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NITRATION OF TYROSINE BY HYDROGEN PEROXIDE AND NITRITE

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Peroxynitrite anion is a powerful oxidant which can initiate nitration and hydroxylation of aromatic rings. Peroxynitrite can be formed in several ways, e.g. from the reaction of nitric oxide with superoxide or from hydrogen peroxide and nitrite at acidic pH. We investigated pH dependent nitration and hydroxylation resulting from the reaction of hydrogen peroxide and nitrite to determine if this reaction proceeds at pH values which are known to occur *in vivo*. Nitration and hydroxylation products of tyrosine and salicylic acid were separated with an HPLC column and measured using ultraviolet and electrochemical detectors. These studies revealed that this reaction favored hydroxylation between pH 2 and pH 4, while nitration was predominant between pH 5 and pH 6. Peroxynitrite is presumed to be an intermediate in this reaction as the hydroxylation and nitration profiles of authentic peroxynitrite showed similar pH dependence. These findings indicate that hydrogen peroxide and nitrite interact at hydrogen ion concentrations present under some physiologic conditions. This interaction can initiate nitration and hydroxylation of aromatic molecules such as tyrosine residues and may thereby contribute to the biochemical and toxic effects of the molecules.

KEY WORDS: peroxynitrite, nitric oxide, superoxide, inflammation, nitrotyrosine, antioxidants.

INTRODUCTION

Nitric oxide is an important intercellular messenger in blood vessels, airways and in the brain. It is also an important toxin produced by macrophages and neutrophils, along with high concentrations of reactive oxygen species such as superoxide and hydrogen peroxide, as a defence against infections. One mechanism by which nitric oxide can be converted from a messenger into a powerful oxidant is through a reaction with the superoxide anion. This reaction proceeds with a rate constant of greater than 10^9 M^{-1} sec^{-1} to produce the peroxynitrite anion.[1] Nitric oxide also reacts directly with hydrogen peroxide to form peroxynitrite.[2,3]

Peroxynitrite is a powerful oxidant which has been shown to be important in the macrophage killing of various pathogens.[4] Peroxynitrite's toxicity is believed to be a result of its ability to directly oxidize sulfhydryls.[5] In addition, peroxynitrite has a pKa of 6.8,[6] and when protonated can decompose by homolytic fission to produce hydroxyl radical and nitrogen dioxide[3,7] (reactions 1 and 2).

$$ONOO^- + H^+ \rightarrow ONOOH \quad (1)$$

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$$ONOOH \rightarrow NO_2 + \cdot OH \quad (2)$$

This fission of peroxynitrite leads to further toxicity by initiating peroxidation of lipids,[8] as well as nitration and hydroxylation of aromatic rings[7] such as tyrosine residues on proteins. The nitration of tyrosine by peroxynitrite can also be catalyzed by superoxide dismutase via the formation of the nitronium cation.[9] There is some debate as to whether or not hydroxyl radical is released directly from peroxynitrite decay[10–12], but it does appear that the molecular product has hydroxyl radical like activity.

The primary oxidation product of nitric oxide in aqueous solutions is nitrite,[13] which can then be further oxidized to nitrate. Nitrite is not an innocuous byproduct of nitric oxide oxidation and has been shown to be cytotoxic in conjunction with hydrogen peroxide in a number of experimental models.[14–17] One mechanism by which these two species may contribute to toxicity is through production of peroxynitrite anion. This reaction proceeds rapidly at acidic pH, and indeed has been used by many investigators to produce peroxynitrite.[3,7,18,19]

Acidic conditions occur *in vivo* under a number of conditions including tissue ischemia. In addition, activated inflammatory cells such as macrophages have been shown to produce an acidic environment (pH ≈ 5–6) in the phagosome.[20] We were interested in determining the pH range in which nitration of an aromatic amino acid, like tyrosine, could be produced from the reaction between nitrite and hydrogen peroxide. Our purpose was to determine if this reaction is plausible during inflammation and other oxidative injuries. In addition we investigated the mechanism by which nitration occurs from this reaction to determine if peroxynitrite is an intermediate.

MATERIALS AND METHODS

Materials

L-tyrosine, sodium nitrite, 3-nitro-L-tyrosine, and salicylic acid were purchased from Sigma (St. Louis, MO). 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were purchased from Aldrich Chemical Company (Milwaukee, WI). Desferol was purchased from CIBA-GEIGY (Summit, NJ).

Reaction Conditions

Nitrotyrosine formation from the reaction of hydrogen peroxide and nitrite was measured at pH values between 2 and 7. For initial experiments the reaction mixtures contained variable concentrations of H_2O_2 and sodium nitrite. The aromatic marker was 0.5 mM L-tyrosine. The buffer used was 100 mM citrate and the pH was adjusted between 2 and 7 using NaOH. The reactions were allowed to proceed at room temperature for 1 hour. The samples were then frozen at −20°C until measurements of nitrotyrosine could be made. Each experiment was repeated two to four times.

Hydroxyl radical formation from the reaction of H_2O_2 with nitrite was also measured at pH values between 2 and 7. The reaction conditions were as described for nitrotyrosine formation, but 5 mM salicylate was used in place of tyrosine to detect hydroxyl radical formation.[21,22] The reaction was allowed to proceed at room temperature for 1 hour. The samples were then frozen at −20°C, and measurements of the dihydroxybenzoic acids made as soon as possible.

The importance of iron as a catalyst in the formation of nitrotyrosine and dihydroxybenzoic acid was evaluated by measuring total iron in the system (see below). In separate experiments, 10 μM deferoxamine was added to the reaction conditions described above. The effect of iron chelation with this compound was determined by assessing differences in nitration and hydroxylation in the presence of the chelator. The deferoxamine concentration (10 μM) was chosen to provide a 10-fold excess of chelator:iron in the reaction mixture as determined by the results of the direct measurements of iron in the mixtures.

Reactions with peroxynitrite

Peroxynitrite was produced as described by Beckman *et al.*[19] Briefly, 0.6 M hydrogen peroxide in 0.5 M HCl and 0.5 M nitrite were injected at 20 ml/min into a T-junction and mixed in a 3-mm diameter by 2.5 cm glass chamber. The reaction was stopped by injecting 1.5 M NaOH at the same rate into a second T-junction at the end of the glass chamber. Unreacted hydrogen peroxide was removed by 2 batch applications of 4 g MnO_2 to the solution. The solution was then frozen at –20°C in a test tube and the yellow peroxynitrite was removed from the top of the tube after freeze fractionation. The concentration of peroxynitrite was determined by measuring the absorbance at 302 nm ($\varepsilon_{302} = 1670\ M^{-1} \cdot cm^{-1}$).

Nitration of tyrosine by peroxynitrite was performed by reacting 10 mM peroxynitrite with 0.5 mM L-tyrosine in 100 mM citrate at pH 3 and 5. The pH of the reaction mixtures after adding the peroxynitrite increased by approximately 0.5 pH units as a result of the basic pH of the stock peroxynitrite. Therefore, the pH of the buffer was adjusted to the pH values indicated after the addition of the peroxynitrite. Nitration of tyrosine was then measured as described below. These experiments were performed in triplicate.

Detection of Nitrotyrosine and Tyrosine

Nitration of tyrosine was analysed by monitoring the formation of 3-nitro-L-tyrosine. Samples from the reaction vials containing 0.5 mM L-tyrosine were filtered through 0.2-μm microcentrifuge tube filters (Sigma, St. Louis, MO). Aliquots of 5–50 μl were injected into a high-pressure liquid chromatography system with a U.V. detector (SM4000 programmable U.V. detector, LDC Riviera Beach, FL). The 3-nitro-L-tyrosine and tyrosine were eluted isocratically onto a 4.6 × 250 mm C-18RP column (Ultrasphere no. 235329, Beckman, Deerfield, IL) with a guard column using a 0.03 M citrate + 0.03 M acetate buffer (pH 3.6) at a flow rate of 0.7 ml/min. The U.V. detector was set at 275 nm. The amount of 3-nitro-L-tyrosine produced was calculated by comparing HPLC peak heights with that of samples containing known concentrations of authentic standards. The amount of tyrosine consumed was calculated by comparing HPLC peak heights with that of samples containing known concentrations of authentic standards. In addition to using retention times to identify peaks corresponding to 3-nitro-L-tyrosine and tyrosine, some samples were spiked with authentic standards resulting in augmentation of the peaks corresponding to the 3-nitro-L-tyrosine and tyrosine.

Detection of Dihydroxybenzoic Acid

Non-enzymatic hydroxylation of salicylate was detected using a modification of

Floyd's method as described previously.[23] Briefly, samples from the reaction vials containing 5 mM salicylate were filtered through 0.2-μm microcentrifuge tube filters. Aliquots of 5–50 μl were injected into a high-pressure liquid chromatography system with an electrochemical detector (HPLCED; Coulochem 5100A, ESA, Bedford, MA). The hydroxyl radical reacts freely with salicylate to form 2,3- and 2,5-dihydroxybenzoic acids and a small amount of catechol. The 2,3- and 2,5-dihydroxybenzoic acids were eluted isocratically on a 4.6 × 250 mm C-18RP column (Ultrasphere, Beckman, Deerfield, IL) using a 0.03 M citrate + 0.03 M acetate buffer (pH 3.6) at a flow rate of 0.7 ml/min. The electrochemical detector was set at an oxidation potential of +0.40, at which 2,3-dihydroxybenzoic acid is fully oxidized. The amount of 2,3- and 2,5-dihydroxybenzoic acid produced was calculated by comparing peak heights from the samples with those of samples containing authentic standards. In addition to using retention times to identify 2,3- and 2,5-dihydroxybenzoic acids, samples were spiked with authentic standards to augment the peaks corresponding to these compounds.

Measurement of total iron

Aliquots from the reaction vials were taken at the end of the reactions to determine the concentration of total iron. Iron concentrations were measured using inductively coupled plasma emission spectroscopy (ICPES; Model P40, Perkin Elmer). Standards of ferric chloride (1.0×10^{-7} to 1.0×10^{-5} M) were prepared in 1 N HCl immediately prior to use. The concentration of iron in both the samples and the standards were quantified as the emission at 238.20 nm. The detection limit for iron in this assay was 0.1 nanomoles. Each experiment was performed in duplicate and each sample was run in triplicate.

Rate of nitration of tyrosine

0.5 mM L-tyrosine was incubated with 50 mM hydrogen peroxide and 50 mM sodium nitrite in 100 mM citrate at pH 3 and 5. The rate of nitro-L-tyrosine formation was followed by monitoring the absorbance at 359 nm. Extinction coefficients of 3040 $M^{-1} \cdot cm^{-1}$ at pH 4, 2760 $M^{-1} \cdot cm^{-1}$ at pH 5 and 2430 $M^{-1} \cdot cm^{-1}$ at pH 6 were established by measuring the absorbance of known concentrations of pure 3-nitro-L-tyrosine at these pH values. Other nitration products of tyrosine produced by this reaction also would increase the absorbance at this wavelength. The spectrophotometric method allows a more complete estimation of the rate of nitration of L-tyrosine because the lack of authentic standards for many of the alternative products prevents quantification using HPLC. Because nitrite absorbs at this wavelength the reaction was also carried out in the absence of L-tyrosine to correct for effects of the loss of nitrite.

RESULTS

Nitrotyrosine formation

The nitration of tyrosine, measured by the production of 3-nitro-L-tyrosine, from the reaction between hydrogen peroxide and nitrite is shown in figures 1a and 1b. Nitration of tyrosine using 10 mM peroxynitrite is shown in figures 1c and 1d for comparison. The nitration of tyrosine from the reaction of hydrogen peroxide and nitrite shows a

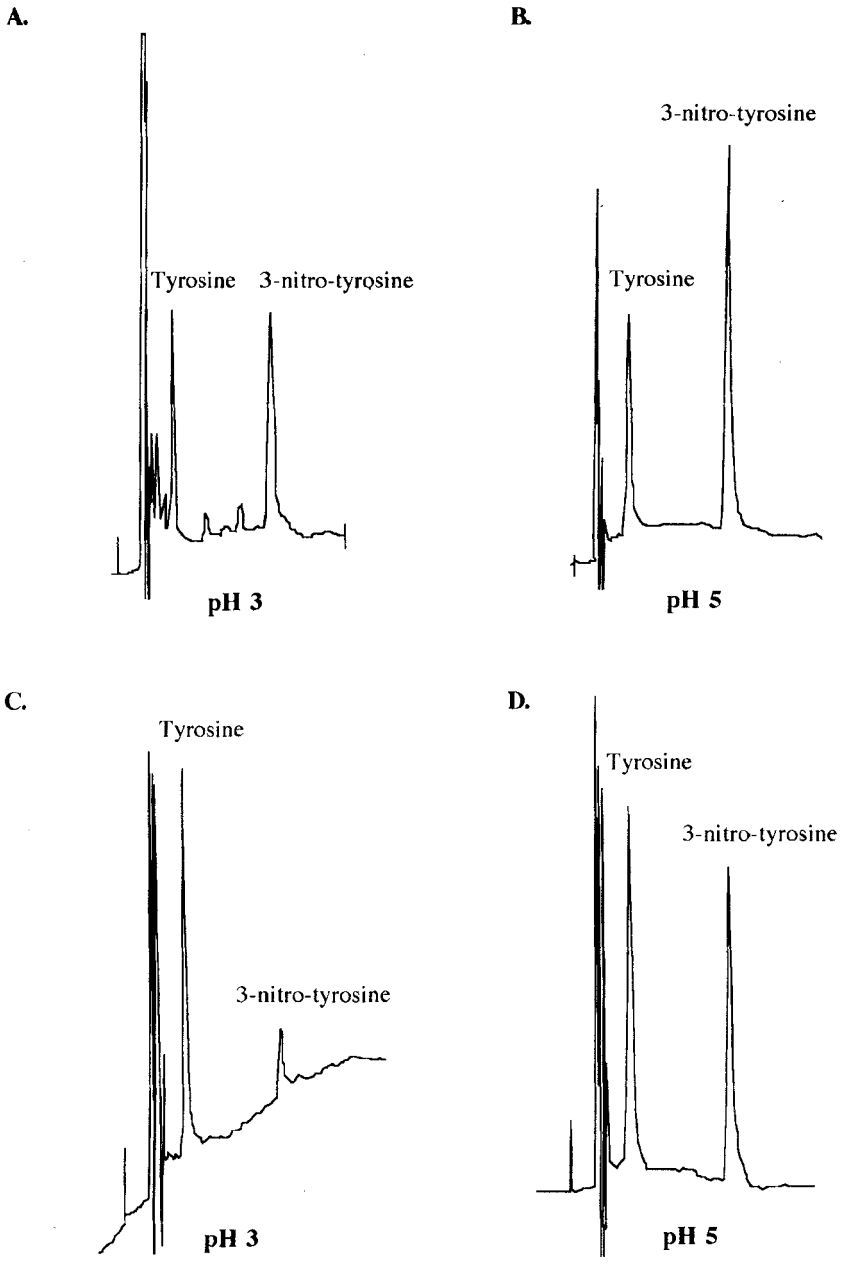

FIGURE 1 Representative tracings of 3-nitro-L-tyrosine formation at pH 3 and pH 5 from the complete reaction of 0.5 mM L-tyrosine with 50 mM hydrogen peroxide and 50 mM sodium nitrite (a and b), or with 10 mM peroxynitrite (c and d). Peaks are identified as tyrosine, or 3-nitro-L-tyrosine based on elution times of authentic standards. Note that the amount of tyrosine consumed, represented by a lower peak height for tyrosine, at both pH values is greater in the reaction with nitrite and hydrogen peroxide than with peroxynitrite.

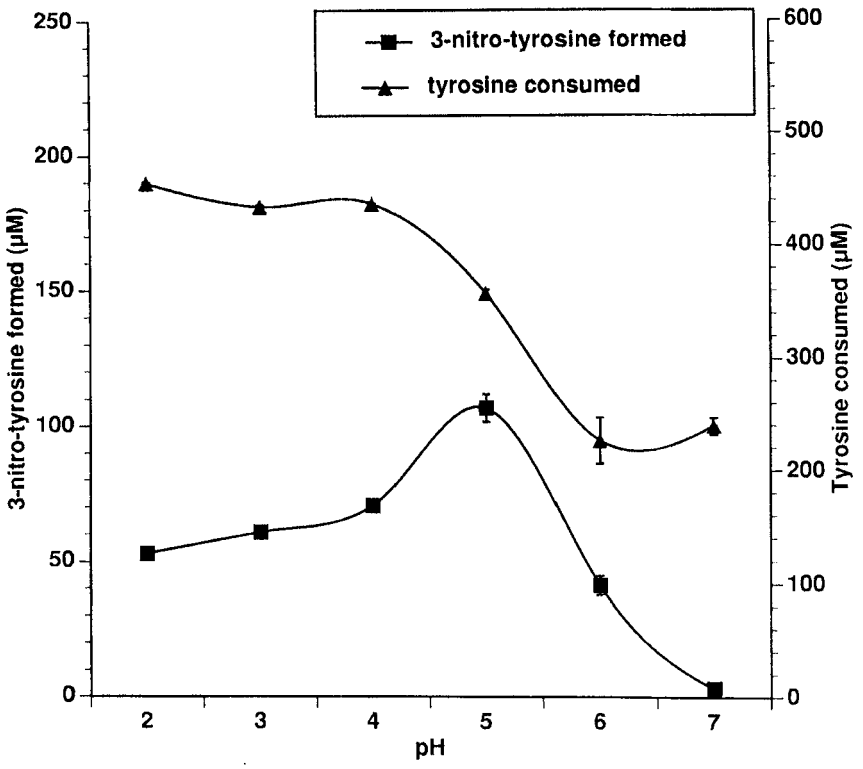

FIGURE 2 Total 3-nitro-L-tyrosine formation and L-tyrosine consumption after reaction of 0.5 mM L-tyrosine with 50 mM hydrogen peroxide and 50 mM sodium nitrite at various pH's until completion. The concentrations of 3-nitro-L-tyrosine and L-tyrosine were calculated from peak heights of samples run through an HPLC column with UV detection using known concentrations of authentic 3-nitro-L-tyrosine and L-tyrosine as standards. Data are presented as mean ± standard error (n = 3).

pH dependence (Figure 2) with maximal nitration occurring at approximately pH 5 (107 μM 3-nitro-L-tyrosine). Less nitration was observed below pH 4 and above pH 6. The disappearance of L-tyrosine from the reaction mixture at various pH values is also shown in figure 2. The figure indicates that the loss of tyrosine was greater at low pH values, and greater than the amount of 3-nitro-L-tyrosine formed at all pH values. These results are consistent with a large quantity of unmeasured products formed at all pH values examined.

Hydroxylation of salicylic acid

The hydroxylation of salicylate, measured by the production of 2,3- and 2,5-dihydroxybenzoic acid, from the reaction between hydrogen peroxide and nitrite is shown in figure 3. In contrast to nitration, the greatest degree of hydroxylation was detected at low pH (maximum between pH 2 and 3) with a rapid decrease in hydroxylation as the pH was increased.

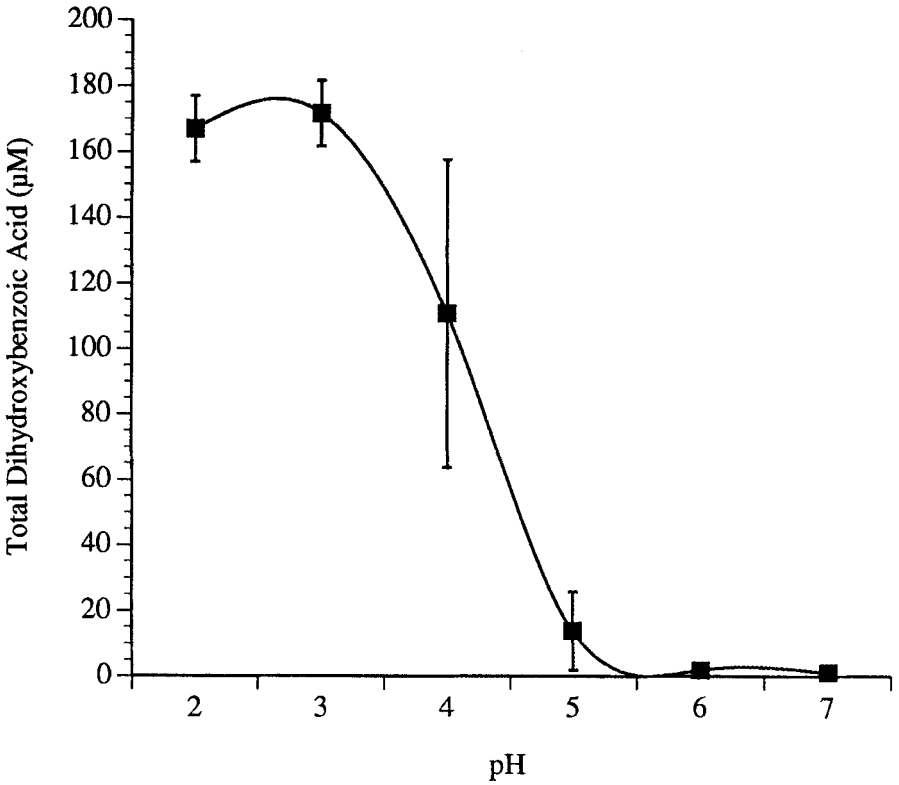

FIGURE 3 Total 2,3- and 2,5-dihydroxybenzoic acid formation after reaction of 5 mM salicylate with 50 mM hydrogen peroxide and 50 mM sodium nitrite at various pH's until completion. The concentrations of product were calculated from peak heights of samples run through an HPLC column with electrochemical detection using known concentrations of authentic 2,3- and 2,5-dihydroxybenzoic acid as standards. Data are presented as mean ± standard error (n = 3).

Peroxynitrite induced nitration of tyrosine

When peroxynitrite was added directly to L-tyrosine at pH 3 or pH 5 it produced 3-nitro-L-tyrosine (Figure 1c and 1d). More nitrotyrosine was produced at pH 5 than at pH 3 as was found in the reaction of tyrosine with hydrogen peroxide and nitrite. In addition to nitrotyrosine, a second peak of unknown identity was produced at pH 3. This unidentified peak is presumed to be a hydroxylation product of tyrosine, as hydroxylation of salicylate by peroxynitrite was shown to occur more readily at the lower pH.

Iron measurements

Iron is capable of catalyzing hydroxyl radical formation from hydrogen peroxide. Total iron levels were measured to ensure that the nitration and hydroxylation were not a result of catalysis by iron in the reaction mixture. ICPES measurements revealed that the reaction systems used contained between 0.08 and 0.7 μM iron. The range of

TABLE 1
Rate of nitration of tyrosine measured by continuous monitoring of absorbance at 359 nm. The reaction mixtures contained 0.5 mM L-tyrosine with 50 mM hydrogen peroxide and 50 mM sodium nitrite in 100 mM citrate buffer.

pH	Nitration Rate (μmoles/min)
4	6.6
5	6.4
6	1.9

concentrations of iron in the reaction mixtures had no effect on the rates of nitration and hydroxylation. In addition, the iron chelator deferoxamine had no effect on nitration or hydroxylation when added to the reaction systems (not illustrated).

Rate of nitro-tyrosine formation

The rate of nitration of L-tyrosine was measured in duplicate at different pH values (Table 1). Under the reaction conditions, nitrotyrosine was produced at a linear rate at pH 5 and at pH 6. However, at pH 4 and below there was an initial decrease in absorbance followed by a linear increase in absorbance. When the absorption was monitored in the absence of L-tyrosine there was no change in the baseline indicating that the initial loss in absorption at low pH was not a result of decomposition of nitrite in the reaction with hydrogen peroxide.

DISCUSSION

The production of peroxynitrite has been shown to lead to sulfhydryl oxidation,[5] and hydroxylation and nitration of aromatic rings such as tyrosine residues in proteins.[7,19,24,25] The hydroxylation and nitration are believed to be a result of hydroxyl radical and nitrogen dioxide release from the homolytic fission of peroxynitrous acid.[7,19] Peroxynitrite has been shown to be an important mediator of macrophage induced killing.[4,26] Nitration of tyrosine residues may be an important part of this toxicity as this nitration has been shown to alter properties of proteins such as surfactant[24] and tyrosine kinases.[27] In addition, nitrotyrosine has also been recently shown to accumulate in the serum of patients with chronic inflammatory diseases[28] suggesting that this reaction may well occur *in vivo*.

Although most of the literature on peroxynitrite production has emphasized the reaction of superoxide with nitric oxide, we have shown that nitration of tyrosine can occur in the presence of hydrogen peroxide and nitrite at pH values that occur *in vivo*. These reactants showed a pH dependence on the degree of nitration and hydroxylation. Low pH values favored hydroxylation, while pH values closer to neutrality favored nitration. A similar ratio of nitration was seen with authentic peroxynitrite at pH 3 and pH 5. This suggests that peroxynitrite formation is the most likely mechanism of nitration and hydroxylation in the reaction of hydrogen peroxide with nitrite.

When the nitration of tyrosine was measured by continuous spectrophotometric monitoring of nitrotyrosine formation, the nitration rate at pH 4 was similar to that found at pH 5. This is in contrast to the results obtained by direct measurement of

3-nitro-L-tyrosine formation using HPLC with UV detection which showed more nitrotyrosine formation at pH 5 than at pH 4. This discrepancy may be a result of greater simultaneous hydroxylation and nitration of tyrosine which occurs at pH 4 and which was not accounted for by the HPLC measurements due to a lack of authentic standards for the multiply substituted products.

The major products from peroxynitrite attack on aromatic rings were described by Halfpenny in 1952.[7] Meta-nitration and ortho-, and para-hydroxylations on aromatic rings were the predominant products found, however, many multiple ring substitutions were produced as well. These included substitutions in which both nitration and hydroxylation occurred on the same ring. We also observed several unidentified products from the reactions of hydrogen peroxide with nitrite and with authentic peroxynitrite in the presence of tyrosine or salicylate. The unidentified peaks found on our chromatograms at low pH presumably represent these alternative hydroxylation and nitration products. These additional reaction products indicate that tyrosine is attacked to a greater extent by this reaction than we were able to measure. This is supported by HPLC measurements of tyrosine consumption in the presence of nitrite and hydrogen peroxide. These studies revealed that more tyrosine was being consumed than could be accounted for by 3-nitro-L-tyrosine formation at all pH values examined with 40% of the tyrosine being consumed even at pH 7 (Figure 2). This result suggests that the reaction of nitrite and hydrogen peroxide with tyrosine is significant at physiologic pH.

While the reaction of nitric oxide with superoxide is extremely rapid, the extracellular matrix of tissues contain high levels of the enzyme extracellular superoxide dismutase (EC SOD)[29,30] which effectively scavenges superoxide in extracellular spaces. The presence of EC SOD would be expected to greatly diminish peroxynitrite formation via this mechanism. EC SOD produces hydrogen peroxide enzymatically, and many extracellular domains lack mechanisms for effectively scavenging hydrogen peroxide. Under these circumstances, detoxification of hydrogen peroxide could depend on its diffusion into intracellular compartments where metabolic enzymes are present. Notably, the concentrations of hydrogen peroxide produced in many injury models are sufficient to be detected *in vivo*[31,32] and the same is true for direct measurement of nitrite.[33,34] The reaction of hydrogen peroxide and nitrite at pH values which occur *in vivo* to produce nitration and hydroxylation, presumably via peroxynitrite formation, may represent an additional mechanism of localized tissue injury which involves both reactive oxygen and nitrogen species.

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