Cellularly Generated Inorganic Oxidants as Natural Microbicidal Agents

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Nature has evolved in the phagocytes of higher organisms a remarkable set of highly specialized cells designed to combat microbial infection. Although the respiration-induced oxidative chemistry of these cells has been actively investigated for over three decades, the identities of the toxins and their microbicidal mechanisms are still not understood. Advances have frequently been driven by periodic discoveries that have caused drastic revision of contemporary working hypotheses. At present, recognition that nitric oxide (•NO) is ubiquitous throughout the peripheral circulation[1] and can be generated by at least certain types of phagocytic cells[2] promises to cause such a "paradigm shift" in our understanding of both phagocyte biochemistry and general mechanisms of oxidative stress. Specifically, nitric oxide and superoxide ($^{\bullet}O_2^-$), both chemically relatively benign radicals, combine at near-diffusion-controlled rates,[3] generating peroxynitrite ion ($ONOO^-$), i.e.

$$^{\bullet}O_2^- + {}^{\bullet}NO \rightarrow ONOO^- \qquad (1)$$

which exhibits widespread oxidizing and nitrating capabilities toward biological compounds.[4]

The following Account summarizes the authors' current viewpoint concerning the nature of these microbicidal processes, which is based primarily upon our own observations. As with any rapidly evolving field, controversies abound and few opinions are universally held. Space limitations prevent full development of our arguments; consequently, numerous important contributions from other laboratories have been omitted. The reader is encouraged to consult the leading references given herein for further information.

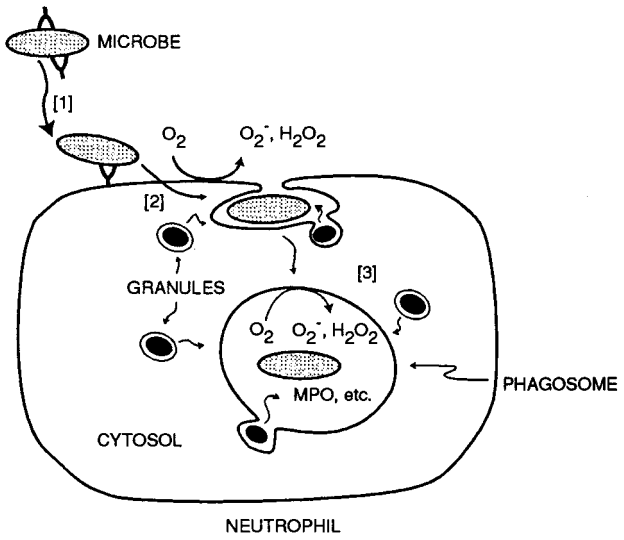

FIGURE 1. Diagram of the phagocytic response of neutrophils illustrating binding and respiratory activation ([1]), phagocytosis ([2]), and degranulation ([3]).

Phagocytosis by Neutrophils[5]

The events associated with the response of a phagocytic cell to foreign bodies is depicted in stylized form in Figure 1. Binding of particles at specific receptor sites on the plasma membrane ([1]) activates a multimeric flavocytochrome called the NADPH oxidase ([2]) that is vectorially organized to transfer electrons across the membrane from cytosolic NADPH to externally localized O_2. Simultaneously, the stimulatory particles are engulfed by membrane invagination, leading ultimately to their isolation within sealed vacuoles (phagosomes). Because the plasma membrane everts during phagocytosis, the NADPH oxidase is now oriented to generate reactive oxygen intermediates directly within the phagosome. In the neutrophil, which is the predominant white blood cell type in our bodies, lysosomal granules migrate to the developing phagosome and discharge their contents into the phagosomal volume ([3]). Prominent among the lysosomal components is the enzyme myeloperoxidase (MPO).

Salient points concerning the reactivities of these cells (summarized in Figure 2) are as follows.

(i) Although NADPH is the immediate source of electrons for O_2 reduction, it is cyclically regenerated in the neutrophil cytosol. Thus, the oxidized respiratory end product is CO_2, which could rise severalfold above its normal physiological level during stimulated respiration.

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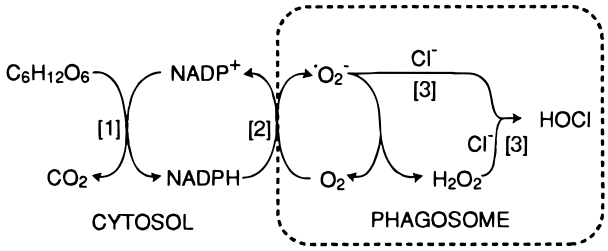


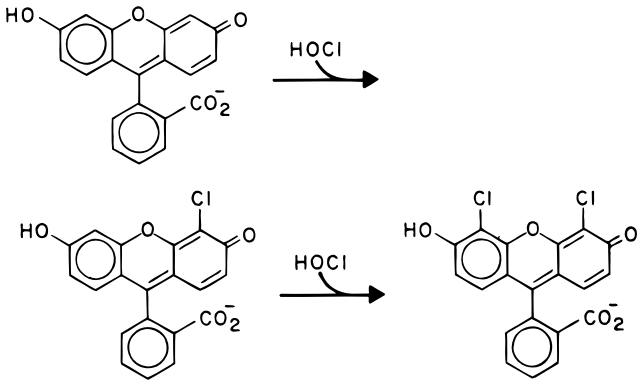
FIGURE 2. Flow diagram for respiratory generation of HOCl and CO_2 with catalysis by the enzymes of the hexose monophosphate shunt ([1]), NADPH oxidase ([2]), and MPO ([3]).

(ii) The heme prosthetic group of the terminal oxygen reductase site in NADPH oxidase is 6-coordinate low spin in both Fe^{II} and Fe^{III} oxidation states.[6] This strong axial ligation precludes direct binding of O_2 to the heme iron, forcing electron transfer to be "outer-sphere", hence, to occur in one-electron steps, and thereby generate $^{\bullet}O_2^-$ as the reaction product.

(iii) MPO has broad specificity, catalyzing both two-electron oxidations of a variety of halides and pseudohalides, including Cl^-, and one-electron oxidations of organic substrates and nitrite ion.[7−9] Hypochlorous acid (HOCl), formed by reaction between Cl^- and MPO compound I (the ferryl π-cation), is freely diffusible from the enzyme active site and, thus, can react at remote biological target sites.

The importance of respiration-derived oxidants to disease resistance is underscored by the observation that individuals whose neutrophils lack a functioning NADPH oxidase suffer chronic life-threatening microbial infections.[5] By utilizing selective inhibitors of MPO and the NADPH oxidase in conjunction with superoxide dismutase-conjugated *Staphylococcus aureus*, Winterbourn and associates have provided strong evidence that the major pathway for killing of this organism is oxidative and involves MPO;[10] arguments supporting the primacy of MPO-dependent mechanisms in other phagocytic reactions have been summarized by Klebanoff.[11] Trapping studies[12] have demonstrated that at least 40% of the O_2 consumed during stimulated respiration can be converted to HOCl; these experiments utilized neutrophils activated with soluble agonists which cause release of the granule components to the external medium.

To gain information on intraphagosomal reactions, we have synthesized bacterial mimics comprising ~1 μm polyacrylamide beads to which fluorescein has been covalently attached via a cystamine linker group.[13] Fluorescein reacts rapidly with HOCl to generate chlorofluoresceins with altered fluorescence properties,[14] i.e.



(2)

Thus, chlorination of the probe by neutrophils can be monitored in real time by using fluorescence spectroscopy. By adding membrane impermeable quenchers such as methylviologen to quench extracellular fluorescence, one can distinguish reactions occurring within sealed phagosomes from reactions in extracellular environments. Additionally, fluorescein can be recovered from the phagosome following reaction by cell lysis and reduction of the cystamine disulfide bond with added thiols, allowing determination of total chlorination yields. Typical results are given in Figure 3, where particle-stimulated O_2 consumption, phagocytosis, and intraphagosomal fluorescein chlorination occur almost simultaneously on time scales that are nearly identical with those reported for bacterial phagocytosis ($t_{1/2} \simeq 5-10$ min).[15] Furthermore, ~6×10^7

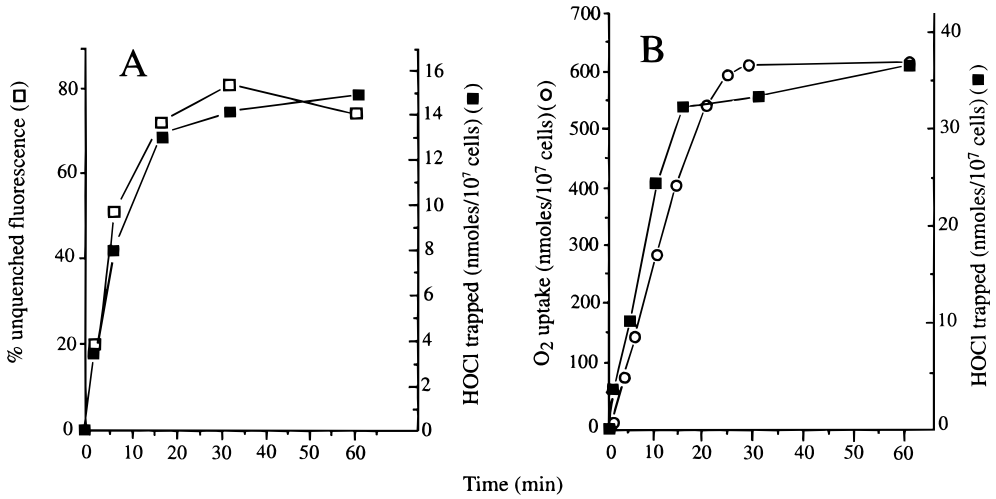


FIGURE 3. Kinetics of neutrophil chlorination (solid squares) of fluorescein-conjugated beads compared to phagocytosis (open squares) and respiratory consumption of O_2 (open circles) (adapted from ref 13). In panel A, the bead/neutrophil ration was 15/1; in panel B, it was 50/1.

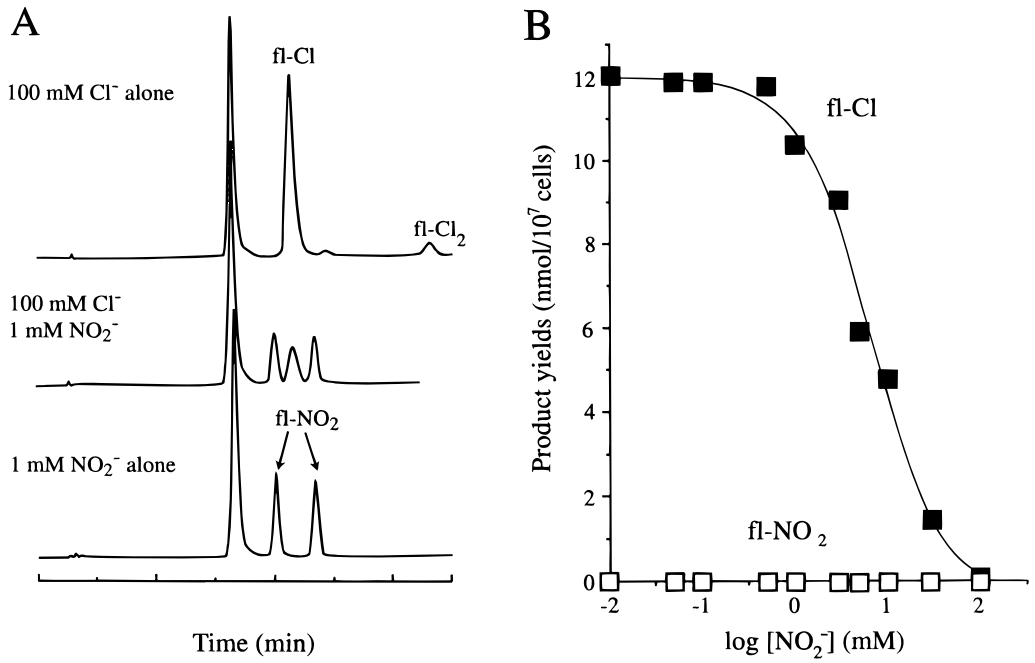
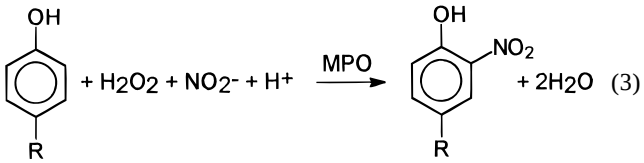


FIGURE 4. MPO-catalyzed reactions: (A) HPLC of reaction products trapped with fluorescein; (B) yields of nitro- and chlorofluorescein products isolated from neutrophil phagosomes for reactions of fluorescein-conjugated beads in serum containing varying [NO_2^-] (adapted from ref 8), The bead/neutrophil ratio was 20/1. Symbols: fl-Cl, chlorofluorescein; fl-Cl_2, dichlorofluorescein; fl-NO_2, isomeric nitrofluoresceins.

HOCl molecules were trapped per bead, a number that is almost identical with the amount required to kill various test bacteria.[16,17] Thus, these studies establish unequivocally that bactericidal levels of HOCl can be generated within neutrophil phagosomes.

A second use of the fluoresceinated beads has been to discriminate between alternative biological substrates for MPO. Normal human fluids contain submillimolar concentrations of NO_2^-, which accumulates as an end product of •NO metabolism.[1] These concentrations can be elevated during infection, possibly to levels sufficient to allow NO_2^- to compete effectively with physiological Cl^- as a substrate for MPO (Figure 4A).[8,9] Thus, MPO-dependent nitration reactions, e.g.



(3)

could also be important in phagocytic killing. However, using the fluorescein-conjugated beads to probe intraphagosomal reactions (Figure 4B), we found no evidence for competitive MPO-catalyzed nitration, indicating that Cl^- was the preferred intraphagosomal substrate.[8]

Chemical Basis for HOCl Toxicity

Hypochlorous acid is highly toxic to bacteria. In macroscopic terms, 1 mL of commercial bleach (5% HOCl) at 10^8 HOCl/cell is sufficient to kill 5 g of *Escherichia coli*. In comparison, it requires greater than 10^{11} molecules of H_2O_2 (in the absence of metal ions) or extracellularly generated •OH to kill the same organism,[18,19] indicating that these more powerful oxidants are at least 10^3-fold less effective. Furthermore, flow studies have established that *E. coli* are killed within 100 ms of exposure to lethal levels of HOCl.[16] Taken together, these observations imply that cellular death is associated with destruction of a relatively small number of vulnerable sites within the bacterium that are highly reactive toward bleach. How then does one identify the sites?

We have approached the problem in two complementary ways, i.e., by probing the inherent chemical reactivity of HOCl, which is the basis for its biochemical selectivity, and by examining the pattern of metabolic dysfunctions and oxidative damage at detectable sites within the bacterium itself. Hypochlorous acid acts as a two-electron oxidant toward most biological compounds, presumably because one-electron reactions are thermodynamically restricted by requiring formation of high-energy •OH or •Cl radicals as products. Rate constants for oxidation of simple compounds by HOCl and similar chlorinating agents are proportionate to the relative electrophilicity of the chlorine atom and nucleophilicity of the reacting partner,[20] indicating that selectivity is dictated by donor−acceptor type interactions. This same selectivity for nucleophilic sites extends to reactions of HOCl with biological partners.[20] Electron-rich π-delocalized centers such as nitrogen heterocycles (hemes and nucleotide bases), iron−sulfur clusters, and conjugated polyenes (e.g., carotenes), as well as amino acids containing highly polarizable sulfur atoms and amines, react extremely rapidly, whereas biomolecules possessing no nucleophilic sites are virtually unreactive.

Plots of cell viability vs oxidant concentration typically exhibit sigmoidal shapes in which bacterial killing occurs over a relatively narrow concentration range. Figure 5

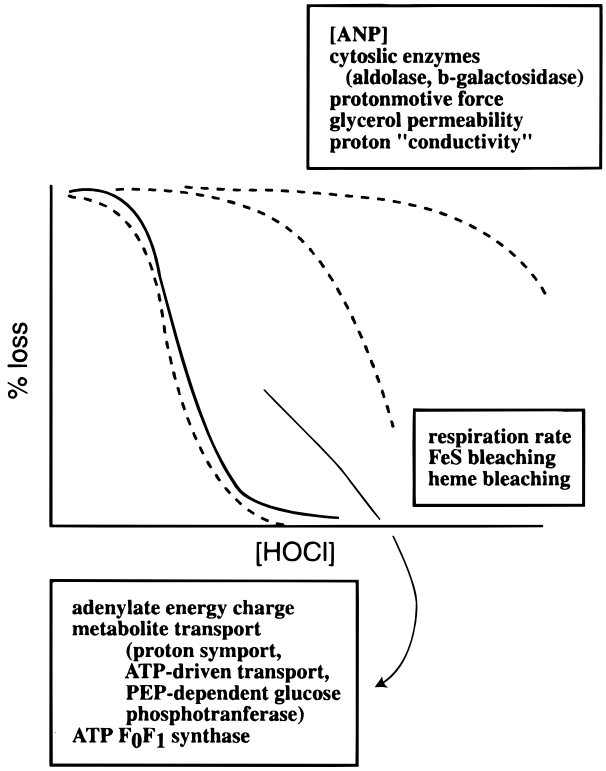


FIGURE 5. Schematic representation of loss of cellular function in *E. coli* upon exposure to HOCl or cell-free MPO−H_2O_2−Cl^- bactericidal systems. The solid line represents the cell survival curve; the dashed lines represent an approximate titrimetric scale for loss of functions indicated in the adjacent boxes. (Reprinted with permission from: Hurst, J. K. In *The Activation of Dioxygen and Homogeneous Catalytic Oxidation*; Barton, D. H. R., Martell, A. E., Sawyer, D. T., Eds.; Plenum Press: New York, 1993; pp 267−286.)

summarizes (in highly stylized form) the results of numerous experiments designed to identify relationships between cellular death and the oxidative damage to *E. coli* inflicted by HOCl.[16,17,20,21] The pattern that emerges indicates that death is accompanied by inhibition of metabolite transport by all available mechanisms and by inhibition of the proton-translocating F_0F_1-ATP synthase, the immediate consequence of which is massive hydrolysis of cellular ATP. The plasma membrane, whose integrity is essential to active transport and ATP synthesis, is not breached since it retains its intrinsic impermeability to ions, nor is there significant damage to cytosolic components since the total adenine nucleotide pool and activities of cytosolic enzymes that are highly susceptible to oxidative inactivation by HOCl are not diminished. In *Pseudomonas aeruginosa*, but not *E. coli*, loss of respiratory function also correlates with cellular death.[21]

The set of metabolic dysfunctions known to accompany cellular death is sufficient to account for the universal bactericidal properties of HOCl;[20] it constitutes no less than complete inhibition of cellular ATP-generating capacity, without which the cells cannot maintain homeostasis, much less undergo biosynthesis and repair, and soon expire. The characteristic shapes of the viability curves (Figure 5) can be understood as follows: the initial reactions of HOCl are confined to relatively innocuous oxidation of sulfhydryl substituents and N-chlorination of amines on the bacterial envelope.[22] Upon loss of these sites, however, the integral membrane proteins associated with energy transduction are exposed and inactivated by HOCl, with catastrophic consequences to the cell. Continued addition of HOCl beyond lethal amounts leads ultimately to damage of susceptible molecules in the cytosol as well, although these are postmortem reactions. Throughout the titration, the extent of damage to the structural components of the cell is minimal, so that the cell morphology is maintained; i.e., the dead cells look normal.

This model is not applicable to eukaryotes, e.g., yeasts and fungi, which are also effectively killed by phagocytes but whose ATP is generated by mitochondria within the relatively protected cell interior. Both mitochondrial respiration and oxidative coupling are retained in the yeast *Saccharomyces cerevisiae* at HOCl doses severalfold in excess of lethal amounts.[23] However, the activity of a proton-translocating ATPase located in the yeast plasma membrane is lost in parallel with cellular viability.[25] Because this H^+-ATPase generates the transmembrane proton gradient that drives active transport of nutrients across the plasma membrane and regulates tonicity and the intracellular ionic composition,[25] it is crucial to cell survival. Thus, lethal lesions occur in both types of cells on the energy-transducing proteins of their plasma membranes.

Reconstitution experiments using the $\alpha_3\beta_3\gamma$-multimeric F_1-ATPase from the F_0F_1-ATP synthase of HOCl-inactivated *E. coli* indicated that loss of ATP-hydrolyzing activity was associated with damage to each of the subunits, although two-dimensional gel electrophoresis indicated that the extent of oxidation was very minor.[26] Examination of succinate dehydrogenases isolated from HOCl-inactivated *E. coli* and *P. aeruginosa* has localized respiratory inhibition to points on the electron transport chains between the Fe/S clusters and the ubiquinone reductase site that are distinct from the highly reactive Fe/S clusters and cytochrome components themselves.[21] Although not identifying the target sites associated with functional loss, these results demonstrate the high selectivity which HOCl shows for them.

Other Oxidative Toxins

The Role of H_2O_2. Phagocytic cells that lack peroxidases but possess active NADPH oxidases (e.g., macrophages, MPO-deficient neutrophils) kill microbes by oxidative mechanisms, indicating the capacity of these cells to generate at least one additional oxidative toxin. Since $^\bullet O_2^-$ is only weakly oxidizing[20] and bacteria, particularly pathogenic ones, are resistant to killing by H_2O_2 in metal-free environments, it has generally been thought that the toxic agents are secondary oxidants formed in reactions catalyzed by redox-active metal ions.[27] Indeed, Fenton-type systems comprising reducing agents, H_2O_2, and iron or copper complexes have been shown to cause oxidative

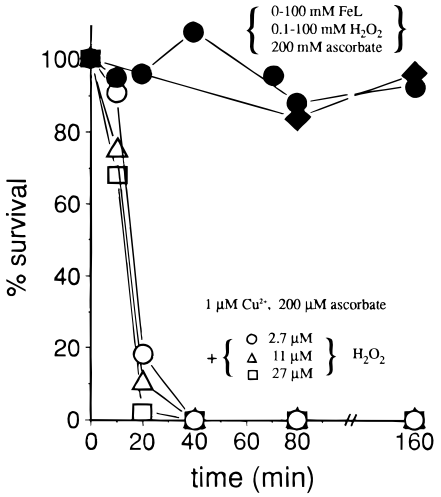


FIGURE 6. Comparative toxicity-enhancing effects of copper and iron toward *E. coli*: L = EDTA, DETAPAC, picolinate, ADP, ATP, lactoferrin, or desferrioxamine (adapted from ref 18).

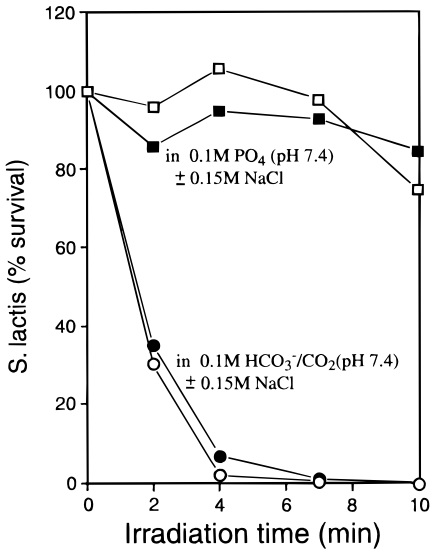
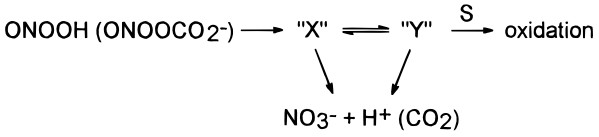


FIGURE 7. Comparative toxicities of radiolytically generated $^{\bullet}OH$ (squares) and $^{\bullet}CO_3^-$ (circles) toward *S. lactis* (adapted from ref 19).

damage to a wide range of biomolecules in homogeneous solutions; whether the causative agents are hydroxyl radical, hypervalent metal ions, or their peroxo complexes is a matter of active debate. Of more immediate concern is whether these oxidative systems are microbicidal. Studies using host-derived metalloproteins, e.g., ceruloplasmin, lactoferrin, and serum protein mixtures, have failed to reveal any potentiation of H_2O_2 toxicity toward *E. coli* in suspension culture.[18] More generally, these bacteria appear oblivious to iron-catalyzed Fenton systems, although media containing physiologically relevant levels of ascorbate and H_2O_2 are potently bactericidal in the presence of trace amounts of Cu^{2+}, with toxicities approximately equal to that of HOCl (Figure 6).[18] Remarkably, cuprous ion alone is 200-fold more toxic than either HOCl or the ascorbate/H_2O_2/Cu^{2+} bactericidal assay system.[18] Since CuCl is neither a strong oxidant nor a strong reductant, death must arise by Cu(I) assimilation by the cells and its subsequent catalysis of intracellular redox reactions. Other recent studies suggest that superoxide dismutase deficient *E. coli*, which are relatively easily killed by H_2O_2,[28] release iron from cytosolic enzymes containing iron−sulfur clusters when exposed to lethal levels of the oxidant, thereby promoting intracellular $^{\bullet}OH$ formation.[29] In contrast, when generated extracellularly by γ-radiolysis, $^{\bullet}OH$ is virtually nontoxic to bacteria in suspension cultures (Figure 7).[19] The slow loss of viability that is observed has been attributed to intracellular $^{\bullet}OH$ generation, because the cells cannot be protected by addition of free radical scavengers to the medium. From the unquenched killing rate, however, it appears that the small fraction of $^{\bullet}OH$ that is formed within cells is at least 10^3-fold more toxic than HOCl. This pattern—apparent potentiation of H_2O_2 toxicity by intracellular but not extracellular redox active metal ions, high toxicity of intracellularly but not extracellularly generated $^{\bullet}OH$—suggests that, if Fenton reactions are important in leukocyte function, the obligatory metal catalysts are derived from the microbe, not the host organism, and, unlike HOCl, the lethal reactions involve damage at intracellular sites.

The Role of ONOOH. Macrophages and at least some neutrophils[2] possess an inducible form of nitric oxide synthase, an enzyme that generates $^{\bullet}NO$ by aerobic oxidation of arginine. Thus, at sites of infection, particularly where mixed populations of neutrophils and macrophages are present, simultaneous generation of $^{\bullet}NO$ and $^{\bullet}O_2^-$ could lead to formation of cytotoxic amounts of $ONOO^-$ (reaction 1). Although $ONOO^-$ decomposes only slowly in alkaline solutions, protonation[30] or binding of other Lewis acids (e.g., CO_2)[31] dramatically increases its decomposition rate and alters its reactivity.[4,32−34] Peroxynitrous acid (ONOOH) can engage in both one-electron and two-electron oxidations. Mechanistic studies to date suggest that the two-electron oxidations are limited to reactions with good nucleophiles and exhibit bimolecular kinetics, whereas the one-electron reactions involve rate-limiting unimolecular conversion of ONOOH to a more reactive form. A distinctive characteristic of the unimolecular (or "indirect") pathway, which has been demonstrated for both ONOOH and the CO_2 adduct ($ONOOCO_2^-$), is that only a fraction of the oxidant is available for reaction with potential reductants, i.e., product yields are always less than the expected stoichiometric limits.[4,32−35] This behavior requires postulation of at least two discrete intermediates (Scheme 1), only one of which oxidizes other compounds (S).

Scheme 1



The CO_2 concentration in respiring phagocytes is sufficiently high that nearly all of the peroxynitrite that is not scavenged by heme proteins[36,37] will be converted to

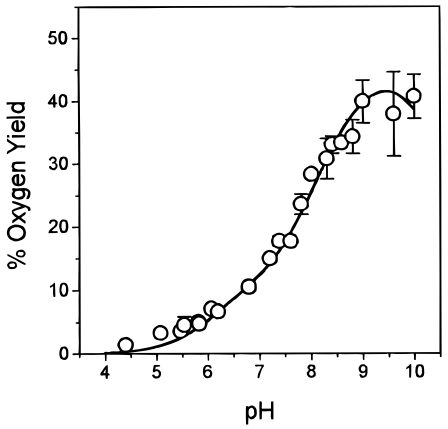


FIGURE 8. Comparison of measured (circles) and calculated (line) O_2 yields based upon Scheme 2 (adapted from ref 41).

$ONOOCO_2^-$ before reacting with target biomolecules.[38] Hence, understanding the biological reactivity of peroxynitrite rests upon identifying "X" and "Y" (Scheme 1). These species cannot be directly examined, however, because they do not accumulate; i.e., the rate-limiting step for reactions involving $ONOOCO_2^-$ is its formation from $ONOO^-$ and CO_2. There are two prevailing opinions concerning the nature of "X" and "Y". One is that they are cis and trans configurational isomers of ONOOH and $ONOOCO_2^-$, and the other is that the reactive species are radicals ("Y") formed by homolytic cleavage of the peroxo O−O bond in the ONOOH and $ONOOCO_2^-$ precursors.[39] To gain information on the identity of "Y", we investigated the indirect reactions of $ONOOCO_2^-$ with a series of simple outer-sphere one-electron reductants.[32] These studies established that "Y" oxidizes compounds with reduction potentials as great as 1.3 V, but that "X" is unreactive toward even easily oxidizable compounds such as $Fe(CN)_6^{4-}$ ($E^\circ = 0.36$ V). Since it is unlikely that the redox potentials for "X" and Y would differ by greater than 0.9 V, the results imply that "X" is unreactive simply because it is too short-lived to engage in bimolecular reactions. These facts are readily accommodated by a mechanism in which "X" is the geminate radical pair $\{^\bullet NO_2, ^\bullet CO_3^-\}$formed by O−O bond homolysis and "Y" is the corresponding separated radicals;[40] note that $E^\circ(^\bullet CO_3^-/CO_3^{2-}) = 1.6$ V. In this scheme, maximal product yields are limited by the cage escape probabilities and should therefore be independent of the reaction partner, as has been observed for these simple one-electron reactions.[32]

An analogous mechanism can be written for ONOOH (Scheme 1), for which "X" is the geminate pair $\{^\bullet NO_2, ^\bullet OH\}$. In acidic solutions, NO_3^- is the sole decomposition product, but in weakly alkaline solutions, where ONOOH and $ONOO^-$ coexist, O_2 and NO_2^- are also produced in a 1:2 proportion. This reaction exhibits the following characteristics:[41] O_2 and NO_2^- yields (i) increase with the medium pH (Figure 8), (ii) are diminished by adding $^\bullet OH$ scavengers, and (iii) are restored in alkaline media containing the free radical scavengers by adding NO_2^-, although (iv) NO_2^- is itself inhibitory in neutral solutions.

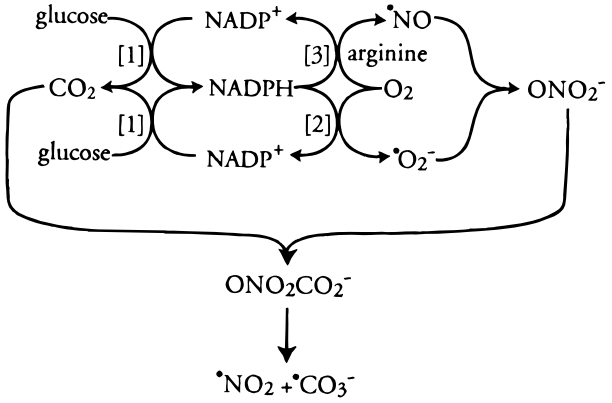
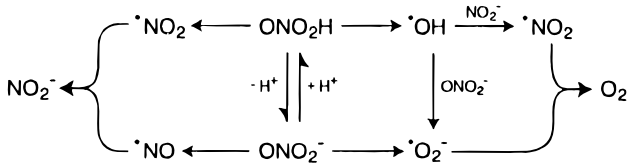


FIGURE 9. Metabolic pathway for respiratory generation of $^\bullet CO_3^-$ in physiological environments with catalysis by the enzymes of the hexose monophosphate shunt ([1]), NADPH oxidase ([2]), and $^\bullet NO$ synthase ([3]).

This complex behavior is accurately predicted by Scheme 2 plus the reactions $N_2O_3 + ONOO^- \rightarrow NO_2^- + 2^\bullet NO_2$ and

Scheme 2



$2^\bullet NO_2 \rightleftharpoons N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$. Calculated oxygen yields based upon this scheme are nearly identical with experimentally determined values (Figure 8). This comparison constitutes a stringent test of mechanism because the rate constants for all of the individual steps have been independently determined; i.e., there are no adjustable parameters. The comparison shows that the data are accurately duplicated by assuming that the intermediary oxidant has a chemical reactivity identical with that of radiolytically generated $^\bullet OH$. Involvement of caged radical pairs is also consistent with the pressure dependence of decomposition rates and limiting product yields,[42] as well as O−O bond energies estimated from ab initio calculations.[43] Typically, we measure $\Delta V^\ddagger = +(6-14)$ cm^3/mol for reactions involving ONOOH,[42] which is the magnitude expected for reactions initiated by O−O bond fission.[44] The calculated bond dissociation energy for ONOOH is ∼22 kcal/mol and for $ONOOCO_2^-$ is ∼9 kcal/mol, compared to HOOH, for which it is 51 kcal/mol.[43] If, as the data imply, $^\bullet OH + ^\bullet NO_2$ is "Y" in reactions involving ONOOH, then almost certainly $^\bullet CO_3^- + ^\bullet NO_2$ is "Y" in the reactions of $ONOOCO_2^-$. Indeed, for several reactions, competition kinetics studies have shown the relative reactivities to be consistent with intermediary formation of $^\bullet CO_3^-$ as the oxidant. [45] Consequently, intraphagosomal formation of $ONOO^-$, e.g., as illustrated in Figure 9, should lead to generation of both $^\bullet OH$ and $^\bullet CO_3^-$ radicals, with the latter strongly predominating in the elevated CO_2 environments of respiring phagocytes.

The toxicity of radiolytically generated $^\bullet OH$ is markedly enhanced when the cellular suspensions contain bicarbonate ion (Figure 7),[19] which is undoubtedly due to

formation of $^{\bullet}CO_3^-$ as a secondary radical. Carbonate radical is expected to exhibit greater toxicity than $^{\bullet}OH$ because its decreased reactivity and longer lifetime allow a higher selectivity for bacterial targets. In contrast, physiological levels of CO_2 completely protected *E. coli* in suspension cultures from ONOOH, which otherwise exhibits a toxicity ~10-fold greater than that of HOCl.[45]

The apparent contradiction between these observations can be reconciled by recognizing that $^{\bullet}CO_3^-$ is produced in the chemical reaction at much higher steady-state concentrations than in the radiolysis experiment, and is accompanied by formation of equimolar $^{\bullet}NO_2$. Because $^{\bullet}CO_3^-$ and $^{\bullet}NO_2$ recombine at nearly diffusion controlled rates, the lifetime (and diffusion distance) of $^{\bullet}CO_3^-$ is orders of magnitude shorter in the $ONOO^- + CO_2$ system, and it simply reacts before it can reach its cellular targets. By the same argument, radiolytically generated $^{\bullet}OH$ is very short-lived, but ONOOH is long-lived and can diffuse over distances as large as several micrometers before reacting, even in media containing physiological levels of CO_2. Since ONOOH can apparently cross bilayer membrane barriers,[37,47] it may be able to enter the bacterial cytosol to generate highly toxic oxidizing radicals. Can we then expect $ONOO^-$ formation within phagosomes to be innocuous or toxic at physiological levels of CO_2?

The Role of Phagosomal Compartmentation

Phagocytosis could significantly alter the dynamics of microbial killing in at least two ways. First, by reducing the reaction volume it may become possible to generate concentration levels of H_2O_2 that are sufficiently high to overwhelm the microbial defense mechanisms without requiring activation by metal ions from an exogenous source. For example, we calculate from the O_2 consumption rate during stimulated respiration that the transitory rate of H_2O_2 production could reach ~1 M/min in the phagosomes of neutrophils lacking active MPO; in contrast, very rapid H_2O_2 consumption by MPO is expected to keep H_2O_2 steady-state levels at very low levels in normal neutrophils.[18] Second, spatial confinement will promote reaction of short-lived oxidants with the entrapped bacterium at the expense of reaction with antioxidants contained within the phagosomal fluid. Mathematical modeling shows that this effect is optimized when the size of the reaction compartment is small relative to the mean diffusion length of the oxidant, i.e., the distance it travels before reacting with an aqueous phase partner.[48] Results of representative calculations are shown in Figure 10 for $^{\bullet}OH$ and $^{\bullet}CO_3^-$ as a function of the phagosomal size. One notes that the probability of $^{\bullet}OH$ reacting with the bacterium is very low unless the phagosomal compartment is small (equivalently, $^{\bullet}OH$ will react with the bacterium only if it is generated immediately adjacent to the bacterial surface); in contrast, a significant fraction of $^{\bullet}CO_3^-$ is predicted to react within relatively large phagosomes, even when the medium contains antioxidants at concentration levels normally found in serum. At present, there is insufficient information on

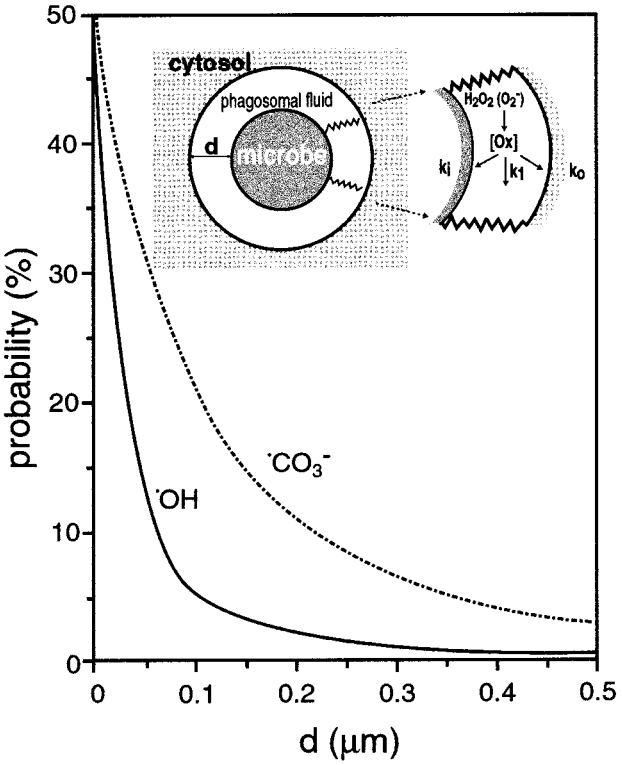


FIGURE 10. Probability of intraphagosomal reaction of bacteria with short-lived oxidants based upon the kinetic model shown in the inset. Oxidants ([Ox]) generated randomly throughout the phagosomal volume react with scavengers (k_1), the phagosomal membrane (k_0) or the bacterium ($k_i = k_0$) at characteristic rates (adapted from ref 48).

the membrane permeability of ONOOH to permit quantitative estimation of how cellularly generated peroxynitrite would partition within phagocytic cells, but both formation of $^{\bullet}CO_3^-$ plus $^{\bullet}NO_2$ in the phagosomal layer and release of oxidizing radicals ($^{\bullet}OH$, $^{\bullet}CO_3^-$) within the bacterial cytosol appear to be plausible mechanisms of toxicity.

Summary

The predominant oxidative microbicidal mechanism of peroxidase-containing phagocytic cells appears to involve respiration-initiated formation of HOCl; cellular death is associated with inactivation of essential transport proteins located in microbial plasma membrane, including (in bacteria) the F_0F_1-ATP synthase. The oxidants produced by phagocytic cells lacking functional peroxidases are more obscure, but evidence is accumulating to suggest that ONOOH and $^{\bullet}CO_3^-$, formed by reaction of CO_2 with $ONOO^-$, may be important toxins. If so, phagosomal compartmentation constitutes an integral component of the microbicidal mechanisms, since $^{\bullet}CO_3^-$ should be effectively scavenged by antioxidants and ONOOH by CO_2 in extracellular environments, but not within the phagosome. There is at present no compelling evidence for metal-mediated H_2O_2 microbicidal mechanisms in which the metal catalysts are derived from the host fluids, although intraphagosomal concentrations of this oxidant might reach sufficient levels in peroxide-free phagocytes to overwhelm endogenous protective systems.

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