesting that the nitrating species could be \(\text{NO}_2^+\), or an \(\text{NO}_2^+\) carrier. However, a free (non-cage) nitronium ion would undergo rapid hydrolysis at neutral pH to give nitrate with a rate constant of \(5 \times 10^8 \text{ s}^{-1}\) (151), and considering the ratio of the concentrations of \(\text{H}_2\text{O}\) (55 M) and phenol (5 mM), the reaction of \(\text{NO}_2^+\) with phenol can not
Scheme 2.3. General Mechanism for the Peroxynitrite Reaction with Phenol.

We show all the possible products derived from decomposition of adduct 1, including the radical pair, $\cdot O_2N/CO_3^-$, the ionic pair, $O_2N^+/CO_3^{--}$, and the nitrocarbonate anion, $O_2NOCO_2^-$ (adduct 2); See references (29,30,39,51).
compete with the reaction of a free $^+$NO$_2$ with water. In order to
observe a yield of nitration such as the one reported here, Zhang et al.
(152) calculate that NO$_2^+$ would have to react with phenol with a second
order rate constant that is at least 4 orders of magnitude higher than the
diffusion-controlled rate. Furthermore, if the nitronium ion were the
nitrating species, the yield of nitration should increase with increasing
initial concentrations of phenol, which is not the case (Fig. 2.7).

Adduct 1 may homolyze to a caged radical-ion pair, CO$_3$$^-$ and
$^+$NO$_2$ (88,89,148,153) with a calculated $\Delta G^\circ = 5.0$ Kcal/mol at pH 7.0
(Scheme 2.3). Lymar et al. (94) observed an increase in the formation of
3,3'-dityrosine under alkaline conditions with excess CO$_2$ and suggested
that this supports a free radical mechanism. The radical-ion pair, CO$_3$$^-$/
$^+$NO$_2$, may recombine to form adduct 1, escape from the cage at a
diffusion-controlled rate, or recombine to form adduct 2 as proposed by
Uppu et al. (89) (Scheme 2.3). The carbonate radical is a good one-
electron oxidant ($E^\circ$ (HCO$_3$/HCO$_3^-$) = 1.67 V) (154) and is known to
oxidize aromatic compounds, with second-order rate constants ranging
from $10^6$ to $10^8$ M$^{-1}$ s$^{-1}$ (155). The reduction potential of the
phenoxy/phenol couple is 0.9 V (156) at pH 7.0. Therefore, it is possible
that the carbonate radical and $^+$NO$_2$ exist in a sticky cage long enough to
react with phenol (41). This sticky cage then oxidizes phenol to the
phenoxyl radical (k = 2.2 x 10^7 M^-1 s^-1 at pH 7.0) (155) and the phenoxyl radical then reacts with \(^\cdot\)NO\(_2\) to yield either \(\alpha\)- or \(\rho\)-nitrophenol (156) (Eq. [2.6]). [The tyrosyl radical, a radical similar to the phenoxyl radical, reacts with \(^\cdot\)NO\(_2\) with a rate constant of 3 x 10^9 M^-1 s^-1 at 25°C (157).]

\[
\text{\begin{align*}
O_2N^*/CO_3^- + ArH & \rightarrow \quad \text{(driving force)} \quad \text{NO}_2^-/HCO_3^- \rightarrow ArNO_2 + HCO_3^- \quad [2.6]
\end{align*}}
\]

The reaction of the phenoxyl radical with \(^\cdot\)NO\(_2\) is expected to produce phenyl nitrate, but we do not detect this product under our experimental conditions. Similarly, the reaction of tyrosyl radicals with \(^\cdot\)NO\(_2\) produces nitrotyrosine as the major product and the formation of tyrosyl nitrate was not detected (157). In both cases, it is possible that the nitrate ester hydrolyzes to give nitrate and phenol or tyrosine.

Under conditions of excess CO\(_2\), there is a limit on the maximum yield of nitrophenols (20 mol% based on the peroxynitrite employed in the reaction) (Fig. 2.6). This can be explained as follows. The radical pair, \(CO_3^*/\cdot\)NO\(_2\) are formed in a sticky cage (41) that can recombine to give either 1 or 2, react with phenol, or escape from the solvent cage to give the free radical products (Scheme 2.3) that react with water to give NO\(_3^-\) (Eq. [2.6]). Thus the yields of the cage-like products (nitrophenols) are limited by competition of the cage partners to diffuse apart and give...
nitrate. This mechanism is similar to the "sticky" cage mechanism that we discussed previously in the context of peroxynitrite cage chemistry (41).

**ONOOh** is a Likely Hydroxylating Species. An activated intermediate of peroxynitrous acid, ONOOh, has been postulated in the pathway of isomerization of ONOOH to nitrate (61,70). The yield of hydroxylation is a maximum around pH 5 where the ratio of ONOOH/ONOOh reaches a maximum and isomerization of ONOOH via ONOOh is fastest (Fig. 2.3). Denicola et al. (142) recently proposed that ONOOh is the species responsible for hydroxylation of benzoate. Kaur et al. (158) also have suggested the participation of a species with reactivity similar to that of the free hydroxyl radical in the nitration and hydroxylation of salicylate and phenylalanine by peroxynitrite. Houk et al. (148) suggest a hydrogen-bonded radical pair (HOh/ONO) as a probable structure for ONOOh. Thus, consistent with the literature, we propose ONOOh as the hydroxylating species in our system (Eq. [2.7]).

\[
\text{ONOOh} + \text{ArH} \rightarrow \left[ \begin{array}{c}
\text{HO} \\
\text{H} \\
\text{O} \\
\text{N} \\
\text{O} \\
\text{H} \\
\end{array} \right] \rightarrow \text{ArOH} + \text{HNO}_2 \]  

[2.7]
Conclusions

The reaction of peroxynitrite with phenol yields mainly hydroxyphenols and nitrophenols. In the presence of added carbonate, both the rates of nitration and the yields of nitroproducts increase while hydroxylation rates and yields decrease. These data require that peroxynitrite mediates hydroxylation and nitration via two different intermediates. We suggest that ONOOH* is the hydroxylating species and that the products of the peroxynitrite anion reaction with CO$_2$ are responsible for nitration (Scheme 2.3). While a detailed mechanism of nitration of phenol still remains to be elucidated, we suggest that likely nitrating species in these reactions include CO$_3^{2-}$/"NO$_2$ and O$_2$N-O-CO$_2^-$.

Derivation of Equation [2.1]

Equation [2.1], which was used to treat the data shown in Fig. 2.9, is derived as follows. In the presence of excess carbonate, the overall disappearance of peroxynitrite can be represented by Eqs. [2.8-2.11], where $k_1$ is a global rate constant for all the zero-order decay processes of peroxynitrous acid, $k_2$ is the rate constant for the reaction of peroxynitrite with CO$_2$, $K_1$ is the acidity constant for peroxynitrite, and $K_2$ is the equilibrium constant for hydration of CO$_2$. 

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From the above equations it is clear that the isomerization of ONOOH and the reaction of ONOO$^-$ with CO$_2$ compete with each other. The overall rate of disappearance of peroxynitrite can be represented by Eq. [2.12]

$$-\frac{d[\text{ONOOH}]_{TOT}}{dt} = k_1[\text{ONOOH}] + k_2[\text{ONOO}^-]\text{[CO}_2\text{]}$$ \hspace{1cm} [2.12]

Equation [2.13] shows the rate of disappearance of peroxynitrite as a function of the total concentrations of peroxynitrite ([ONOOH]$_{TOT}$) and carbonate ([HCO$_3^-$]$_{TOT}$), where [ONOOH]$_{TOT} = [\text{ONOOH}] + [\text{ONOO}^-]$ and [HCO$_3^-$]$_{TOT} = [\text{HCO}_3^-] + [\text{CO}_2]$. At the pH where the maximum rate occurs, the most abundant species are ONOOH, ONOO$^-$, CO$_2$ and HCO$_3^-$. [The pK$_a$ of ONOOH is 6.8 (see pK$_{1}$) (41,61) and the hydration of CO$_2$ (Eq. [2.10]) has pK$_a = 6.1-6.3$ (see pK$_2$) (88,146).]
\[
\frac{d[\text{ONOOH}]}{dt} = \frac{k_1[H^+]}{[H^+] + K_i} [\text{ONOOH}]_{\text{Tot}}
\]

\[
- \frac{k_2K_i[H^+]}{(K_1 + [H^+])(K_2 + [H^+]}) [\text{HCO}_3^-]_{\text{Tot}} [\text{ONOOH}]_{\text{Tot}} \tag{2.13}
\]

From Eq. (2.13), the observed rate constant \(k_{\text{obs}}\) for the overall decay of peroxynitrite can be obtained as given in Eq. (2.14), where the second term on the right side is the apparent second-order rate constant \(k_{\text{app}}\) for the reaction of peroxynitrite with carbonate, shown in Eq. (2.1).

\[
k_{\text{obs}} = \frac{k_1[H^+]}{[H^+] + K_i} + \frac{k_2K_i[H^+]}{(K_1 + [H^+])(K_2 + [H^+]}) [\text{HCO}_3^-]_{\text{Tot}} \tag{2.14}
\]

\[
k_{\text{app}} = \frac{k_2K_i[H^+]}{(K_1 + [H^+])(K_2 + [H^+]}) \tag{2.1}
\]
CHAPTER 3. THE CATALYTIC ROLE OF CARBON DIOXIDE IN THE DECOMPOSITION OF PEROXYNITRITE
Introduction

We here report a mechanistic probe of the peroxynitrite/CO₂ reaction. Lymar and Hurst (88), using a pH jump method, were the first to show that the reaction of peroxynitrite, ONOO⁻, with CO₂ is fast and that the adduct(s) formed cause(s) the accelerated decomposition of peroxynitrite. We confirmed those observations using both pH jump and carbonic anhydrase, and suggested that ONOO⁻/ CO₂ adducts are responsible for many of the reactions attributed to peroxynitrite or peroxynitrous acid (89). The studies by Lymar and Hurst, our group, Radi et al. (144), and Denicola et al. (142) were done under conditions where CO₂ was present in excess over peroxynitrite; under these conditions it is not possible to tell if CO₂ is consumed in the reaction or if it acts as a catalyst. That is, when CO₂ is present in excess relative to peroxynitrite, its concentration remains nearly constant during the decomposition of peroxynitrite, and the recycling of CO₂, although it occurs, can not be detected kinetically. If CO₂ is a true catalyst, then the peroxynitrite/CO₂ reaction results in the regeneration of CO₂ (not CO₃⁻ or HCO₃⁻), and this

---

4The term peroxynitrite refers to the sum of both peroxynitrite anion (ONOO⁻) and its conjugated acid, peroxynitrous acid (HOONO). The IUPAC nomenclature recommends oxoperoxonitrate (1-) for peroxynitrite and hydrogen oxoperoxonitrate for peroxynitrous acid. The term carbonate is used for the sum of all carbonated species (CO₂, H₂CO₃, HCO₃⁻, and CO₃²⁻). If a particular carbonated species is referred to, it is represented by its chemical formula.
is an important (and as we will show, revealing) restriction on any mechanism that is proposed to explain the peroxynitrite/CO$_2$ interaction.

In order to probe the role of CO$_2$ in this reaction system, we have studied systems in which the initial concentration of CO$_2$ is less than that of peroxynitrite. The data demonstrate that the CO$_2$-accelerated decomposition of peroxynitrite, under some conditions, results in the reformation of CO$_2$, which therefore is a true catalyst. This is a fundamental insight, which can only be detected in the presence of limiting amounts of CO$_2$, and it must be accommodated in any mechanism for the action of CO$_2$ in vivo as well as in vitro. In addition, the catalytic role of CO$_2$ has practical consequences in in vitro studies using non-carbonate buffers, conditions that have been used by most workers in the field (including ourselves). Buffer solutions absorb CO$_2$ from the air (dry clean air contains ca. 330 ppm CO$_2$, and indoor air a higher amount), and these low, but unregulated and irreproducible CO$_2$ impurities can have profound catalytic effects in systems that do not contain purposefully-added carbonate. We here report the decomposition of peroxynitrite in the presence of limiting amounts of CO$_2$ and the comparison of experimental data with simulations.

**Experimental Procedures**

**Reagents.** Sodium azide and sodium dibasic phosphate were purchased from EM (Gibbstown, NJ). Sodium bicarbonate was from
Mallinckrodt (Paris, KY), diethylenetriaminepentaacetic acid (dtpa) and
tetrasodium pyrophosphate were from Sigma (St. Louis, MO), and sodium
monobasic phosphate was from Baker (Phillipsburg, NJ). All solutions
were prepared using deionized water.

**Synthesis of peroxynitrite.** Peroxynitrite was synthesized according
to the method of Uppu *et al.*, in which sodium azide is reacted with ozone
(97). A Sander 200 ozonizer from Erwin Sander (Uetze-Eltze, Germany)
was used to ozonize the azide solution. The ozonation was continued
after the maximum yield of peroxynitrite was obtained to minimize the
contamination by unreacted azide. The concentration of the peroxynitrite
solution was measured using a Hewlett Packard Model 8415A diode array
spectrophotometer \( \epsilon_{302} = 1670 \text{ M}^{-1} \text{ cm}^{-1} \) (64).

**Stopped-flow studies.** Kinetics measurements at pH 7.4 were
performed on a stopped-flow spectrophotometer manufactured by On-
Line Instrument Services (Jefferson, GA) with a mixing time of less than
1.5 ms and a 2-cm long mixing cell. The stock peroxynitrite solution was
diluted to the appropriate concentration with deionized water adjusted to
pH 11.0 with 0.1 N NaOH immediately before loading into the stopped-
flow syringe. Equal volumes of the solution containing peroxynitrite and
of the solution containing a 0.1 M buffer, 0.1 mM dtpa and various
amounts of carbonate (0 or 1 mM) were mixed in the cell of the stopped-
flow apparatus. The decomposition of 0.4 mM peroxynitrite in the
presence of limiting amounts of CO$_2$ is not significantly affected by 0.05 mM dtpa (Table 3.1). Buffer contamination from atmospheric CO$_2$ is unavoidable even in freshly prepared solutions. Therefore, unless specified, our buffers were purged with argon while sonicated for about 20 min and used immediately. (Sonication is used to purge solutions of dissolved gases, e.g., in high pressure liquid chromatography (159).)

Further sonication and purging did not affect the rate of decomposition of peroxynitrite. The rate of peroxynitrite disappearance was followed at 302 nm while maintaining the temperature at 25.0 ± 0.1°C. The pH of the solution containing the phosphate buffer was recorded before mixing and the final pH was measured at the outlet. Each run was repeated six times in order to ensure reproducibility of the measurements.

**Kinetic simulations.** The kinetic simulations of the decay of peroxynitrite were performed using the Gepasi kinetic simulation software package (160). The mechanism used for simulations is shown in Figure 3.1, and the reactions and their rate and equilibrium constants are shown in Table 3.2. The equilibrium constants were calculated at 298 K from the respective standard Gibbs energies corrected for an ionic strength of 0.13 M, which is similar to the ionic strength of an isotonic saline solution (141), and $K_w$ is taken as $1.38 \times 10^{-14}$. The simulations used the rate constants shown in Table 3.2. The initial concentration of CO$_2$ is determined by the pH of the carbonate-containing buffer since the rate of
Table 3.1. Half-Life of Peroxynitrite as a Function of the Initial Concentrations of Carbonate, Nitrite, and dtpa.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Purge</th>
<th>Added Carbonate (mM)</th>
<th>dtpa (mM)</th>
<th>Nitrite (mM)</th>
<th>Half-life (s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>yes</td>
<td>0</td>
<td>0.05</td>
<td>-</td>
<td>2.56 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>no</td>
<td>0</td>
<td>0.05</td>
<td>-</td>
<td>2.42 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>yes</td>
<td>1</td>
<td>0.05</td>
<td>-</td>
<td>2.00 ± 0.11</td>
</tr>
<tr>
<td>4</td>
<td>yes</td>
<td>1</td>
<td>0.05</td>
<td>-</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>yes</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>6</td>
<td>simul.</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1.81</td>
</tr>
<tr>
<td>7</td>
<td>yes</td>
<td>1</td>
<td>0</td>
<td>9.0</td>
<td>0.64 ± 0.04</td>
</tr>
</tbody>
</table>

Peroxynitrite (0.4 mM) was decomposed at 25°C in a 0.05 M phosphate buffer pH 7.3 in the presence of various concentrations of carbonate, nitrite, and dtpa to give a final pH 7.4.

* Since the decays do not fit single exponential kinetics, we used half-life for a qualitative comparison of the peroxynitrite decomposition curves observed or simulated.

b pH jump experiment (from pH 11.0 to pH 7.4) in which carbonate (1 mM) was allowed to equilibrate at pH 11.0 with 0.4 mM peroxynitrite. The solution obtained was then rapidly mixed in the stopped-flow apparatus with a 0.05 M phosphate buffer to give a final pH of 7.4.

c Half-life obtained when the decay of peroxynitrite at pH 7.4 in the presence of 1 mM carbonate pre-equilibrated at pH 7.3 was simulated with no recycling of CO₂.
Table 3.2. Kinetic Equations and their Forward and Reverse Rate Constants Used for the Simulation of the Decay of Peroxynitrite in the Presence of CO₂.

<table>
<thead>
<tr>
<th>Kinetic equations</th>
<th>Rate constants*</th>
<th>Equilibrium constantsb</th>
<th>Refs.</th>
</tr>
</thead>
</table>
| a. H₂O + CO₂ ⇌ H₂CO₃ | \( k_1 = 0.03 \text{ s}^{-1} \)  
\( k_1 = 11.60 \text{ s}^{-1} \) | \( K_r = 2.59 \times 10^{-2} \) | (146,161) |
| b. H₂CO₃ ⇌ HCO₃⁻ + H⁺ | \( k_2 = 4.0 \times 10^2 \text{ s}^{-1} \)  
\( k_2 = 1.4 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1} \) | \( K_2 = 2.86 \times 10^{-4} \) | (146,161, 162) |
| c. HCO₃⁻ ⇌ CO₃²⁻ + H⁺ | \( k_3 = 19.18 \text{ s}^{-1} \)  
\( k_3 = 1.4 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1} \) | \( K_3 = 1.37 \times 10^{-10} \) | (146,161, 162) |
| d. HOONO ⇌ ONOO⁻ + H⁺ | \( k_4 = 2.21 \times 10^8 \text{ s}^{-1} \)  
\( k_4 = 1.4 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1} \) | \( K_4 = 1.58 \times 10^{-7} \) | (70,162) |
| e. ONOO⁻ + CO₂ ⇌ 1 | \( k_5 = 30000 \text{ M}^{-1} \text{ s}^{-1} \) | | (88,89) |
| f. HOONO ⇌ H⁺ + NO₃⁻ | \( k_6 = 1.38 \text{ s}^{-1} \) | | (69,70) |
| g. CO₂ + OH⁻ ⇌ HCO₃⁻ | \( k_7 = 8500 \text{ M}^{-1} \text{ s}^{-1} \)  
\( k_7 = 1.99 \times 10^{-4} \text{ s}^{-1} \) | | (161) |
| h. [1 or X] − CO₂ + NO₃⁻ | \( k_8 = 0.92 \times 10^{6} \text{ s}^{-1} \) (f = 0.92)  
or \( k_8 = 0 \text{ s}^{-1} \) (f = 0)  
or \( k_8 = 1 \times 10^{6} \text{ s}^{-1} \) (f = 1) | | |
| i. [1 or X] − Y⁻ | \( k_9 = 0.08 \times 10^{6} \text{ s}^{-1} \) (f = 0.92)  
or \( k_9 = 1 \times 10^{6} \text{ s}^{-1} \) (f = 0)  
or \( k_9 = 0 \text{ s}^{-1} \) (f = 1) | | |

* For each equation, the forward rate constant is noted as \( k_r \), and \( k_r \) is the corresponding reverse rate constant.

b Equilibrium constants were calculated at 298 K from the standard Gibbs energies corrected for an ionic strength of 0.13 M (146).

c Y includes all possible products from pathways that do not produce CO₂.
Figure 3.1. A Simplified Mechanism for the Decomposition of Peroxynitrite in the Presence of CO$\text{}_2$. X represents all the possible decomposition products of adduct 1, including the radical pair, •NO$_2$/CO$_3$$^\cdot$, the ionic pair, NO$_2$$^+$/CO$_3$$^-$, and the nitrocarbonate anion, O$_2$NOCO$_2$$^-$ (2); see references (88,89,92,148).
hydration/dehydration of CO₂ is slow compared to the rate of proton transfer and the reaction of ONOO⁻ with CO₂. Therefore, the concentrations of the various carbonate species at time 0 are set to be half of those at equilibrium before mixing, while the pH is fixed at the final value that would occur in our typical stopped-flow experiment once the reaction is complete.

Results and Discussion

Introduction—a hint of difficulties. A troubling feature of peroxynitrite decompositions is that they often can not be fit by a single exponential decay curve. Both our group and that of Beckman had observed this, and had offered differing ad hoc explanations (see the discussion in appendix A in reference¹⁰). We now suggest that this deviation from a single exponential decay that is observed in unpurged solutions is due to the absorption of CO₂ from the air into the buffer solutions used, leading (partly) to a CO₂-catalyzed rate of peroxynitrite decomposition. After mixing in the stopped-flow instrument, the CO₂ concentration decreases from its initial value in the mixed solutions to one that is slightly less, since the carbonate in adduct ONOO⁻CO₂⁻ (1) is not regenerated as CO₂ in 100% yield (i.e., some is regenerated as HCO₃⁻ or CO₃²⁻; see the discussion below). Consequently, the rate gradually
slows from the initial value to a slower one. Fig. 3.2 shows a simulation of the time course for the concentration of \( \text{CO}_2 \) in the mixed solutions corresponding to a representative stopped-flow experiment.

**pH-Jump from 7.0 to 9.0.** The rates for the disappearance of peroxynitrite via its spontaneous decay or via its bimolecular reaction with micromolar concentrations of \( \text{CO}_2 \) are very similar at pH 7.4, so the change in the rate of decay of peroxynitrite as \( \text{CO}_2 \) is consumed is smooth and not easy to detect. In order to observe a more dramatic effect, the rate of peroxynitrite’s isomerization must differ noticeably from that for its reaction with \( \text{CO}_2 \); this occurs at more basic pH, where the rate of spontaneous decay of peroxynitrite is considerably smaller than its bimolecular reaction with \( \text{CO}_2 \). However, at these basic pH, \( \text{CO}_3^- \) is the predominant species and \( \text{CO}_2 \) is a negligible portion of the total carbonate. Therefore, to study the reaction of peroxynitrite at pH 9.0 with finite, low levels of \( \text{CO}_2 \), we have used a pH jump from pH 7.0 (where the \( \text{CO}_2/\text{carbonate} \) ratio is higher) to pH 9.0 (where the isomerization of peroxynitrite to nitrate is slow). Figure 3.3 shows experimental data for the decay of peroxynitrite in the presence of various limiting initial concentrations of \( \text{CO}_2 \). For each concentration of \( \text{CO}_2 \), the decomposition is clearly biphasic; the steep left hand portion of each of the three curves (0-4 s) corresponds to the reaction of peroxynitrite with \( \text{CO}_2 \) until \( \text{CO}_2 \) is consumed. The right hand portion of each of the three decay curves (>4 s) corresponds to the much slower,
Figure 3.2. Simulated Profile of the Concentrations of CO\textsubscript{2} versus Time during the Decomposition of 0.4 mM Peroxynitrite at pH 7.4. The simulated experimental conditions were: 0.05 M phosphate buffer containing 1 mM carbonate pre-equilibrated at pH 7.3, and assuming 92% recycling of CO\textsubscript{2}. Note the change of scale on the time axis. (A) CO\textsubscript{2} is used in reaction with ONOO\textsuperscript{-} and is only partially regenerated. (B) Slow regeneration of CO\textsubscript{2} from H\textsubscript{2}CO\textsubscript{3} $\rightleftharpoons$ H\textsubscript{2}O + CO\textsubscript{2}.

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non-CO₂ dependent isomerization of peroxynitrite at pH 9.0. We approximated the amount of peroxynitrite consumed in its initial fast reaction with CO₂ as the difference between the initial concentration of peroxynitrite and the concentration corresponding to the y-intercept of a line extrapolated from the right hand, slow decay curves. (One such extrapolation is shown as trace E in Fig. 3.3) The difference between the extrapolated intercept and the initial concentration of peroxynitrite approximates the concentration of peroxynitrite that reacted with CO₂. This value is plotted versus the initial concentration of CO₂ in Fig. 3.4 for each initial concentration of CO₂; this plot shows that the number of moles of peroxynitrite consumed increases linearly with the initial concentration of CO₂. The slope of the line in Fig. 3.4 is 4.6, and this value represents the number of peroxynitrite molecules decomposed by one CO₂, proving that CO₂ is recycled from the peroxynitrite/CO₂ adduct. The biphasic nature of the curves in Fig. 3.3 indicates that the CO₂ recycling is incomplete and/or that CO₂ is consumed in a reaction with HO⁻. The latter reaction, which occurs with a rate constant of \( k = 8500 \text{ M}^{-1} \text{s}^{-1} \) (Table 3.2), is significant at pH 9.0. To select between these two possibilities, we have simulated the decay of peroxynitrite in the presence of a limiting amount of CO₂ at the physiologically relevant pH of 7.4.

**Reactions and simulations at pH 7.4.** When the decay of peroxynitrite is studied in thoroughly degassed solutions produced by simultaneous sonication and argon sparging, the data shown in curve A,
Figure 3.3. Decay of Peroxynitrite in the Presence of Limiting Amounts of CO$_2$ at pH 9.0. A pH jump was used during which the carbonate buffer was pre-equilibrated at pH 7 and then, in a pH jump, the reaction carried out at pH 9.0 in a 0.1 M tetrasodium pyrophosphate buffer and 25.0 ± 0.1°C. (A) Peroxynitrite decay when no CO$_2$ was added. (B) Peroxynitrite decay in the presence of an initial concentration of 14 μM CO$_2$. (C) Peroxynitrite decay in the presence of 27 μM CO$_2$. (D) Peroxynitrite decay in the presence of an initial concentration of 55 μM CO$_2$. (E) Extrapolation of the right hand slow decay curve.
Figure 3.4. Plot of Peroxynitrite Consumed by Reaction with CO₂ (see Fig. 3.3) as a Function of the Initial Concentration of CO₂. The concentration of peroxynitrite consumed was estimated as the difference between the initial concentration of peroxynitrite (0.4 mM) and the concentration corresponding to the y-intercept of a linear regression fit to the decay observed after 4 s.
Figure 3.5, are obtained. If the solutions are not purged, the decay is observed to be 5-10% faster, due to catalysis by CO₂; curve B, Fig. 3.5 shows experimental data for solutions that are not purged. From the rate of decay of peroxynitrite in curve B, we estimate that the concentration of CO₂ in the unpurged buffer we used was 8 μM; the concentration of CO₂ in a buffer in equilibrium with clean, dry air is calculated to be 11 μM (using thermodynamic parameters(146)), in good agreement with observation.

Figure 3.5 also shows experimental data for the decomposition of peroxynitrite in the presence of 1 mM carbonate pre-equilibrated at pH 7.3 (curve D) and simulated curves for the decomposition of peroxynitrite corresponding to no reformation of CO₂ (curve C), 92% reformation of CO₂ (curve E), and 100% reformation of CO₂ (i.e. complete catalytic recycling) (curve F). We simulated the decay of peroxynitrite using the mechanism shown in Figure 3.1 and the rate constants listed in Table 3.2. The simulated decay curves are not single-exponent curves, except curve F, where 100% reformation of the CO₂ results in a constant, steady-state concentration for CO₂ and a single-exponent fit.

The results from a simulation performed assuming adducts 1 or X decompose with no reformation of CO₂ (f = 0; k₇ = 0 s⁻¹; k₈ = 1x10⁶ s⁻¹) is shown in curve C. [Note that X represents products formed from 1]
Figure 3.5. Simulated and Observed Curves for the Disappearance of 0.4 mM Peroxynitrite in a 0.05 M Phosphate Buffer at pH 7.4 and 25.0 ± 0.1°C. The concentrations given are final concentrations after mixing. Simulations generally included 100 time points and are displayed as continuous traces. Although stopped-flow traces consist of at least 100 time data points, a selection of equally spaced points is shown to allow easy distinction between experimental and simulated traces. Curve A: (●) Decay of peroxynitrite in a purged 0.05 M phosphate buffer. Curve B: (▲) Same as curve A, but using a buffer that is not purged. Curve C: Simulated decay of peroxynitrite (0.4 mM) in the presence of 1 mM carbonate at pH 7.4 if CO₂ is not regenerated. Curve D: (■) Decay of peroxynitrite in the presence of 1 mM carbonate equilibrated at pH 7.3 in a 0.05 M phosphate buffer (final pH 7.4). Curve E: Simulated decay of peroxynitrite (0.4 mM) in the presence of 1 mM carbonate at pH 7.4 if 92% of the CO₂ is regenerated. Curve F: Same as curve E, but assuming 100% of the CO₂ is regenerated.
by O-O bond homolysis (\( ^1\text{NO}_2/\text{CO}_3^- \)) or heterolysis (\( ^1\text{NO}_2/\text{CO}_3^- \)) along with the recombination product of the geminate radical pair or ion pair, the nitrocarbonate anion (\( \text{O}_2\text{NOCO}_3^- \)) \( (89,148) \). Intermediates 1 or X are thought to decompose rapidly \( (88,92,148) \), and therefore \( k_8 + k_9 \) is set as \( 1 \times 10^8 \text{ s}^{-1} \); increasing the rate of decomposition of 1 or X beyond this value has no effect on the rate of decomposition of peroxynitrite or the catalytic effect of CO\(_2\). Note that reaction f in Table 3.2 is the sum of two consecutive processes that give nitrate, the first involving formation of the activated intermediate, \( \text{HOONO}^* \), from ground state \( \text{HOONO} \), and the second the formation of nitrate from \( \text{HOONO}^* \) \( (41,70,163) \).

Curve C predicts that a biphasic curve should be observed if \( \text{CO}_2 \) is not regenerated (i.e. is not a catalyst). The first phase is a combination of the fast \( \text{CO}_2 \)-catalyzed decay of the peroxynitrite anion \( (k_5, \text{see Table 3.2}) \) and the uncatalyzed isomerization of peroxynitrous acid \( (k_6) \); the second phase appears after \( \text{CO}_2 \) is nearly exhausted, and the simulation indicates it mainly involves the uncatalyzed decomposition of peroxynitrous acid \( (k_6) \) with a minor contribution from catalysis due to \( \text{CO}_2 \) arising from the dehydration of \( \text{H}_2\text{CO}_3 \). However, the experimental data (curve D) show a smooth transition, not a biphasic curve. The half-life of peroxynitrite observed experimentally, 0.45 s, is much shorter than that obtained in a simulation in which no regeneration of \( \text{CO}_2 \) is assumed, 1.81 s (see Table 3.1).
When we simulate the decay of peroxynitrite in the presence of 1.0 mM carbonate pre-equilibrated at pH 7.3 and assume that carbonate is 100% regenerated as CO$_2$ ($f = 1$; $k_8 = 1 \times 10^6$ s$^{-1}$; $k_9 = 0$ s$^{-1}$), the simulated decay (curve F) is slightly faster than that observed experimentally (curve D, Fig. 3.5). A simulation performed assuming 92% of the CO$_2$ is reformed and 8% is converted to other carbonated species fits the experimental data (curve E, Fig. 3.5).

This 8% difference we observe from the value predicted if CO$_2$ were regenerated in 100% yield (i.e., is a perfect catalyst) could arise because the rate constants used in our simulation are slightly in error or because less than a 100% regeneration is an inherent property of this reaction system. We believe that the latter is correct, and that species that are present in the system trap some of the intermediates 1 or X and reduce the yields of nitrate and CO$_2$. Note, for example, that hydrolysis of 1 would be expected to give NO$_3^-$ and HCO$_3^-$. In addition, we believe that nitrite can intercept 1 or X (see Table 3.1); since nitrite is a common, virtually unavoidable impurity in peroxynitrite preparations (115), if nitrite does intercept these intermediates, CO$_2$ generally will not be regenerated in 100% yields.

The source of CO$_2$. The simulations in Figure 3.5 take into account CO$_2$ arising both from regeneration from its adducts with peroxynitrite and from the dehydration of H$_2$CO$_3$. An additional set of experiments was performed at pH 7.4 to study the contribution of the amount of CO$_2$
that arises from the dehydration of H$_2$CO$_3$. We carried out pH-jump experiments in which carbonate (1.0 mM after mixing) was pre-equilibrated with 0.40 mM peroxynitrite at pH 11.0, and then rapidly mixed with 0.05 M phosphate buffer containing 0.05 mM dtpa so that the final pH was 7.4. When carbonate is equilibrated at pH 11.0, it exists mostly as CO$_3$\(^-\), and the concentration of CO$_2$ is approximately 1 nM. Upon mixing with the buffer, the pH changes rapidly to 7.4, but the concentration of CO$_2$ remains near 1 nM since the hydration/dehydration reactions of CO$_2$/H$_2$CO$_3$ are slow. Under these conditions, the decay of peroxynitrite is about 20% faster than that observed in the absence of carbonate (2.00 s versus 2.56 s half-life; see Table 3.1). This is due, in part, to dehydration of H$_2$CO$_3$ (k,7) occurring simultaneously with the spontaneous decay of peroxynitrite at pH 7.4. Thus, although the contribution from the dehydration of H$_2$CO$_3$ is small, we account for it in all our simulations.

**Biphasic curves?** As noted above, a simulation that assumes no regeneration of CO$_2$ is biphasic (Figure 3.5, curve C). The initial fast phase in curve C is due to acceleration by CO$_2$, which is consumed and not recycled. The experimental curve, D, does not show a second slower phase, implying that CO$_2$ is not entirely consumed (as was assumed in
curve C) and is partially or entirely regenerated. This represents a confirmation of the complexities described in this paper.

**Mechanism of CO\(_2\) regeneration.** The reformation of CO\(_2\) can be envisioned as occurring by the net exchange of an oxygen radical-anion O\(^•−\), which has an overall apparent rate constant (164) of 1 x 10\(^9\) M\(^−1\)s\(^−1\), as shown in reaction [3.1].

\[
*\text{NO}_2/\text{CO}_3* \rightarrow \text{NO}_3^- + \text{CO}_2 \quad [3.1]
\]

It seems likely that this reaction does not occur in one step, but rather occurs via a metastable adduct, O\(_2\)N-O-CO\(_2\)^− (2) (88,89,148,164).

Alternatively, 1 or X can react with water to give nitrate, as shown in reactions [3.2a] and [3.2b]:

\[
\text{ONOOCO}_2^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + \text{H}^+ + \text{HCO}_3^- \quad [3.2a]
\]

\[
\text{X} + \text{H}_2\text{O} \rightarrow \text{H}^+ + \text{NO}_3^- + \text{HCO}_3^- \quad [3.2b]
\]

The reaction of X with nitrite could convert carbon dioxide to carbonate, which, under the conditions of these experiments, is only slowly converted back to CO\(_2\), reactions [3.3] and [3.4].

\[
\text{X} + \text{NO}_2^- \rightarrow \text{N}_2\text{O}_4^- + \text{CO}_3^- \quad [3.3]
\]

\[
\text{N}_2\text{O}_4^- + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + \text{NO}_3^- + 2\text{H}^+ \quad [3.4]
\]
Table 3.1 shows that adding nitrite to peroxynitrite solutions causes an increase in the half-life for the decomposition of peroxynitrite, as expected if nitrite traps $X$ and thus reduces the amount of CO$_2$ that is regenerated.

**Summary.** We suggest the mechanism shown in Fig. 3.1 to explain the decomposition of peroxynitrite in the presence of CO$_2$. This mechanism, which is observed at limiting concentrations of CO$_2$, also holds for physiological carbonate concentrations and is crucial to the understanding of the role of the peroxynitrite/CO$_2$ adduct *in vivo*. We do not attempt to distinguish between possible intermediate(s) formed from further reactions of the initial peroxynitrite/CO$_2$ adduct, 1, represented here by $X$, since the data presented here do not allow us to select between these possibilities. (As shown in Figure 3.1, $X$ includes the radical pair, *$^{\cdot}$NO$_2$/CO$_3^-$*, the ionic pair, *$^{+}$NO$_2$/CO$_3^-$*, and the nitrocarbonate anion O$_2$NOCO$_2^-$ (88,89,142).) However, the homolysis of 1 to give *$^{\cdot}$NO$_2$/CO$_3^-$* is calculated to be fast (148), noted above, the exchange of an oxygen radical anion ($O^-$) between *$^{\cdot}$NO$_2$ and CO$_3^-$* has been reported to be very fast (164), which may occur via formation of O$_2$NOCO$_2^-*$, and this may be the mechanism for reformation of CO$_2$.

These considerations suggest that the radical pair is a likely candidate for...
a structure involved in the intermediates represented by $X$. (The radical pair is shown in reaction [3.1].)

**Implications of the recycling of CO$_2$.** The reformation and catalytic role of CO$_2$ in the decomposition of peroxynitrite is an important mechanistic aspect of the *in vivo* as well as *in vitro* chemistry of peroxynitrite. The recycling of CO$_2$ has the most noticeable effect when CO$_2$ concentrations are low. This occurs, for example, when non-carbonate buffers are used and dissolved CO$_2$ arises from absorption of CO$_2$ into aqueous solutions from the atmosphere. However, it is important to recognize that CO$_2$ regeneration/recycling is an essential step of the mechanism of reaction of the peroxynitrite/CO$_2$ adduct and must be accommodated by any mechanism that is proposed. As we have discussed in the section above, this insight also can be used to select which derivatives of $1$ are the reactive species. Of course, these insights apply both *in vivo* and *in vitro*.

Under the experimental conditions that have generally been used by all investigators in *in vitro* studies involving peroxynitrite in non-carbonate buffers, CO$_2$ is limiting and is regenerated and recycled during the decomposition of peroxynitrite. Therefore, some of the reports dealing with the chemistry of peroxynitrite may need to be reconsidered in light of the reformation and recycling of CO$_2$. For example, the yields and kinetics of peroxynitrite-mediated oxidation and nitration reactions have been shown to be CO$_2$-dependent (89,94,142,165).
REFERENCES


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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Jean-Noel Lemercier

Major Field: Chemistry

Title of Dissertation: Peroxynitrite Mediated Oxidations: Spin Trap Studies; Nitration and Hydroxylation of Phenol Result from Different Intermediates; CO2 Catalyzes the Decomposition of Peroxynitrite.

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

April 9, 1997

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