The capabilities of stimulated neutrophils to initiate intraphagosomal and extracellular chlorination, nitration, and other oxidative reactions has been evaluated using a fluorescent particle and soluble phenolic compounds as target molecules. Neutrophils activated by the soluble stimulus, phorbol myristate acetate, both chlorinated fluorescein that was covalently attached to polyacrylamide microspheres and initiated tyrosine dimerization. When nitrite ion was present at millimolar concentration levels in the medium, nitration of the phenolic rings also occurred; the relative extent of nitration increased as the nitrite concentration was increased. Myeloperoxidase (MPO) also catalyzed nitration and chlorination of fluorescein and the fluorescein-conjugated particles in cell-free solutions; the relative nitration yields increased with increasing \([\text{NO}_2^-]/[\text{Cl}^-]\) ratios. Nitration did not involve intermediary formation of nitrating agents derived from reaction between MPO-generated HOCl and \(\text{NO}_2^-\); because this reaction also occurred in chloride-free media and direct addition of HOCl to solutions containing \(\text{NO}_2^-\) and fluorescein gave only chlorinated products. In marked contrast to these extracellular reactions, intraphagosomal nitration of the fluorescein-conjugated particles could not be detected (even at \([\text{NO}_2^-]\) as high as 0.1 M), whereas chlorination of the probe was extensive. These data indicate that intraphagosomal aromatic nitration in neutrophils is negligible, although extracellular nitration of phenolic compounds by secreted MPO could occur at physiological concentration levels of \(\text{NO}_2^-\).

Hypochlorous acid (HOCl) is generally regarded as a primary microbial (1–4) produced by stimulated neutrophils (5, 6) via myeloperoxidase (MPO)\(^1\)-catalyzed oxidation of chloride ion (7). However, recent research has suggested that other oxidants that are either derived from HOCl or formed in other MPO-catalyzed reactions may also play significant roles in microbicial action. Specifically, nitrite ion has been shown to be a substrate for several peroxidases (8, 9), including MPO (10), whose reactions generate a bactericidal (11) strong oxidant, presumably nitrogen dioxide (\(\text{NO}_2\)) (10); \(\text{NO}_2^-\) can also react with HOCl to form transitory species, e.g., nitryl chloride (\(\text{NO}_2\text{Cl}\)) (12), which are capable of both chlorinating and nitratating phenolic rings (13); and HOCl can apparently react with the immediate product of the respiratory “burst,” superoxide anion (\(\text{O}_2^-\)) (5, 6), to generate hydroxyl radical (\(\text{OH}\)), viz., \(\text{HOCl} + \text{O}_2^- \rightarrow \text{OH} + \text{Cl}^- + \text{O}_2\) (14). Neutrophils activated with soluble stimuli to secrete MPO catalyzed extracellular aromatic nitration when the medium contained \(\text{NO}_2^-\) at concentration levels approximating those found in biological fluids (10), establishing that physiologically derived \(\text{NO}_2^-\) could be an alternative MPO substrate. Extracellularly generated ‘OH is probably too short lived to function as an effective bactericidal agent in biological environments (15). Nonetheless, conversion of a substantial fraction to the longer lived (and bactericidal) carbonate (\(\text{CO}_3^-\)) radical should occur in the \(\text{CO}_3^-\)-rich environment of respiring phagosomes via the reaction: \(\text{OH} + \text{HCO}_3^- \rightarrow \text{H}_2\text{O} + \text{CO}_3^-\) (16). Thus, each of the reactions involving \(\text{NO}_2^-\) and \(\text{O}_2^-\) are potentially important contributors to host defense mechanisms against pathogenic organisms.

These reactions might also be major contributors to the pathogenesis of diseases associated with oxidative stress. Neutrophil activation at sites of infection is generally accompanied by some degranulation at the plasma membrane, releasing MPO into the extracellular environment, which could then participate in oxidative reactions leading to damage of surrounding tissues. For example, extracellular MPO has been shown to accumulate within human atherosclerotic lesions (17) which also contain elevated levels of chlorotyrosines (18). Since MPO and stimulated neutrophils are capable of chlorinating peptide tyrosyl groups (19, 20), it follows that the reactions leading to protein tyrosyl chlorination in the lesions probably involve MPO-generated HOCl. Similarly, high levels of nitrotyrosine are often found in damaged tissues of individuals with respiratory or neurological diseases (21). Although these findings have often been attributed to involvement of peroxynitrous acid (\(\text{ONO}_2\text{H}\)), which could be formed by radical coupling of \(\text{O}_2^-\) with nitric oxide (\(\text{NO}\)) (22), alternative possibilities in environments containing accumulated neutrophils are MPO-catalyzed nitration (10) or reaction of MPO-generated HOCl with \(\text{NO}_2^-\) to form nitrating intermediates (13).

In this study, we have utilized a particulate chemical probe that acts as a bacterial mimic (23) to determine relative contributions of the various possible MPO-mediated reactions by stimulated neutrophils. The probe contains a fluorescein derivative as a reporter group, which gives easily distinguishable products when reacted with chlorinating (23), nitrating (23), or other putative neutrophil-generated oxidizing agents (16). When opsonized with serum-derived proteins, the probe stimulates neutrophil respiration and is simultaneously phagocytosed; unopsonized particles, however, elicit no cellular response (23). Opsonization therefore provides a means to control the reaction locus, either within the phagosome or in extracellular environments. Results described herein will show that the reactivity patterns differ in the two environments, with chlorination...
predominating within the phagosome, but being accompanied by appreciable nitration when the reactions are extracellular.

EXPERIMENTAL PROCEDURES

Materials—Human neutrophils were isolated, counted, and stored as described previously (23). Myeloperoxidase, purified from bovine spleens (24), had an A430/A280 = 0.85 and a specific activity of 284 guaiac units/mg of protein (25); enzyme concentrations were determined spectrophotometrically using \( \epsilon_{430} = 91 \text{ M}^{-1} \text{cm}^{-1} \) (26). Commercial HOCl was prepared by vacuum distillation; HOCl concentrations were determined spectrophotometrically using \( \epsilon_{280} = 100 \text{ M}^{-1} \text{cm}^{-1} \) (27). Stock peroxynitrite solutions were prepared by reacting potassium nitrite with hydrogen peroxide (28). 3,3-Dithiobis was prepared by horseradish peroxidase-catalyzed oxidation of tyrosine by hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) (29). Fluorescein-conjugated 1-μm diameter polyacrylamide microspheres containing cystamine linker groups were synthesized as described previously (23). The particles used in this study contained 3.5 × 10⁷ fluorescein groups/bead and ~8 × 10⁸ unreacted carboxyl end-groups/bead. Other chemicals and biochemicals were the highest grade commercially available and were used as supplied. All solutions were prepared from deionized water that had been further purified by passage through a laboratory reverse osmosis/ion exchange unit.

Enzymatic Reactions—Peroxidase-catalyzed nitration reactions were generally carried out at 37 °C in 25 mM sodium phosphate, pH 7.4, containing 105 μM fluorescein, 120 μM tyrosine, or 5 mg/ml bovine serum albumin and varying amounts of \( \text{H}_2\text{O}_2, \text{NO}_2^- \), and the peroxidase (MPO, horseradish peroxidase, or lactoperoxidase). For some reactions involving MPO, 0.10 mM NaCl was also added to the reaction medium. Reactions were terminated after 30 min either by adding 20 μM catalase or by cooling on ice; these methods gave equivalent results. For studies with fluorescein-conjugated beads, 1.5 × 10⁶ beads/ml (fluorescein = 80 μM) were incubated for 30 min at 37 °C in the same buffer with 70 nM MPO, 100 μM \( \text{H}_2\text{O}_2 \), 0.10 mM \( \text{Cl}^- \), and varying amounts of nitrite ion.

Reactions with Neutrophils—For most experiments with neutrophils, 2 × 10⁵ cells/ml were incubated in human serum with 5 × 10⁶ fluorescein-conjugated beads/ml. During the incubation period, the reaction mixtures were gently rotated (23). Reactions were terminated after 30 min at 37 °C by cell homogenization. Comparative studies were made using preosonized beads to stimulate neutrophils suspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM \( \text{Na}_2\text{HPO}_4 \), 1.5 mM KH₂PO₄ (PBS)); rates and extents of phagocytosis under these conditions are nearly identical to phagocytosis in serum (23). Intracellular reactions involving neutrophil-secreted MPO were investigated by using unopsonized beads in PBS containing 30 mg/ml phorbol myristate acetate (PMA) as a soluble stimulant. Following the reaction, the fluorescein was solubilized for chemical analysis by adding ~0.25 M dithiothreitol to cleave the cystamine disulfide bond and centrifuging to remove cell debris (25). Experiments in which extracellular reactions involving tyrosine and intracellular reactions of fluorescein were simultaneously measured were conducted in PBS containing 1 mM tyrosine; immediately following incubation of the fluoresceinated particles with neutrophils, the mixture was centrifuged at 90,000 × g, and the tyrosine-containing supernatant was analyzed by HPLC. The pellet was subsequently homogenized, and the fluorescein was recovered for analysis by adding dithiothreitol.

Product Analyses—Products formed in the various reactions were identified by HPLC on a Gilson 305/306 system equipped with a 5-μm Microsorb C-18 column and UV-visible detector. For reactions involving tyrosine, components were separated by isocratic elution with 20 mM phosphate, pH 3.0, containing 8% methanol, and were detected at 276 nm; for reactions involving fluorescein or the fluorescein-conjugated particles, the eluant was 20 mM phosphate, pH 7.4, containing 32% methanol, and detection was at 498 nm. In all cases, peak identities were confirmed by co-chromatography with authentic samples and by optical absorption spectroscopy. Product yields were determined from the peak areas by comparison to reference standards, which were prepared by reacting fluorescein or the fluorescein-conjugated beads with HOCl, MPO-H₂O₂-Cl⁻, or ONOO⁻ and isolating the products by HPLC (23). Concentrations of the purified standards were determined by peak-height integration. Concentrations of chlorinated standards prepared from free fluorescein were determined spectrophotometrically at pH 9.0 using \( \epsilon_{500} = 7.5 \times 10^4 \text{ M}^{-1} \text{cm}^{-1} \) for monochlorofluorescein and \( \epsilon_{500} = 8.0 \times 10^4 \text{ M}^{-1} \text{cm}^{-1} \) for dichlorofluorescein (23). Two optically distinguishable mononitrofluorescein isomers were obtained, which gave band maxima at 490 nm for the more rapidly eluting isomer and 500 nm for the other; for both compounds, \( \epsilon_{	ext{max}} = 7.6 \times 10^4 \text{ M}^{-1} \text{cm}^{-1} \). Concentrations of reference standards for the chlorinated and nitrated fluorescein derivatives isolated from the beads were determined assuming \( \epsilon \) values identical to the other prepared chlorofluorescein and nitrofluorescein standards.

RESULTS

MPO-catalyzed Reactions Involving Fluorescein—Incubation of MPO with fluorescein in solutions containing \( \text{H}_2\text{O}_2 \), \( \text{Cl}^- \), and \( \text{NO}_2^- \) resulted in formation of three major products (Fig. 1), one of which had previously been identified by mass spectrometry as a monochlorofluorescein (23). The other two peaks co-eluted with nitrofluoresceins formed by reacting fluorescein with ONOO⁻. The latter were identified as isomeric mononitrofluoresceins by Ionspray mass spectrometry, for which each product gave a major ion peak at \( m/z = 378.0 \) atomic mass units (\( [\text{M} + \text{H}]^+ \)) with additional minor peaks at 361 (\( [\text{M} + \text{H}]^+ - \text{[OH]} \)), 332.0 (\( [\text{M} + \text{H}]^+ - \text{[NO}_2^-] \)), and 303.2 atomic mass units (\( [\text{M} + \text{H}]^+ - \text{[NO}_2^- + \text{CO}] \)). Nitrature required the presence of MPO, \( \text{H}_2\text{O}_2 \), and \( \text{NO}_2^- \), but not \( \text{Cl}^- \), and was nearly completely blocked by addition of the MPO inhibitor, azide ion (Table I and Fig. 1). The nitrofluorescein yields increased with increasing \( \text{NO}_2^- \) concentration up to about 1 mM (Fig. 2a), then decreased slowly at higher concentrations; at the maximal yield, ~30% of the initially added \( \text{H}_2\text{O}_2 \) was converted to nitrofluoresceins. The isomer distribution was unaffected by the \( \text{NO}_2^- \) concentration. However, the chlorofluorescein yield rapidly declined, reaching a level of ~4% of the added \( \text{H}_2\text{O}_2 \) at the nitrofluorescein maximum and becoming negligible at higher \( \text{NO}_2^- \) concen-

![Fig. 1. HPLC chromatograms of reaction product solutions.](image)

Conditions: 105 μM fluorescein, 20 nm MPO, 100 μM \( \text{H}_2\text{O}_2 \), and 0.1 mM \( \text{Cl}^- \) and/or varying \( \text{NO}_2^- \) in 25 mM phosphate, pH 7.4, incubated at 37 °C for 30 min. Upper trace, \( \text{Cl}^- \) alone; peaks identified as \( \beta, \beta'-\text{Cl}, \) and \( \beta'-\text{Cl}_2 \) are fluorescein, and the mono- and dichlorofluorescein derivatives. Middle trace, \( \text{Cl}^- \) and 0.5 mM \( \text{NO}_2^- \). Lower trace, 1.0 mM \( \text{NO}_2^- \) alone; peaks identified as \( \beta^-\text{NO}_2^- \) are isomeric mononitrofluoresceins.

| Compound | Formula | Mass (amu) |
|----------|---------|------------|
| fluorescein | \( \text{C}_15\text{H}_11\text{O}_5\text{N} \) | 317.2 |
| monochlorofluorescein | \( \text{C}_15\text{H}_11\text{ClO}_5\text{N} \) | 331.2 |
| dichlorofluorescein | \( \text{C}_15\text{H}_11\text{Cl}_2\text{O}_5\text{N} \) | 345.2 |
| mononitrofluorescein | \( \text{C}_15\text{H}_11\text{NO}_5\text{N} \) | 329.2 |

Other compounds and intermediates formed in these reactions were identified as isomeric mononitrofluoresceins (23).
trofluorescein yields. The complete system corresponds to 30 nM maximal nitration yield was considerably less, with only was increased (Fig. 3). Quantitative differences were: (i) the incubation of free fluorescein, with chlorination yields decreasing and nitration yields increasing as the NO₂ concentration was increased (Fig. 3). Quantitative differences were: (i) the maximal nitration yield was considerably less, with only 9% of the added H₂O₂ being converted to nitrofluoresceins under optimal conditions, and (ii) the maximal yield was attained at much higher [NO₂], i.e. ≥ 10 mM. In contrast, the optimal chlorofluorescein yield was the same as for free fluorescein within experimental uncertainty, i.e. 52–67% (cf. Figs. 2 and 3 with [NO₂] = 0). Enzymatic nitration of the conjugated fluorescein was partially inhibited by preopsonization of the beads in 25% serum and completely inhibited when unopsonized beads were reacted in an assay medium comprising 100% serum. Specifically, the nitrofluorescein yield obtained following incubation of 1.5 × 10⁶ opsonized beads/ml containing 100 μM fluorescein with 70 mM MPO, 100 μM H₂O₂, and 3–10 mM NO₂ in PBS gave only ~50% of the nitration product obtained using unopsonized beads. Reaction of unopsonized beads in serum with [NO₂] as high as 100 mM gave no nitrofluorescein product. **Reactions of Neutrophils with Fluorescein-conjugated Beads**—Human neutrophils were incubated with the fluoresceinated beads in 100% human serum at a bead:neutrophil ratio of ~15:1. Under these conditions, 80–90% of the beads and nearly all of the MPO are entrapped within sealed phagosomes (23). Addition of NO₂ to the medium had no effect upon chlorination yields until its concentration exceeded ~1 mM, at which point the chlorination yield declined progressively with increasing NO₂, becoming negligible at [NO₂] = 100 mM (Fig. 4). Very similar results were obtained using serum-opsonized beads in PBS, i.e. addition of up to 1 mM NO₂ had no effect upon the chlorination yields, which in this case were ~8 nmol/10⁷ cells. Thus, in marked contrast to the MPO-catalyzed reactions of the fluorescein-conjugated beads in solution (Fig. 3), nitrofluorescein was not formed under any experimental conditions in the intraphagosomal reactions of stimulated neutrophils (Fig. 4). However, the neutrophils were capable of catalyzing aromatic nitration reactions, at least in the extracellular medium. Stimulation of neutrophils with PMA in media containing tyrosine and NO₂ caused formation of both nitrotyrosine and dityrosine; the relative yield of nitrotyrosine increased with increasing NO₂ concentration and only dityrosine was formed when NO₂ was deleted (Table II). Similarly, unopsonized fluorescein-conjugated beads reacted extracellularly (23) with PMA-stimulated neutrophils to give both chloro- and nitrofluorescein products (Table II). In this case, the relative chlorination of the chlorinated product decreased and the nitratated products increased with increasing [NO₂] in a manner similar to the cell-free reaction of the beads with MPO-H₂O₂-Cl⁻-NO₂ (Fig. 3), although the overall nitration yields were less. For these reactions, the rate of O₂ consumption by the PMA-stimulated neutrophils (~20 nmol/10⁷ neutrophils/min) was approximately the same as previously measured for cells stimulated with opsonized beads in PBS (~16 nmol/10⁷ neutrophils/min) and unopsonized beads in 100% serum (~30 nmol/10⁷ neutrophils/min) at a 15:1 bead:neutrophil ratio (23). When the buffer contained both opsonized beads (bead:neutrophil = 44:1) and tyrosine, both chlorofluorescein and dityrosine products were obtained. Although only ~40% of the beads were phagocytosed, previous studies have established that nearly all of the chlorination is intracellular under these conditions (23). The reduced yield of dityrosine (2.7 nmol/10⁷ neutrophils) compared with analogous reactions using PMA-stimulated cells in the absence of the beads (18 nmol/10⁷ neutrophils) (Table II) is consistent with other data indicating only limited secretion of MPO under phagocytosing conditions (23). Addition of NO₂ to the medium gave rise to nitrotyrosine formation, enhancement of dityrosine yields and, at [NO₂] ≥ 1 mM, partial inhibition of

**TABLE I**

| Reaction            | Nitration yield | Chlorination yield |
|---------------------|-----------------|--------------------|
| Complete system     | 30 μM           | 4.2 μM             |
| −MPO                | 0 μM            | 0 μM               |
| −H₂O₂               | 0.1 μM          | 0 μM               |
| −Cl⁻                 | 31.5 μM         | 0 μM               |
| −NO₂                 | 0 μM            | 52 μM              |
| +N₃ (1 mM)           | 3.1 μM          | 0 μM               |

**Fig. 2. Nitrite ion dependence of product distributions.** Panel a, MPO-catalyzed reactions, conditions identical to Fig. 1, except [NO₂] as indicated. Panel b, reaction of 105 μM fluorescein with 100 μM HCl for 20 min at 37 °C in the same medium. Symbols: closed squares, chlorofluorescein yield; open squares, nitrofluorescein yields.
chlorofluorescein formation (Table II). These patterns of reactivity are qualitatively the same as were observed when the intracellular and extracellular reactions were studied separately.

**Enzyme-catalyzed Tyrosine Nitration**—The extent to which phenolic nitration might be a general attribute of peroxidase-catalyzed reactions was briefly explored. Tyrosine was chosen as the substrate because nitrotyrosine formation is thought to be a specific marker for participation of reactive nitrogen species in *in vivo* oxidative damage (21). Incubation of 5 mg/ml bovine serum albumin with 20 nM MPO, 200 μM H₂O₂, 0.10 M Cl⁻, and 5 mM NO₂⁻ gave spectrophotometric evidence of extensive nitration of its tyrosyl residues, i.e., upon reaction, a strong peak appeared at λ₃₆₀ nm, which further intensified and shifted to λ₄₃₀ nm upon increasing the medium alkalinity to pH 11 (data not shown). These spectral changes are very similar to changes measured for nitrotyrosine in PBS, where λ₅₃₆ nm at pH 5 and λ₄₃₂ nm at pH 11. Incubation of 120 μM tyrosine with 30 nM MPO, 100 μM H₂O₂, and 5 mM NO₂⁻ gave a conversion of 32% to nitrotyrosine. Nitrotyrosine yields increased with increasing [NO₂⁻] over the experimentally investigated range of 0–10 mM. Dityrosine also formed when the concentration of tyrosine was increased, and was accompanied by a corresponding reduction in the nitrotyrosine yield. Comparable amounts of nitrotyrosine were formed when the MPO was replaced with 500 nM horseradish peroxidase, but replacement with 260 nM lactoperoxidase gave a product yield that was about 10-fold lower than the other peroxidases. No products were formed when 300 nM catalase was the catalyst.

**Fluorescein Recovery from Neutrophil Phagosomes**—The amount of dye that was recovered from 17:1 bead:neutrophil suspensions in 100% serum is plotted in Fig. 5 as a function of incubation time. In these reactions, mono-, di-, and trichlorofluoresceins are formed (23); the chlorination yield, plotted on the same time scale, is the total yield of chlorinated products determined by HPLC. Previous studies have established that phagocytosis of the beads, respiratory uptake of O₂, and bead

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**TABLE II**

**Reactions of stimulated neutrophils**

| Reactants | [NO₂⁻] | Products | Nitration yield | Dityrosine | NO₂-Tyr⁺ |
|----------|--------|----------|----------------|------------|----------|
| A. Tyrosine + NO₂⁻: | mM | nmol | | | |
| 0 | 11 | 0 | 0 | |
| 0.10 | 12.5 | 0.97 | 0 | |
| 0.50 | 13 | 3.9 | 0 | |
| 1.0 | 13 | 5.8 | 0 | |

| B. Unopsonized beads + NO₂⁻: | mM | nmol | Chlorination yield | Dityrosine | NO₂-Tyr⁺ |
|----------------------------|----|------|-------------------|------------|----------|
| 0 | 34 | 0 | 0.03 | 0 | |
| 0.30 | 12 | 0 | 0.4 | 0 | |
| 1.0 | 8.7 | 0.03 | 0.4 | 0 | |
| 5.0 | 0.6 | 0.4 | 0 | 0 | |

C. Opsonized beads + tyrosine + NO₂⁻: | mM | nmol | Chlorination yield | Dityrosine | NO₂-Tyr⁺ |
|---------------------------------|----|------|-------------------|------------|----------|
| 0 | 11 | 2.4 | 0 | 0 | |
| 0.10 | 11 | 6.8 | 0.05 | 0 | |
| 0.50 | 11 | 8.4 | 1.6 | 0 | |
| 1.0 | 9.4 | 8.4 | 2.0 | 0 | |
| 5.0 | 6.8 | 5.6 | 4.9 | 0 | |

* Nitrotyrosine.
chlorination are approximately parallel events (23); these relationships hold for both opsonized beads in PBS and unopsonized beads in 100% serum as stimuli. Furthermore, respiratory activity was not influenced by NO2 ion, i.e. at 15/1 unopsonized beads/neutrophil in 100% serum, the initial rates of O2 uptake were 31 and 33 nmol/107 neutrophils/min in the presence of 1 mM and 10 mM NO2, respectively, compared with −30 nmol/107 neutrophils/min when NO2 was absent (23). Chlorination therefore tracks both phagocytosis and respiratory activity, which are essentially complete after 15–20 min. The data at zero time indicate that −95% of the fluorescein bound to the beads can be recovered prior to phagocytosis; the data obtained at 10 and 30 min in the presence of 1 mM NO2 (triangles, Fig. 5), for which phagocytosis and respiration are normal (23), indicate that the same amount of dye can be recovered from the neutrophil phagosomes when MPO activity is inhibited. However, in neutrophils containing active MPO, phagocytosis was accompanied by partial loss of recoverable fluorescein, e.g. at completion of the respiratory burst, only −70% of the initially added dye was recovered (Fig. 5).

DISCUSSION

General Considerations—Neutrophils are thought to possess multiple microbicidal mechanisms, both oxidative (1–4) and nonoxidative (30, 31). Recent studies by Winterbourn and associates using selective inhibitors of the neutrophil NADPH oxidase and MPO in conjunction with superoxide dismutase conjugated Staphylococcus aureus provide evidence that the major pathway for killing of this organism is oxidative and involves MPO catalyzed reactions (32), and Klebanoff (1) has summarized indirect arguments supporting the primacy of MPO-dependent mechanisms in other phagocytic reactions. As discussed in the Introduction, the actual toxins might include HOCI formed by MPO catalyzed oxidation of Cl−, secondary oxidants formed by reaction of HOCI with, e.g. endogenous amines (33), NO2− (13), or O2− (11), or other oxidants formed by direct reaction of other substrates with MPO (3, 10). Use of fluorescein as a chemical probe offers an unusual opportunity to evaluate the relative chlorinating, nitrating, and other oxidative capabilities of stimulated neutrophils because these reactions give rise to distinct reaction products (3). When attached to 1-μm polystyrene beads via cystamine spacer groups, the fluorescein can be used to monitor the dynamics of intraphagosomal reactions and can be recovered in near-quantitative yield for subsequent chemical analyses (23).

Intraphagosomal Chlorination Versus Nitration—The most remarkable finding of these studies is that intraphagosomal nitration of fluorescein in neutrophils simply does not occur, even when the reaction medium contains 100 mM NO2 (Fig. 4). The reason for preferential chlorination of the probe within the phagosome is not apparent. It is not attributable to the intrinsic reactivity of fluorescein, which undergoes highly selective MPO catalyzed ring nitration. For example, the amount of nitrofluorescein formed in a solution containing 1 mM NO2 and 100 mM Cl− was 5–6-fold greater than the amount of chlorofluorescein formed (Figs. 1 and 2a). The selectivity for fluorescein nitration over chlorination decreased slightly when the dye was attached to polystyrene beads, but nitration was the predominant product when [NO2−] ≥ 1 mM (Fig. 3). Likewise, NO2 is not excluded from the phagosome since intraphagosomal MPO-catalyzed chlorination was inhibited at high NO2 concentrations (Fig. 4). The apparent inhibition constant (Kd) at pH 7.4 was −5 mM, based upon the amount of NO2 required to inhibit chlorination by 50%. This value is very similar to the ligand dissociation constant (Kd) for NO2 binding to the peroxidase heme extrapolated from EPR data, i.e. Kd ~ 2 mM (pH 7.4) (34) and from spectrophotometric determinations at other pH values, i.e. Kd = 0.6 mM (pH 6.0); 55 mM (pH 8.5) (35). Axial heme ligation blocks reaction with H2O2, preventing formation of the reactive ferryl cation, compound I (7); consequently, one expects that Kd ≥ Kf. The correspondence between Kf and the measured intraphagosomal Kd strongly suggests that the effective intraphagosomal NO2 concentrations are approximately the same as in bulk solution. Inhibition of intraphagosomal MPO reactions by N3−, an anion with solution properties similar to NO2−, is well established (36). Finally, the possibility that the cellular environment presents additional target molecules that react preferentially with MPO-generated NO2 or other reactive nitrogen species is not in quantitative accord with the experimental results. Our data indicate that the medium does contain alternative reactants, i.e. fluorescein nitration was −50% inhibited when the beads were opsonized and completely inhibited when the reactions were carried out in serum; fluorescein chlorination by HOCI is also completely inhibited in serum. If intraphagosomal fluorescein nitration were blocked by intracellular scavengers, one would expect a sharp decline in chlorination yields similar to that observed in solution at [NO2−] ≥ 0.5 mM (Figs. 2 and 3); this occurs because enzymatic reaction of NO2 reduces the HOCI yield. However, no significant decrease in chlorination yield was observed in reactions with neutrophils until the NO2 concentration reached levels that inhibited the enzyme (Fig. 4). Consequently, the data suggest that NO2 is not competitive with Cl− as an MPO substrate within the neutrophil phagosome.

Experiments utilizing both opsonized fluorescein-conjugated beads and tyrosine (Table II) were designed to measure simultaneously the extent of intracellular and extracellular MPO generation of oxidants during phagocytosis. Chlorination was not inhibited until the NO2 concentration reached −5 mM, consistent with a reaction localized to the phagosome (Fig. 4).

2 Q. Jiang, unpublished observations.
and Table II), although extracellular nitration of tyrosine became appreciable at NO$_2^-$ concentrations as low as 500 μM (Table III). The results are consistent with a prior report that PMA-stimulated neutrophils are capable of nitrating 4-hydroxyphenylacetic acid (10); our data further establish that sufficient MPO is secreted from actively phagocytosing neutrophils to catalyze extracellular nitration of tyrosine. Thus, these MPO-catalyzed reactions may contribute to oxidative injury in these and related diseases where peroxidase-containing leukocytes are involved, as previously discussed (10).

**HOCl-mediated Nitration—Clorination of soluble fluorescein by HOCl was inhibited by NO$_2^-$ without formation of appreciable nitrofluorescein (Fig. 2b). This inhibition was only partial, even at NO$_2^-$ concentrations as high as 10 mM, and appeared to plateau at ~20% yield. Very similar behavior has been reported for competitive nitration and chlorination of 4-hydroxyphenylacetic acid by HOCl in NO$_2^-$-containing media, although in these reactions ring nitration was more extensive (13). The general behavior suggests that reaction between NO$_2^-$ and HOCl to form NO$_2^-$Cl (12) competes with direct chlorination of the phenolic ring by HOCl and, when [NO$_2^-$] ≥ 1 mM, the predominant pathway is through the nitro chloride intermediate. Although this (and other putative successor intermediates) (13) are potentially capable of both aromatic chlorination and nitration, fluorescein chlorination is apparently strongly favored. This mechanistic interpretation is consistent with available kinetic data. The reaction of NO$_2^-$ with HOCl is complex and, in neutral solutions at millimolar NO$_2^-$ concentrations, is retarded by Cl$^-$ (12); under our experimental conditions, the HOCl rate constant is $k$(HOCl) $\leq$ 6 $\times$ 10$^{-2}$ M$^{-1}$ s$^{-1}$. A lower limit for the rate constant for monochloro-fluorescein formation from HOCl and fluorescein, based upon stopped-flow measurements, is $k_f$ $\geq$ 10$^4$ M$^{-1}$ s$^{-1}$ (37). Thus, only when [NO$_2^-$]/[fluorescein] $\geq$ 20 can NO$_2^-$ effectively compete with fluorescein for HOCl. In our experiments, [fluorescein] = 105 μM, requiring [NO$_2^-$] ≥ 2 mM for substantial NO$_2^-$Cl formation; the data (Fig. 2b) are consistent with these constraints. Assuming the validity of this mechanism, the data indicate that NO$_2^-$Cl and any reactive species formed from it are insufficient MPO is secreted from actively phagocytosing neutrophils to catalyze extracellular nitration of tyrosine. Thus, these and related diseases where peroxidase-containing leukocytes are involved, as previously discussed (10).

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