Nitration and Hydroxylation of Phenolic Compounds by Peroxynitrite

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The kinetics and products of the reaction of peroxynitrite with the phenolic compounds phenol, tyrosine, and salicylate were studied as a function of pH. All reactions are first-order in peroxynitrite and zero-order in the phenolic compound. Relative to the hydroxyl group, electrophilic substitution in the 2- and 4-positions (if available) leads to hydroxylated and nitrated products. The total yield of the products is proportional to the concentration of peroxynitrite. The sum of the rates of hydroxylation and nitration of phenol, determined by the stopped-flow technique, is approximately equal to the rate constant for the isomerization of peroxynitrite to nitrate. The rate vs pH profiles of the nitration and hydroxylation reactions parallel the yield vs pH profile with nitration maxima at pH 1.8 and 6.8, while hydroxylation is dominant between these two pH values. The activation energies for both hydroxylation and nitration are 18.8 ± 0.3 kcal mol^{-1}, identical to that of the isomerization of peroxynitrite to nitrate. Ethanol decreases the yield of hydroxylation, but has less effect on the nitration. The rate of reaction in the presence of metal complexes is first-order in metal complex and peroxynitrite and zero-order in the phenolic compound. The enhancement of the nitration of phenol by Fe(III)−edta and −nta is pH-dependent, with a maximum near pH 7, while Fe(III)−citrate, Cu(II)−edta, and $CuSO_4$ affect the nitration much less. The second-order rate constants for Fe(III)−edta at pH 4.8 and 7.2 are 1.4×10^3 and 5.5×10^3 M^{-1} s^{-1}, respectively, at 25 °C. The activation energies for the nitration reaction in the presence of Fe(III)−edta are 11.5 and 12.2 kcal mol^{-1} at pH 4.8 and 7.2, respectively. The nitration of tyrosine and salicylate by peroxynitrite is maximally enhanced by Fe(III)−edta.

Introduction

Nitrogen monoxide (*1*) has been identified as a biologically important molecule involved in a number of physiological processes, including relaxation of vascular smooth muscle, neurotransmission, platelet inhibition, and immune regulation (*2*−*4*). One of the important reactions of nitrogen monoxide is that with superoxide to form the toxic compound peroxynitrite [oxoperoxonitrate(1−), $O{=}NOO^-$] (*1*) (eq 1). The rate constant for this reaction is close to diffusion-controlled, 6.7×10^9 (*5*):

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

Oxoperoxonitrate(1−) is a relatively stable species, but its protonated form, O═NOOH, readily isomerizes (*6*, *7*) to NO_3^- and H^+ at a rate of 1.3 s^{-1} at 25 °C (*8*). Raman studies indicate (*9*) that the oxoperoxonitrate(1−) anion is present in alkaline solutions in the *cis* form. The isomerization to nitrate is thought to require a conformational change to the *trans* form (*8*−*10*). Most higher level ab initio calculations place the *trans* forms of the acid and the anion 1 and 3 kcal above the respective *cis* forms; the activation energy for interconversion between these two forms is considerable, approximately 12−15 kcal mol^{-1} for the acid and 21−24 kcal mol^{-1} for the anion (*9*). Both hydrogen oxoperoxonitrate and its anion are powerful oxidants (*8*) that damage biological compounds (*8*, *11*−*14*). The reaction of hydrogen oxoperoxonitrate with aromatic compounds produces both nitrated and hydroxylated products. These reactions proceed as fast as the isomerization to nitrate and do not depend on the concentration of the aromatic compound. As will be shown here, these reactions have the same activation energy as the isomerization to nitrate, 18 kcal mol^{-1}. In contrast, oxoperoxonitrate(1−) oxidizes methionine (*15*), sulfhydryls (*16*), and ascorbate (*17*) in second-order reactions. Given the respective bimolecular rate constants, these compounds have to be present in the millimolar range in order to compete with the first-order isomerization reaction of oxoperoxonitrate(1−). At lower concentrations, these compounds can be expected to undergo one-electron oxidations by the same intermediate that is responsible for the nitration and hydroxylation of phenolic compounds and that, in the absence of a substrate, would form nitrate. Indeed, one-electron oxidations of ascorbate (*17*) and methionine (*15*) have been observed.

The first evidence for the nitration of aromatic compounds by peroxynitrite comes from the work of Trifononow (*18*). He devised a test for nitrite in which aniline and hydrogen peroxide were added to the solution to be analyzed, followed by acidification; the yellow color of nitroaniline indicated nitrite. Mutatis mutandis, he developed tests for hydrogen peroxide and aromatic compounds (*18*). In 1952, Halfpenny and Robinson (*19*, *20*) reported the nitration and hydroxylation of aromatic compounds by "pernitrous acid" (hydrogen oxoperoxonitrate) at pH 1.4 and proposed a radical mechanism for these reactions. Beckman and co-workers showed that

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4-hydroxyphenylacetate, a tyrosine analog, is nitrated most efficiently at pH 7.5 (*21*). Van der Vliet et al. (*22, 23*) studied the reaction of phenylalanine and tyrosine with peroxynitrite and concluded that hydroxylation and nitration are caused by hydroxyl and nitrogen dioxide radicals obtained from homolysis of hydrogen oxoperoxonitrate. However, Koppenol et al. (*8*) used thermodynamic calculations and kinetic considerations to show that hydrogen oxoperoxonitrate is unlikely to yield free hydroxyl radical and nitrogen dioxide and proposed that O=NOOH itself is a strongly oxidizing agent. The oxidizing species is believed to be an intermediate closely related to the transition state for the isomerization of hydrogen oxoperoxonitrate to nitrate (*8*).

Complexes of transition metals such as iron and copper catalyze the formation of oxyradicals, and low molecular weight complexes of iron may be present in vivo (*24–26*). Such complexes may enhance the nitration of phenolic compounds. In fact, Fe(III)–edta enhances the nitration of 4-hydroxyphenylacetate quite effectively (*21*). Furthermore, bovine Cu,Zn superoxide dismutase carries out a similar reaction, which results in the modification of its tyrosine 108 to 3-nitrotyrosine (*27, 28*). Removal of the copper from the Cu,Zn superoxide dismutase prevented this reaction. The chemistry of peroxynitrite has recently been reviewed (*29, 30*).

With the stopped-flow technique, we have been able to directly measure the formation of nitrated and hydroxylated products from phenol, tyrosine, and salicylate. Here we report on the kinetics and products of these reactions in the presence and absence of iron and copper complexes.

Experimental Procedures

General. Oxoperoxonitrate(1−) was prepared by ozonizing a solution of sodium azide in sodium hydroxide (*31, 32*). The concentration of oxoperoxonitrate(1−) was determined with a Beckman (Irvine, CA) DU-7HS spectrophotometer at 302 nm ($\epsilon = 1670 \pm 50\ M^{-1}\ cm^{-1}$) (*33*). Phenol was obtained from Sigma Chemical Co. (St. Louis, MO), and sodium azide was from EM Science (Gibbstown, NJ). The pH of the solutions was adjusted with hydrochloric acid (pH 1.0–2.5), acetate buffer (pH 3.6–5.6), phosphate buffer (pH 6.0–8.0), and glycine/sodium hydroxide buffer (pH 8.0–10.0). Deionized water was used for preparing solutions. The metal complexes Fe(III)–edta[1] and Cu(II)–edta were prepared by mixing solutions of the metal ions and edta in a 1:1.1 ratio, Fe(III)–citrate was prepared by adding citric acid in 4-fold excess, and Fe(III)–nta was prepared in a 1:2 ratio. In experiments with Fe(III)–atp and Fe(III)–adp involving tyrosine, the ligand was present in 4-fold excess. Where indicated, the excess ligand was necessary to ensure complete complexation of iron(III).

Analysis of Reaction Products. Reaction products were identified and quantified by HPLC and GC–MS. We used a Milton Roy (presently Thermo Separation Products, Riviera Beach, FL) CM 4000 liquid chromatograph with an Alltech (Deerfield, IL) Microsphere 300 C18 250 × 4.6 mm reverse-phase column. The flow rate was 1 mL per minute. Hydroxylated products derived from phenol and tyrosine were detected at 280 nm. In the case of phenol, the mobile phase consisted of a 30 mM sodium citrate and 27 mM sodium acetate solution in water with acetonitrile (80:20), adjusted to pH 3.2 with 1 M H_2SO_4. Peaks were identified on the basis of coelution and mass spectrometry and quantified by peak area using external standards. The retention times, relative to phenol, of 4-hydroxyphenol (hydroquinone), 2-hydroxyphenol (catechol), 4-nitrophenol, and 2-nitrophenol are 0.45, 0.61, 1.71, and 2.37, respectively. Removal of solvent refers to the use of a rotary evaporator operating at aspirator pressure. Mass spectra were obtained with a Hewlett-Packard 5971A GC–MS. Products of the reaction of oxoperoxonitrate(1−) with tyrosine were detected at 280 nm, like those of phenol, and those of salicylate were detected at 310 nm. The mobile phase consisted of a 30 mM sodium citrate and 27 mM sodium acetate solution in water, adjusted to pH 5.1 for tyrosine or to pH 4.7 for salicylate with 1 M H_2SO_4. 2,5-Dihydroxy- and 2,3-dihydroxybenzoate elute within 10 min with this mobile phase. After 10 min, the mobile phase was gradually changed to 40% acetonitrile, which resulted in the elution of 2-hydroxy-5-nitrobenzoate within 20 min.

Kinetics. We studied the kinetics of the reactions with an OLIS stopped-flow instrument equipped with an OLIS-RSM 1000 rapid scanning monochromator (OLIS, Bogart, GA). With the help of this instrument, 1000 spectra are collected per second. The decay of oxoperoxonitrate(1−) was monitored from 250 to 350 nm. Rates of hydroxylation were monitored at 290 nm and rates of nitration at 380 nm. During all kinetic experiments the phenolic compound was present in 5–10-fold excess. The kinetics of metal-enhanced nitrations were followed by monitoring the buildup of nitrophenol at 380 nm, because the iron(III) complexes absorb strongly in the region 280–340 nm. The data were analyzed by OLIS RSM 1000 global fit software. Results from six experiments were averaged to obtain each rate constant. Temperatures were maintained to ±1 °C with a VWR Model 1160 circulating water bath.

Reaction Products of Oxoperoxonitrate(1−) with Phenol. Oxoperoxonitrate(1−) was added dropwise (final concentration if unreacted: 0.87 mM) to a solution containing excess phenol (5.0 mM), dtpa (0.2 mM), and a buffer solution (0.25 M) with stirring for 30 min at room temperature. Experiments in the presence of ethanol were carried out as follows. Oxoperoxonitrate(1−) (final concentration if unreacted: 3.0 mM) was added dropwise to a mixture of phenol (5.0 mM), phosphate buffer (125 mM), dtpa (0.2 mM), and ethanol (0.0–0.7 M) with stirring. The final pH was 6.8. The yield of *p*-hydroxyphenol decreased from 3% to 1.5% in the presence of 0.7 M ethanol. Similarly, the yields of *o*-hydroxyphenol (0.2% → 0.0%), *p*-nitrophenol (2.2% → 1.5%), and *o*-nitrophenol (3.0% → 2.2%) decreased as indicated. The total yield of reaction products, relative to oxoperoxonitrate(1−), decreased from 8% to 5% at 0.7 M ethanol. Preparative HPLC resulted in four different products. These were extracted with ether, dried over magnesium sulfate, and concentrated in a rotary evaporator. Three of the products (R_f values of 0.45, 0.61, and 1.71) were derivatized with bstfa before GC–MS spectra were recorded.

Reaction of Oxoperoxonitrate(1−) with Phenol in the Presence of Metal Complexes. Oxoperoxonitrate(1−) was added dropwise (final concentration if unreacted: 0.9 mM) to a solution of phenol (5.0 mM), metal complex (0.0–2.5 mM), and a buffer solution (0.08 M) with stirring for 30 min at room temperature.

Reaction of Oxoperoxonitrate(1−) with Tyrosine. Oxoperoxonitrate(1−) was added dropwise (final concentration: 0.74 mM) to a solution containing tyrosine (1.2 mM), dtpa (0.2 mM), and a buffer solution (0.13 M) with stirring for 30 min at room temperature.

Reaction of Oxoperoxonitrate(1−) with Tyrosine in the Presence of Metal Complexes. Oxoperoxonitrate(1−) was added dropwise (final concentration if unreacted: 0.8 mM) to a solution of tyrosine (1.2 mM) and metal complex (0.0–0.55 mM) at pH 7.3 ± 0.3 with stirring for 30 min at room temperature. The concentration of the phosphate buffer was 0.16 M.

Reaction of Oxoperoxonitrate(1−) with Salicylate. Oxoperoxonitrate(1−) was added dropwise (final concentration if unreacted: 8.0 mM) to a solution containing salicylate (10.0 mM), dtpa (0.2 mM), and a buffer solution (0.1 M) with stirring for 30 min at room temperature.

[1] Abbreviations: adp, adenosine 5′-diphosphate anion; atp, adenosine 5′-triphosphate anion; bstfa, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; dtpa, [[(carboxymethyl)imino]bis(1,2-ethanediylnitrilo)]tetraacetate anion; edta, 1,2-ethylenedinitrilotetraacetate anion; nta, nitrilotriacetate.

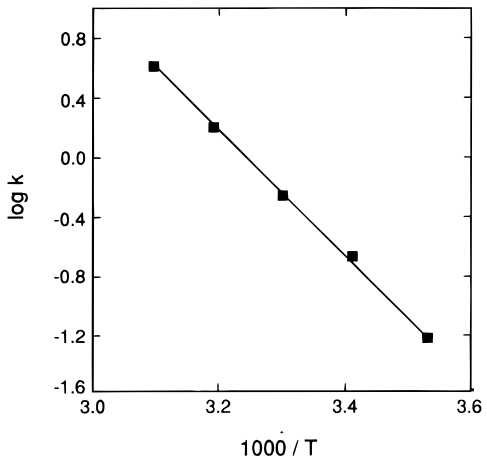

Figure 1. Arrhenius plot for the reaction of oxoperoxonitrate(1−) with phenol at pH 7.2: [$ONOO^-$] = 0.5 mM, [phenol] = 5.0 mM, and a buffer concentration of 50 mM.

Reaction of Oxoperoxonitrate(1−) with Salicylate in the Presence of Metal Complexes. Oxoperoxonitrate(1−) was added dropwise (final concentration if unreacted: 6.2 mM) to a solution of salicylate (8.0 mM), metal complex (0.0−8.0 mM), and phosphate buffer (0.1 M) at pH 6.5 ± 0.3 with stirring for 30 min at room temperature.

Results

Kinetics of Phenol. The reaction of oxoperoxonitrate(1−) with phenol was studied at pH 4.0 and pH 7.2 by monitoring the buildup of nitrophenol at 380 nm and that of hydroxyphenol at 290 nm. The rate of the reaction is first-order in oxoperoxonitrate(1−) and independent of phenol concentration over the entire concentration range of 5.0−25.0 mM. The effect of temperature was studied by monitoring the buildup of nitrophenol at pH 7.2 over the temperature range 283−323 K. The activation energy calculated from the slope of the Arrhenius plot (Figure 1) is 18.8 ± 0.3 kcal mol^{-1}, which is identical to that of the isomerization to nitrate. The activation energy of the hydroxylation reaction, determined in a similar fashion, is the same within the error.

At all pH values, the sum of the rate constants of hydroxylation and nitration is close to the rate constant for the isomerization of hydrogen oxoperoxonitrate to nitrate. A rate law to express the observed rate data is given in eq 2:

$$-\mathrm{d[O{=}NOOH]}/\mathrm{d}t = k[\mathrm{O{=}NOOH}]^1[\mathrm{phenol}]^0 \quad (2)$$

Hydrogen oxoperoxonitrate has a pK_a of 6.8 at 25 °C (*8*). Below pH 3.0, the rate of isomerization of hydrogen oxoperoxonitrate increases (*34*). The variation in the rates of hydroxylation and nitration with pH at 25 °C is shown in Figure 2. Over the pH range 1.0−10.0, the rate of nitration is maximal at pH 1.5 and 6.8, with a minimum in between. In the pH range 1.5−3.5, no hydroxylated products are observed by UV detection in the stopped flow (Figure 2), although product analysis showed that some are formed. Monitoring of these products was difficult due to the absorption of hydrogen oxoperoxonitrate and the weak absorption of the hydroxyphenols. The rate of hydroxylation is maximal at pH 4.8. Beyond pH 4.8 the rate decreased, and no product buildup was observed above pH 8.0. The rate equations to explain the observed data are given in

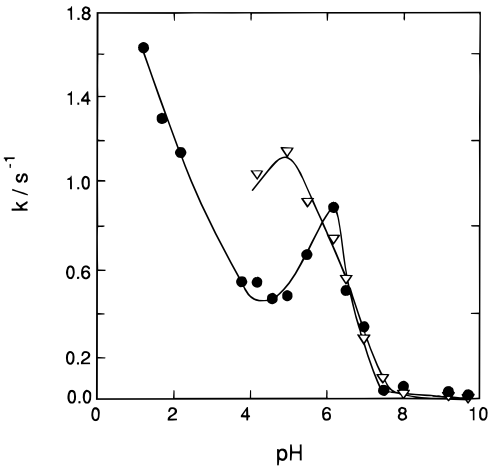

Figure 2. Rate of nitration (●) and hydroxylation (▽) vs pH for the reaction of oxoperoxonitrate(1−) with phenol at 298 K: [$ONOO^-$] = 0.5 mM, [phenol] = 5.0 mM, and a buffer concentration of 50 mM.

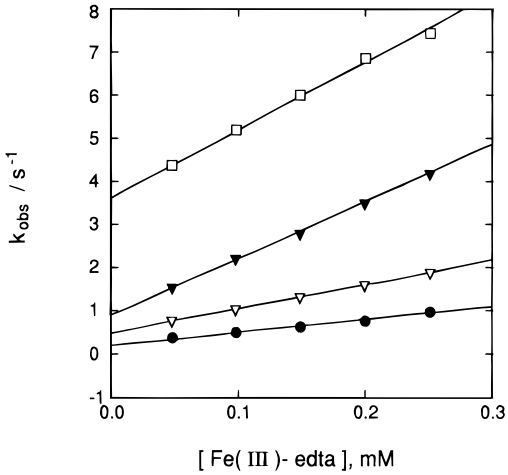

Figure 3. Plot of k vs concentration of Fe(III)−edta for the reaction of $ONOO^-$ with phenol at various temperatures. Conditions: [$ONOO^-$] = 0.5 mM; [phenol] = 5.0 mM; buffer concentration 50 mM (pH = 4.8). Symbols: ●, 288 K; ▽, 298 K; ▼, 308 K; □, 318 K.

$$+\mathrm{d[hydroxyphenol]}/\mathrm{d}t = k_h[\mathrm{O{=}NOOH}] \quad (3)$$

$$+\mathrm{d[nitrophenol]}/\mathrm{d}t = k_n[\mathrm{O{=}NOOH}] \quad (4)$$

Thus, the overall rate constant k is equal to the sum of k_h and k_n and is close to the rate of isomerization of hydrogen oxoperoxonitrate at that pH. Above pH 8.0, the rate of nitration is essentially zero.

Kinetics of Phenol in the Presence of Metal Complexes. The rate of nitration is first-order in metal complex and in oxoperoxonitrate(1−), but zero-order in phenol. Figure 3 shows the dependence of the pseudo-first-order rate constant (k_{obs}) on the metal complex concentration at pH 4.8 at different temperatures. The rate constants are calculated from the slopes of the lines (Figure 3) at pH 4.8 and 7.2 (Table 1). The activation energies are 11.5 and 12.2 kcal mol^{-1} at pH 4.8 and 7.2, respectively.

The pH dependence of the rate constant in the presence of Fe(III)−edta is shown in Figure 4. The rate of nitration is maximal at pH 7.0 ± 0.2. The rate constants at room temperature in the presence of other Fe(III) and Cu(II) complexes are shown in Table 2. Clearly, all of these complexes affect the rate of nitration, even though

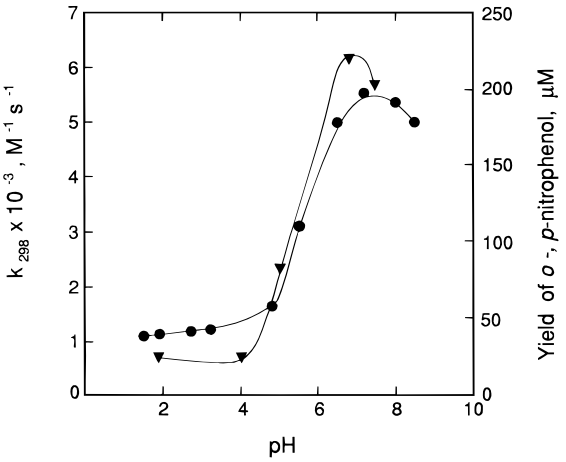

Figure 4. (●) Rate constant vs pH for the reaction of $ONOO^-$ with phenol in the presence of Fe(III)−edta. Conditions: [$ONOO^-$] = 0.5 mM; [phenol] = 5.0 mM; [Fe(III)−edta] = 0.1−0.5 mM; buffer concentration 50 mM. (▼) Yield vs pH phenol in the presence of Fe(III)−edta. Conditions: [phenol] = 5.0 mM; [$ONOO^-$] = 0.8 mM; [Fe(III)−edta] = 4.0 mM; buffer concentration 125 mM.

Table 1. Second-Order Rate Constants for the Reaction of Oxoperoxonitrate(1−) with Phenol in the Presence of Fe(III)−edta at pH 4.8 and 7.2

	k (M^{-1} s^{-1})	
T (K)	pH 4.8	pH 7.2
288	$(5.5 \pm 0.1) \times 10^2$	$(2.9 \pm 0.2) \times 10^3$
298	$(1.4 \pm 0.1) \times 10^3$	$(5.5 \pm 0.1) \times 10^3$
308	$(2.5 \pm 0.2) \times 10^3$	$(1.2 \pm 0.3) \times 10^4$
318	$(4.8 \pm 0.2) \times 10^3$	$(1.7 \pm 0.5) \times 10^4$

Table 2. Rate Constants for the Reaction of Oxoperoxonitrate(1−) Phenol in the Presence of Various Metal Complexes at pH 7.2

metal complexes	k_{298} (M^{-1} s^{-1})
Fe(III)−edta	$(5.5 \pm 0.1) \times 10^3$
Fe(III)−nta	$(1.5 \pm 0.1) \times 10^3$
Cu(II)−edta	$(9.3 \pm 0.1) \times 10^2$
$CuSO_4$	$(6.5 \pm 0.1) \times 10^2$

they behave differently. Fe(III)−edta and Fe(III)−nta enhance the rate without showing any saturation at pH 7.0 ± 0.2. The influence of Fe(III)−citrate on the rate of the reaction at pH 7.0 ± 0.2 is very small compared to other complexes. A control experiment was carried out at pH 7.0. A known amount of Fe(III)−edta and phenol in one syringe of the stopped flow was mixed with oxoperoxonitrate(1−) in the other syringe. The rate of the reaction was determined. The reactants were also mixed outside the stopped flow, and the stopped-flow experiment was repeated by reacting oxoperoxonitrate-(1−) with this solution. The pseudo-first-order rate constant was smaller. By repeating this experiment we observed that the pseudo-first-order rate constant decreased continuously and reached the same value as that of the decay of oxoperoxonitrate(1−) in the absence of metal complexes.

Products of Phenol. Products of the reaction of phenol with oxoperoxonitrate(1−) in the pH range 1.0−10.0 were determined by HPLC and mass spectrometry. The mass spectrum of the derivatized sample with R_f = 0.45 yields peaks at m/z 254 ($M^{\bullet+}$), 239 ($M - CH_3^{\bullet+}$), and 73 [$(Si(CH_3)_3^{\bullet+}$]. R_f = 0.61: m/z 254 ($M^{\bullet+}$), 239 ($M - CH_3^{\bullet+}$), 91 ($C_7H_7^{\bullet+}$), 77 ($C_6H_5^{\bullet+}$), and 73 [$Si(CH_3)_3^{\bullet+}$]. R_f = 1.71: m/z 211 ($M^{\bullet+}$), 196 ($M - CH_3^{\bullet+}$), 91 ($C_7H_7^{\bullet+}$), 77 ($C_6H_5^{\bullet+}$), and 73 [$Si(CH_3)_3^{\bullet+}$]. The mass spectrum of the

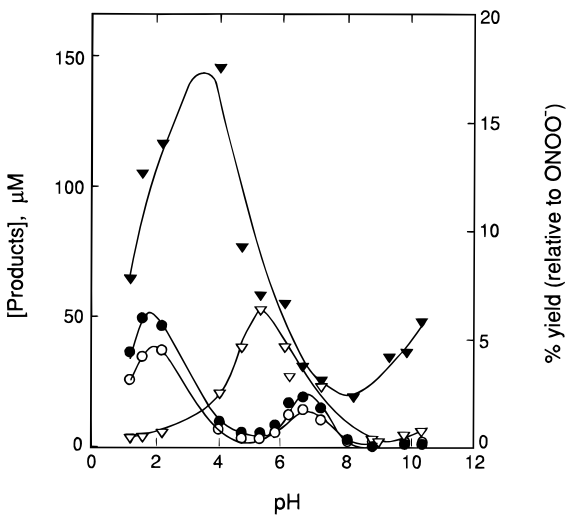

Figure 5. Yield of hydroxylated and nitrated products vs pH for the reaction of oxoperoxonitrate(1−) with phenol: ▼, *o*-hydroxyphenol; ▽, *p*-hydroxyphenol; ●, *o*-nitrophenol; ○, *p*-nitrophenol. Conditions: [$ONOO^-$] = 0.8 mM; [phenol] = 5.0 mM; buffer concentration 125 mM.

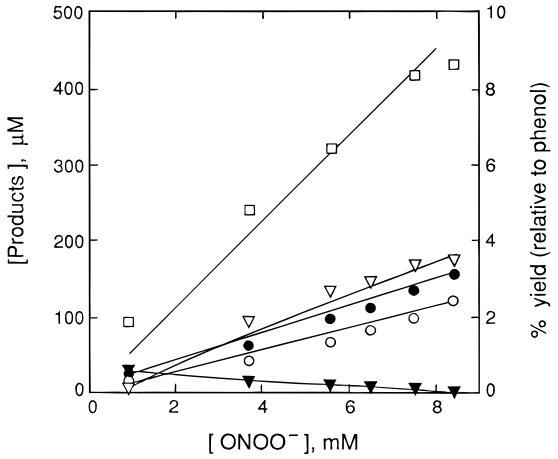

Figure 6. Yield of hydroxylated and nitrated products vs concentration of $ONOO^-$ for the reaction of oxoperoxonitrate-(1−) with phenol at pH 6.8 ± 0.2. Conditions: [phenol] = 5.0 mM; buffer concentration 125 mM. Symbols: □, total yield; ▼, *o*-hydroxyphenol; ▽, *p*-hydroxyphenol; ●, *o*-nitrophenol; ○, *p*-nitrophenol.

underivatized sample with R_f = 2.37 yields peaks at m/z 139 ($M^{\bullet+}$), 109($M - NO^{\bullet+}$), 93 ($M - NO_2^{\bullet+}$), 81 [$M - (NO,-CO)^{\bullet+}$], 78 ($C_6H_6^{\bullet+}$), and 65 ($C_5H_5^{\bullet+}$). The following compounds were identified: *p*-hydroxyphenol (R_f = 0.45), *o*-hydroxyphenol (R_f = 0.61), *p*-nitrophenol (R_f, = 1.71), and *o*-nitrophenol (R_f = 2.37).

The yield of *o*-hydroxyphenol increases rapidly with increasing pH to a maximum near pH 4.0, followed by a decrease and another rise above pH 8.0 (Figure 5). *o*- and *p*-nitrophenol show maxima near pH 2.0 and 6.6, while little is formed near pH 4.7. Formation of *p*-hydroxyphenol is maximal around pH 5.0, while above pH 9.0 only trace amounts are observed. *o*-Hydroxyphenol appears to be the only product formed at alkaline pH. The maximal yield of reaction products is 24% near pH 2.0, relative to oxoperoxonitrate(1−) (Figure 6). Around this pH the yields of nitrated and hydroxylated products are similar. Overall, the yields of *o*-hydroxyphenol and *o*-nitrophenol are greater than those of para-substituted compounds (Figure 5). Beckman et al. (*21*) reported maximal nitration of 4-hydroxyphenylacetate at pH 7.4. Because the phosphate concentration in their experi-

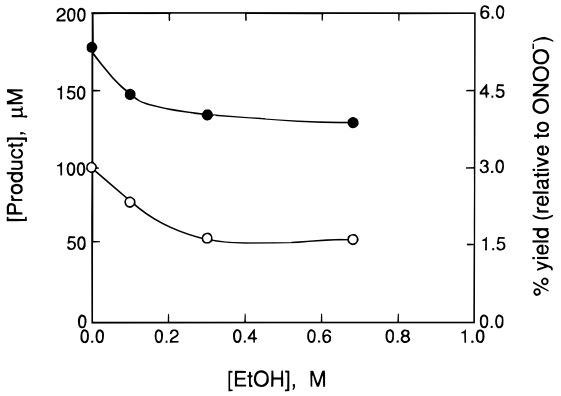

Figure 7. Total yield of hydroxylated and nitrated products vs concentration of ethanol for the reaction of oxoperoxonitrate(1−) with phenol at pH 6.8 ± 0.2. Conditions: [$ONOO^-$] = 3.0 mM; [phenol] = 5.0 mM; buffer concentration 125 mM. Symbols: ●, *o*- and *p*-nitrophenol; ○, *o*- and *p*-hydroxyphenol.

ments was lower than that in ours, we varied the phosphate concentration from 50 to 250 mM. However, no change in the pH maximum was observed (not shown).

The effect of the concentration of oxoperoxonitrate(1−) on the total yield of products is shown in Figure 6. Generally, the yield increases linearly with the oxoperoxonitrate(1−) concentration. However, the yield of *o*-hydroxyphenol decreases with increasing concentrations of oxoperoxonitrate(1−). It is possible that *o*-hydroxyphenol is further oxidized by oxoperoxonitrate(1−). The ratio of *o*-nitrophenol to *p*-nitrophenol (1.4:1) remained the same throughout all experiments.

Figure 7 presents the effect of ethanol on the yield of products at pH 6.8 ± 0.2. The yield of *o*- and *p*-hydroxyphenol decreases with increasing ethanol concentration. At concentrations of ethanol greater than 0.4 M, only trace amounts of *o*-hydroxyphenol are formed. Formation of nitrophenol products also decreases, but the effect of ethanol on hydroxylation is much greater than that on nitration.

Since nitrate is the product of isomerization of oxoperoxonitrate(1−), a control experiment was carried out. Phenol was mixed with nitrate at various pH values between 1.0 and 7.0, but no nitration was observed. Similar experiments with nitrite near neutral pH also yielded negative results. At low oxoperoxonitrate(1−) concentrations (50−100 μM) and neutral pH, we observed the formation of small amounts of 4-nitrosophenol. These findings are currently under investigation.

Products of Phenol in the Presence of Metal Complexes. By coelution the following compounds were identified: *p*-hydroxyphenol, *o*-hydroxyphenol, *p*-nitrophenol, and *o*-nitrophenol. Nitration as a function of pH in the presence of Fe(III)−edta is shown in Figure 4. The yield remains the same from pH 1.0 to 4.0 and then increases, with a maximum at pH 7.0 ± 0.2. At pH 7.0 ± 0.2, when the ratio of oxoperoxonitrate(1−), phenol, and Fe(III)−edta is approximately 1:1:3.5, 45% of all phenol is nitrated. The increase in yield of nitrated products is at the expense of hydroxylated products.

The yield of nitration for the reaction of oxoperoxonitrate(1−) with phenol in the presence of other metal complexes at pH 7.0 ± 0.2 is shown in Figure 8. At this pH the yields increase with the concentrations of Fe(III)−nta and Fe(III)−edta (also shown in Figure 8). However, Fe(III)−citrate, copper sulfate, and Cu(II)−edta have little effect.

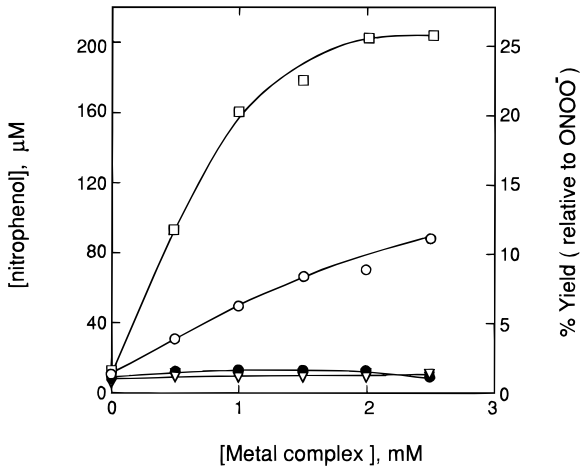

Figure 8. Yield of nitrophenol as a function of the concentration of metal complex for the reaction of $ONOO^-$ with phenol. Symbols: ○, Fe(III)−edta; □, Fe(III)−nta; ●, $Cu^{II}SO_4$; ▽, Cu(II)−edta. Conditions: pH 7.0 ± 0.2; [phenol] = 5.0 mM; [$ONOO^-$] = 0.8 mM. The buffer concentration was 125 mM.

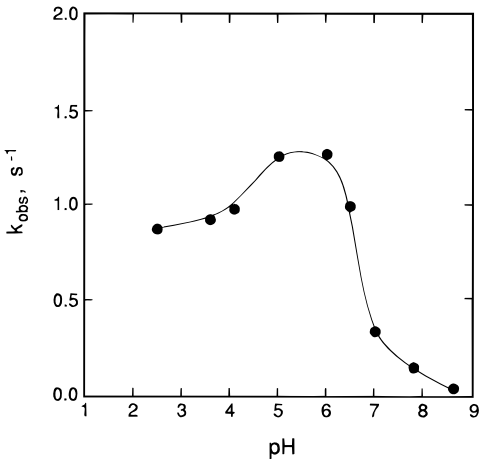

Figure 9. First-order rate constant vs pH for the reaction of oxoperoxonitrate(1−) with salicylate to form 2-hydroxy-5-nitrobenzoate. Conditions: [$ONOO^-$] = 1.0 mM; [salicylate] = 20 mM; buffer concentration 50 mM.

Kinetics of Tyrosine and Salicylate. The kinetics of the reaction of oxoperoxonitrate(1−) with tyrosine could not be studied due to the limited solubility of tyrosine. The kinetics of the reaction of oxoperoxonitrate(1−) with salicylate was studied at pH 7.1, with the latter present in excess. The reaction is followed by monitoring the buildup of the product, 2-hydroxy-5-nitrobenzoate, at 400 nm. The rate of the reaction is independent of the concentration of salicylate over the entire concentration range, and the observed first-order rate constant is approximately the same as that of the decomposition of oxoperoxonitrate(1−) at that pH.

The pH dependence of the rate of the reaction at room temperature was studied over the pH range 2.0−9.0. The rate of nitration is highly pH-dependent. The rate vs pH curve (Figure 9) shows a maximum around pH 6.0, while above pH 8.0 the rate of nitration is almost zero. No hydroxylated product could be detected by UV absorption during the stopped-flow experiment, and therefore the rate of hydroxylation could not be measured. This may be due to the very low yield of hydroxylated products.

Products of Tyrosine and Salicylate. The reaction of peroxynitrite with salicylate and tyrosine in the pH range 4.0−9.0 leads to three major products from salicylate, 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate,

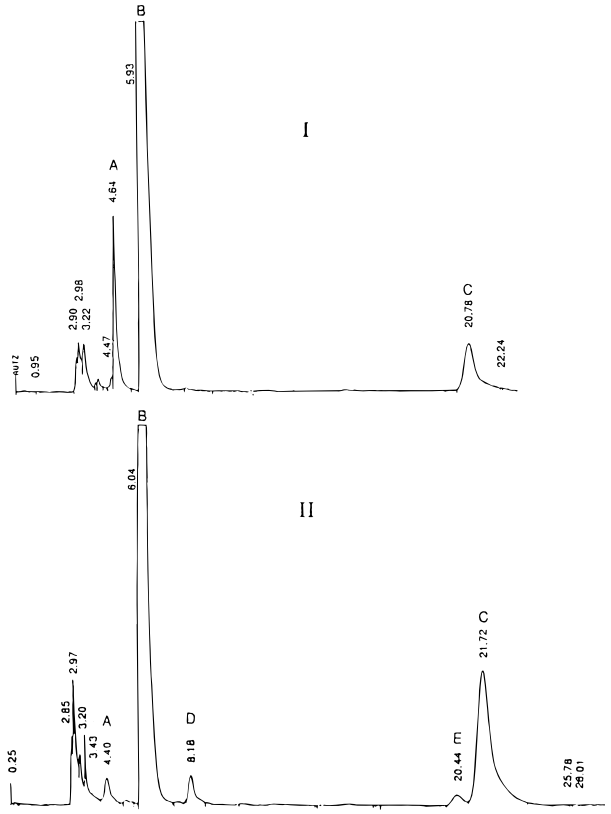

Figure 10. HPLC chromatograms of reaction products of oxoperoxonitrate(1−) (0.74 mM) with tyrosine (1.2 mM): A, 3-hydroxytyrosine; B, tyrosine; C, 3-nitrotyrosine; D and E, unidentified compounds; I, pH 4.0−5.0; II, pH 7.0.

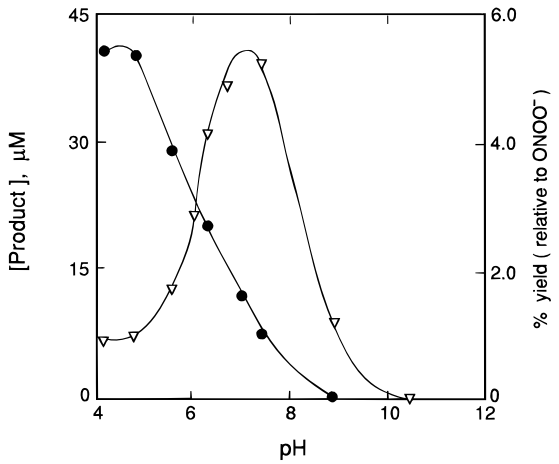

Figure 11. Yield vs pH for the reaction of oxoperoxonitrate-(1−) with tyrosine. Conditions: [$ONOO^-$] = 0.74 mM; [tyrosine] = 1.2 mM; [dtpa] = 0.2 mM; buffer concentration 0.13 M. Symbols: ●, 3-hydroxytyrosine; ▽, 3-nitrotyrosine.

and 2-hydroxy-5-nitrobenzoate, and to two major products from tyrosine, 3-hydroxytyrosine and 3-nitrotyrosine.

The HPLC chromatogram (Figure 10, panel I) shows that, between pH 4.0 and 5.0, only 3-hydroxy- and 3-nitrotyrosine are formed. The distribution of products is pH-dependent: formation of 3-hydroxytyrosine is maximal at pH 4.2, while 3-nitrotyrosine is formed mostly at pH 7.4 (Figure 11). At pH greater than 4.2, two unidentified products (Figure 10, panel II) were also observed. These unidentified compounds may be isomers of hydroxy- and nitrotyrosine. No products were detected above pH 9.0. The effect of pH on the reaction of salicylate with peroxynitrite is shown in Figure 12. At

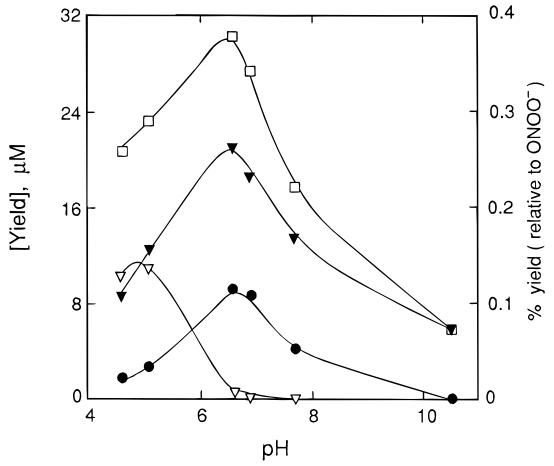

Figure 12. Yield vs pH for the reaction of oxoperoxonitrate-(1−) with salicylate. Symbols: □, total yield; ▼, 2-hydroxy-5-nitrobenzoate; ▽, 2,3-dihydroxybenzoate; ●, 2,5-dihydroxybenzoate. Conditions: [$ONOO^-$] = 8.0 mM; [salicylate] = 10.0 mM; [dtpa] = 0.2 mM^2; buffer concentration 0.10 M.

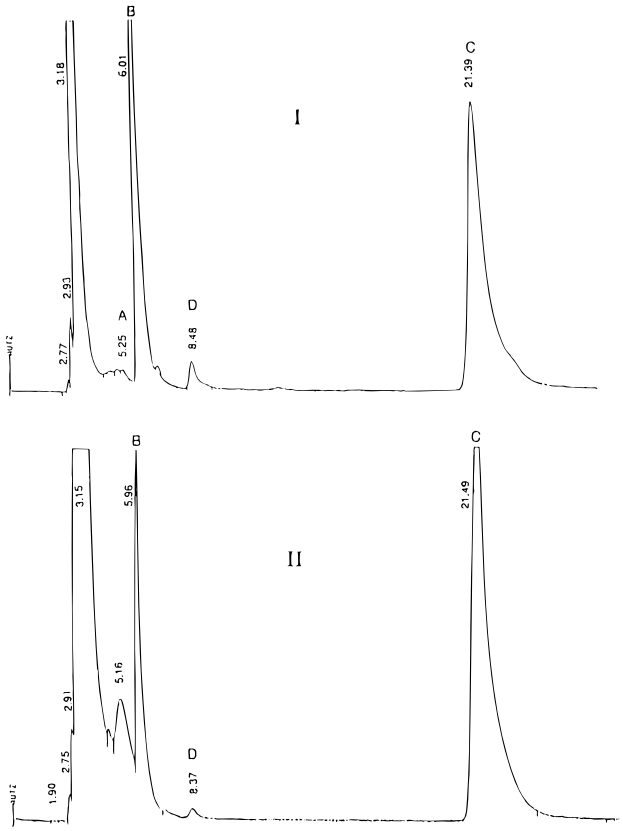

Figure 13. HPLC chromatograms of the reaction products of oxoperoxonitrate(1−) with tyrosine in the presence of Fe(III)−edta at pH 7.3 ± 0.3: A, 3-hydroxynityrosine; B, tyrosine; C, 3-nitrotyrosine; D and E, unidentified compounds; I, 0.11 mM Fe(III)−edta; II, 0.55 mM Fe(III)−edta.

all pH's more nitrated than hydroxylated products were formed. 2,3-Dihydroxybenzoate is formed between pH 4.0 and 5.0, but not above pH 6.0. The yields of 2-hydroxy-5-nitrobenzoate and 2,5-dihydroxybenzoate were maximal at pH 6.6 and decreased at higher pH.

Products of Tyrosine and Salicylate in the Presence of Metal Complexes. Transition metal complexes, especially Fe(III)−edta, enhance the nitration of tyrosine and salicylate by oxoperoxonitrate(1−). Figure 13 shows the effect of Fe(III)−edta. At 0.11 (Figure 13, panel I) and 0.55 mM (Figure 13, panel II) Fe(III)−

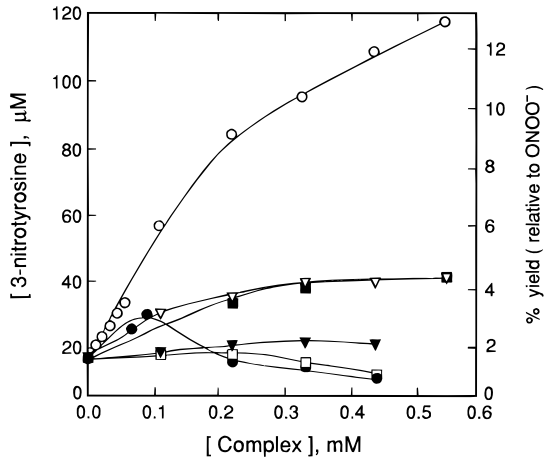

Figure 14. Yield as a function of the metalcomplex concentration for the reaction of oxoperoxonitrate(1−) with tyrosine. Symbols: ○, Fe(III)−edta; ■, Cu(II)−acetate; ▽, Fe(III)−nta; ●, Fe(III)−citrate; □, Fe(III)−adp; ▼, Fe(III)−atp. Conditions: [$ONOO^-$] = 0.8 mM; [tyrosine] = 1.2 mM; pH 7.3 ± 0.3; buffer concentration 0.16 M.

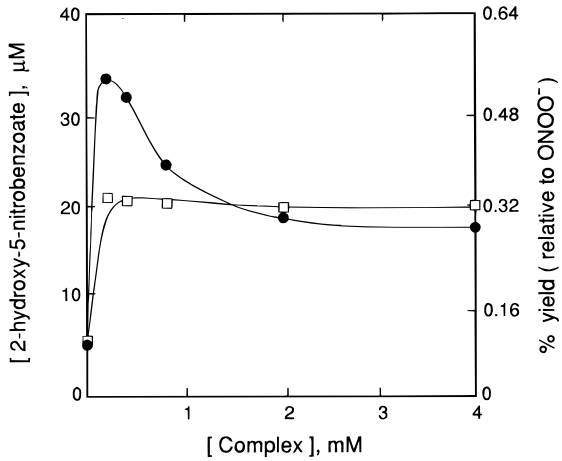

Figure 15. Yield as a function of the metal complex concentration for the reaction of oxoperoxonitrate(1−) with salicylate. Symbols: ●, Fe(III)−edta; □, copper sulfate. Conditions: [$ONOO^-$] = 6.2 mM; [salicylic acid] = 8.0 mM; pH 6.5 ± 0.3; buffer concentration 0.1 M.

edta, the yield of 3-nitrotyrosine increases (compare Figure 10), while there is almost no effect on the yield of hydroxylated and unidentified products. 3-Nitrotyrosine was obtained in 15% yield relative to oxoperoxonitrate(1−) when the ratio of oxoperoxonitrate(1−) to tyrosine to Fe(III)−edta was 1:1.2:5.5. Figure 14 shows the effect of iron and copper complexes on the reaction of oxoperoxonitrate(1−) with tyrosine at pH 7.3 ± 0.3. The yield of 3-nitrotyrosine increases sharply with increasing concentrations of Fe(III)−edta. Fe(III)−nta, Cu(II)−acetate, and Fe(III)−citrate also enhance the yield of nitrotyrosine, but Fe(III)−atp and Fe(III)−adp have little effect. Fe(III)−edta and copper sulfate, up to 0.2 mM, increase the yield of salicylate nitration at pH 6.5 ± 0.3, as shown in Figure 15. However, at higher concentrations of metal complexes the yield decreases. We have no explanation for this observation.

Discussion

Phenol. Hydrogen oxoperoxonitrate is a nitrating and hydroxylating agent capable of reacting by multiple mechanisms, which depend on the pH. Given the total yield of products, which in the absence of metal complexes is, at best, 24% relative to oxoperoxonitrate(1−), the bulk of this anion isomerizes to nitrate, since the rate of its disappearance does not depend on the phenol concentration. Thus, the mechanism of the reaction involves a rate-limiting conformational change to an intermediate, which reacts with phenol or isomerizes further to nitrate. In the pH range 1.0−2.5, where we find an increase in nitrated products, the isomerization of hydrogen oxoperoxonitrate is catalyzed by acid (*34*) and may involve the following equilibrium:

$$O{=}NOOH + H^+ \leftrightarrow O{=}NOOH_2^+ \leftrightarrow NO_2^+ + H_2O \quad (5)$$

The species $O{=}NOOH_2^+$, or even NO_2^+, could be responsible for nitration. Thermodynamic calculations suggest that heterolytic cleavage cannot occur spontaneously, because the energy required to produce an initial charge separation into NO_2^+ and HO^- in water requires approximately 45 kcal mol^{-1}. However, protonation would reduce this barrier considerably (*8*). Since at low pH the reaction is still first-order in oxoperoxonitrate(1−) and zero-order in phenol, one must assume that formation of the nitrating species is rate-limiting. Alternatively, since our oxoperoxonitrate(1−) preparations contain significant amounts of nitrite (*32*), it is conceivable that at low pH the phenol is first nitrosated by nitrous acid and that nitrosophenol is subsequently oxidized by hydrogen oxoperoxonitrate. We have found that 4-nitrosophenol is oxidized to 4-nitrophenol by oxoperoxonitrate(1−).[2] However, one would expect to observe small amounts of nitrosophenol, which we did not. For this reason, we prefer the first explanation.

In the pH range 4.0−6.0, the rate and yield of hydroxylation are higher than the rate of nitration (Figures 4 and 5). Since homolysis of the O−O bond of hydrogen oxoperoxonitrate is unlikely, we propose that an intermediate structurally similar to the transition state postulated for the isomerization to nitrate (*8*) reacts with phenol. Again, the formation of this intermediate is rate-limiting. The ratio of *o*- to *p*-nitrophenol, 1.4:1, is very similar to that found during the nitration of phenol in sulfuric acid, which presumably involves NO_2^+, the nitryl cation, and to that observed when phenol is nitrated by nitrogen dioxide in carbon tetrachloride and cyclohexane (*35*). In those cases it is assumed that the nitryl cation and nitrogen dioxide oxidize phenol to the phenoxyl radical. The standard one-electron reduction potentials of the $NO_2^+/NO_2^\bullet$ and the $NO_2^\bullet/NO_2^-$ couples are 1.6 and 0.99 V, respectively, while that of the $O{=}NOOH/NO_2^\bullet$ (H_2O) couple is estimated to be 1.4 V at pH 7 (*8*). These values are valid in water, but they do indicate that oxoperoxonitrate(1−) is oxidizing enough: the reduction potential of the phenoxyl/phenol couple is 0.9 V at pH 7 (*36*). The one-electron reduction of hydrogen oxoperoxonitrate results in water and nitrogen dioxide; the latter is likely to react with the phenoxyl radical to form 4-nitrocyclohexa-2,5-dien-1-one and 6-nitrocyclohexa-2,4-dien-1-one. This reaction is likely to be very fast, in analogy to the reaction of phenoxyl radicals with other radicals (*37*). The conversion to *p*- and *o*-nitrophenol is relatively slow (*35*), and it is possible that the nitrohexadienone intermediates hydrolyze to form catechol and hydroquinone. In order to preserve the ratio of *o*- to *p*-nitrophenol, one must assume that the rates of hy-

[2] M. S. Ramezanian, S. Padmaja, and W. H. Koppenol, Unpublished results.

drolysis of the two nitrohexadienone intermediates are the same. This mechanism does not suggest a pH dependence of the rates of hydroxylation and nitration, as observed. However, it is possible that either the rate of hydrolysis or the rate of isomerization of the nitrohexadienones to nitrophenols is pH-dependent. This mechanism accounts for the observation that the activation energies of the hydroxylation and nitration reactions are identical. While the yields of *o*- and *p*-nitrophenol as a function of pH parallel one another, as discussed earlier, this is not the case for the hydroxylated products: the yield of *p*-dihydroxybenzene is maximal at pH 5.5 and that of *o*-dihydroxybenzene at pH 3.5. One must assume that both are formed in the same ratio as the nitrophenols and that, below pH 5.5, *p*-dihydroxybenzene disappears through unknown oxidation processes.

As mentioned earlier, we observed a maximum in nitration at pH 6.6. An explanation for the nitration maximum has been offered by Beckman et al. (*10*), based on the different pK_a values of *cis*- and *trans*-hydrogen oxoperoxonitrate. These investigators observed a maximum for the yield of nitration, not at pH 6.6 but at pH 7.5, in between the pK_a values of 6.8 for the *cis* form and 8.0 for the *trans* form. Since the maximum we observed lies below the pK_a of the *cis* form, the hypothesis of Beckman et al. (*10*) does not explain our results.

At pH 6.8 ± 0.2 the hydroxyl radical scavenger ethanol reduced the yield of hydroxyphenol products by 50%, but that of nitrophenol only by 30%. In the case of the reaction of oxoperoxonitrate(1−) with phenylalanine, 100 mM mannitol decreased the yield of hydroxylated products by 56% (*22*). If O═NOOH were to undergo homolysis and form hydroxyl and nitrogen dioxide radicals, and given the rate constant for the reaction of the hydroxyl radical with mannitol ($k = 1.7 \times 10^9$ M^{-1} s^{-1}) (*38*), the 100 mM mannitol should have scavenged all hydroxyl radicals and the 40% yield of hydroxylated products (*22*) should not have been observed. Furthermore, after the reaction of oxoperoxonitrate(1−) with phenol, neither biphenol nor any other byproduct resulting from a radical mechanism was observed. Beckman and co-workers also oberved that hydroxyl radical scavengers minimally affected the yield of nitrated products (*21*).

Phenol and Metal Complexes. Transition metal complexes can increase the rate yield of nitration of phenol by oxoperoxonitrate(1−). When there is such an increase, the yield of hydroxylated products is concomitantly decreased. The effect of Fe(III) complexes on the nitration of phenol by oxoperoxonitrate(1−) is larger than that of Cu(II) complexes. Our studies on the reaction of oxoperoxonitrate(1−) with phenol show that the rate constants vary from 5.5×10^3 M^{-1} s^{-1} for Fe(III)−edta to 6.5×10^2 M^{-1} s^{-1} for $CuSO_4$ at pH 7.0 ± 0.2 and at room temperature. The activities of complexes vary with the pH of the solution. The rate vs pH curve of the nitration reaction is very similar to that of the yield vs pH curve from pH 1.0 to 9.0 in the presence of Fe(III)−edta (Figure 4). Over the pH range 4.0−9.0, the effect of pH on nitration shows the same trend as that observed in the absence of metal complexes. This indicates that the same intermediate is responsible for the nitration of phenol.

As mentioned earlier, the rate is zero-order in phenol, and therefore the interaction between oxoperoxonitrate(1−) and the metal complex is the rate-limiting step. The activation energy for the Fe(III)−edta reaction is 11.5 kcal mol^{-1}, which is close to the value reported by Beckman et al. (*21*) in the reaction of oxoperoxonitrate(1−) with phenylacetic acid in the presence of the same metal complex. The metal withdraws electron density and may promote the formation of a species that reacts like NO_2^+. This would be analogous to the protonation of O═NOOH proposed earlier.

One could argue that these metal complexes are catalysts, because they lower the activation energy from 18.8 (see above) to 11.5 kcal mol^{-1}. However, we have also shown that the activity of these metal complexes decreases upon repeated exposure to oxoperoxonitrate(1−). As the heterolysis would leave the iron(III) with a coordinated hydroxide anion, it is possible that the formation of (HO^-)Fe(III)-L inhibited the activity of the Fe(III)−edta complex. Given the pK_a of the water molecule in the seven-coordinate H_2O−Fe(III)−edta complex (*39*), this is quite feasible. It is possible that a coordination site occupied by water is a requirement for the enhancement of nitration: Fe(III)−edta is seven-coordinate and active, while Cu(II)−edta is six-coordinate and shows no activity. The increases in rate and yield are small for the Cu(II) complexes compared to those for the Fe(III) complexes.

Tyrosine and Salicylate. As observed with phenol, the reaction of hydrogen oxoperoxonitrate with tyrosine and salicylate involves at least two intermediates: one that leads to nitration and a second that results in hydroxylation. Again, the formation of these intermediates is rate-limiting. The yield of the reaction of oxoperoxonitrate(1−) with salicylate is much smaller than with phenol and with tyrosine. While salicylate is the best aromatic scavenger to detect hydroxyl radicals in vivo (*40*, *41*), the high concentrations required to obtain detectable yields make this scavenger less valuable for obtaining evidence for in vivo oxoperoxonitrate(1−) formation. It is also troublesome that the reaction of the hydroxyl radical and hydrogen oxoperoxonitrate with this scavenger yield both 2,3- and 2,5-dihydroxybenzoate. Were it not for the low yield, one could argue that previous reports (*42*, *43*) on hydroxyl radical formation in vivo have more to do with oxoperoxonitrate(1−) detection.

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