LEUKOCYTIC OXYGEN ACTIVATION AND MICROBICIDAL OXIDATIVE TOXINS

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I. INTRODUCTION

This review is concerned with the oxidative chemistry of phagocytes (Gk. *phagos* = devouring, *kytos* = container), the motile cells in animals that constitute the primary cellular defense against invading pathogens. Special attention will be given to the polymorphonuclear leukocyte, also called granulocyte or neutrophil, which is by far the most prevalent form of white blood cell and which is directed primarily against bacteria and other unicellular organisms. As such, its physiology and biochemistry are relatively well understood. The neutrophil also possesses or is capable of generating a diverse set of microbial toxins, both oxidative and nonoxidative, and may, therefore, be a paradigm for the microbicidal reactions of the phagocytic group.

This topic has been reviewed frequently in the past few years[1-7] and has been the subject of an admirable monograph.[8] Nonetheless, areas concerned with the mechanisms of formation of active oxygen species in leukocytes and the molecular basis of bactericidal action of these toxins have been developing rapidly, and their further consideration at this time is appropriate. We attempt to provide the chemist's perspective, emphasizing where possible the principles of reactivity inherent in the chemical systems. It should be recognized, however, that in phagocytic cells ultrastructural organization and compartmentation probably play as important roles in controlling reactions as the intrinsic chemical selectivity of the oxidants. To place the chemistry in its proper context, one must, therefore, have a conceptual understanding of the biochemical and physiological response of these cells to infection. Some basic introductory information relevant to the chemistry being considered is presented in the next section, and the monograph by Klebanoff and Clark is particularly recommended for additional reading.

A. PHAGOCYTOSIS BY LEUKOCYTES — A THUMBNAIL SUMMARY[8]

The inception of cellular immunology is attributed[9] to the zoologist Elie Metchnikoff, who in 1882, while experimenting with mobile, amoeboid cells in starfish larvae, first conceived the idea that these types of cells might function in host defense systems. This notion was initially opposed by others, who believed that bactericidal action was attributable to "humoral" factors, i.e., the soluble glycoproteins residing in serum. Subsequent research has established that phagocytes and serum-derived protein work cooperatively, at least in part, with binding of antibody and complement to bacteria and other foreign bodies serving to aid recognition and assimilation by phagocytes. The process in which foreign bodies are labeled by serum-derived glycoproteins is termed opsonization (Gk. *opsono* = to cater for)

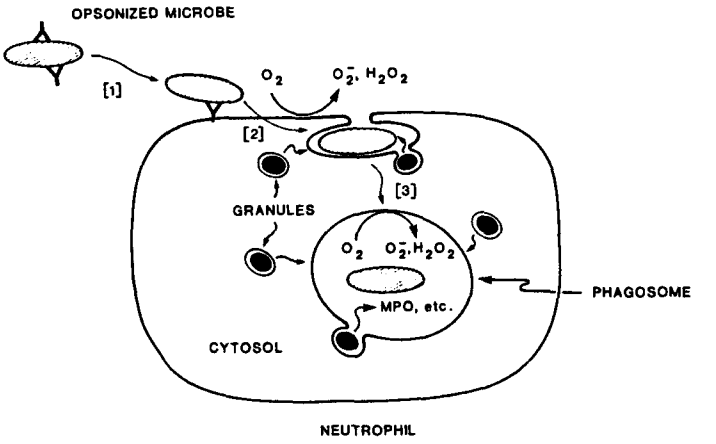

FIGURE 1. Diagram of phagocytosis by neutrophils. (1) Binding of antibody-coated bacterium elicits the respiratory burst; (2) the neutrophil membrane invaginates; and (3) ultimately pinches off, forming the phagolysosome. Simultaneous degranulation leads to both extracellular secretion and intraphagosomal compartmentation of granule components.

and, although not always essential to obtaining a response, often strongly enhances phagocytosis.

Neutrophils arise from presursor cells located in the bone marrow of higher animals. During early stages of development, the cells synthesize a diverse set of enzymes and other biopolymers which are packaged in special granules for later use in killing and clearing pathogens. The granular enzymes are primarily digestive, comprising acid hydrolases, neutral proteases, alkaline phosphatases, and lysozyme. The granules also contain cationic proteins which possess bactericidal properties, lipopolysaccharides, and two biopolymers that could be involved in oxidative microbicidal reactions, namely, a highly unusual peroxidase capable of oxidizing Cl^- to HOCl, called myeloperoxidase (MPO), and lactoferrin. In mammals, the quantity of MPO in neutrophils is truly staggering, comprising 1 to 5% of the dry weight of the cell,[7,10,11] but this enzyme is absent from the avian neutrophils that have been examined.[11] Lactoferrin is predominantly in its apo, or demetalated form, with only about 10 to 20% of its iron-binding sites occupied. Over the course of differentiation, glycogen also accumulates, and mitochondria, prevalent in the cell at early immaturity, progressively decline until they are virtually absent in the mature neutrophil. Once released to the bloodstream, neutrophils do not divide and maintain very low basal respiratory rates. Until activated, metabolic energy needs are apparently minimal, i.e., just those required for cellular maintenance reactions.

Neutrophils migrate to sites of infection in tissues by responding to chemotactic factors, i.e., chemical signals, generated by reaction at those sites. Particles encountered that are recognized as foreign are bound tightly to the plasma membrane, eliciting a complex series of physiological and metabolic changes within the neutrophil leading to their encapsulation by phagocytosis. Once compartmented within the neutrophil, bacteria are rapidly killed and, subsequently, extensively digested.

The sequence of events comprising phagocytosis is illustrated stylistically in Figure 1. Binding of an opsonized microbe activates a dormant pyridine-nucleotide-dependent oxidase located within the plasma membrane, causing marked enhancement of oxygen consumption. Once initiated, this "respiratory burst", as it is called, lasts 15 to 20 min and generates O_2^- and H_2O_2 as one- and two-electron-reduced products, respectively. Unlike the terminal oxidase of mitochondria, the neutrophil oxidase is not inhibited by CN^- or N_3^- anions.

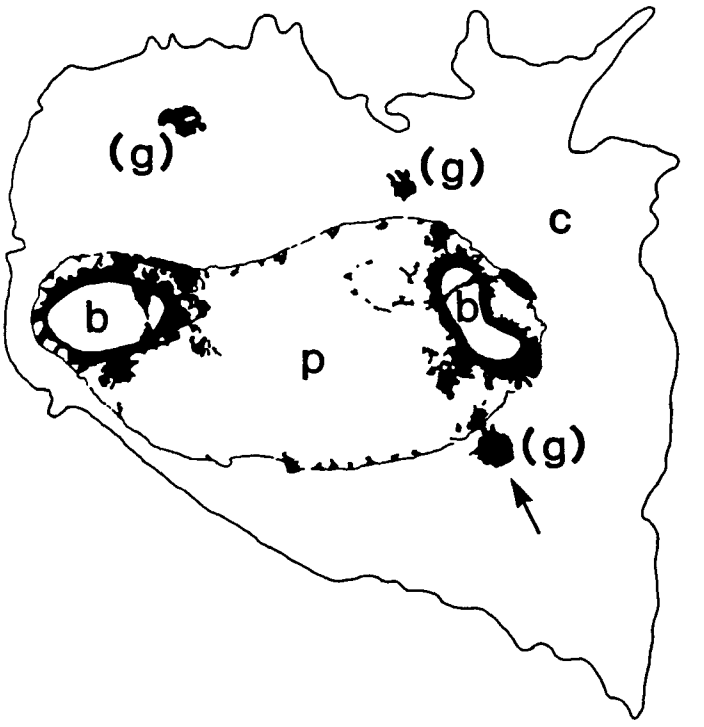

FIGURE 2. Tracing of an electron micrography of a human neutrophil containing two *Lactobacillus acidophilus* within a phagocytic vacuole. Only the plasma and phagosomal membranes and regions of the cell staining for peroxidase are shown. (b) Bacteria; (p) phagosome; (c) neutrophil cytosol; (g) azurophilic granules. (Adapted from Figure 2 in Hurst, J. K., *Oxygen Complexes and Oxygen Activation by Transition Metals*, Martell, A. E. and Sawyer, D. T., Eds., Plenum Press, New York, 1988.)

Simultaneously, the plasma membrane invaginates at the binding site, eventually surrounding the microbe and pinching off, isolating it within a special lysosome called a phagolysosome, or phagosome. During and subsequent to these events, the granular lysosomes migrate to the phagosome, and their membranes fuse in such a manner that the granular contents are discharged into the phagosomal membrane and surrounding medium. This process is termed degranulation.

Phagocytosis is further illustrated in Figure 2, which is a tracing taken from an electron micrograph[8] of a single neutrophil containing two bacteria within its phagosome. In this tracing, much of the internal structure of the cell has been omitted. Only the plasma and phagosomal membranes and cellular regions staining positively for peroxidase activity are shown. These include the MPO-containing granules located in the cytosol, one granule which appears to be in the process of fusing with the phagosome (arrow), and the intraphagosomal regions immediately surrounding the encapsulated bacteria. Since MPO is a cationic protein and bacterial cell walls are negatively charged, they are bound strongly by electrostatic forces.

The entire process from recognition and binding to degranulation is quite rapid, requiring at most a few minutes. Bacteria are generally killed within the same time frame.[12,13] Gram-negative organisms, whose cell walls are resistant to cleavage by lysozyme, give no indication of morphological changes accompanying cellular death.[14] Subsequent digestion occurring over a period of several hours[15-17] leads to eventual disruption of cellular integrity. There is strong evidence indicating that neutrophils possess both oxidative[1-8] and nonoxidative[6-8,18,19]

microbicidal systems, which at times appear to be redundant. Although there is currently an active debate concerning the primacy of one or the other, pathogenic organisms present a wide variety of ultrastructural composition and metabolic capability, so that a diversity in response may be essential to host survival. Two types of observations underscore the importance of oxidative reactions, however. First, many bacteria are killed by phagocytes much more effectively in aerobic than in anaerobic environments.[20] Second, there exists among individuals a set of congenital defects[21] known collectively as chronic granulomatous disease (CGD), which are characterized by the inability of their neutrophils to mount a respiratory burst, although other aspects of phagocytosis appear normal. These individuals suffer from chronic infection, particularly by pathogens that are catalase-positive and/or do not generate endogenous H_2O_2.

Finally, the respiratory burst can also be elicited by various soluble stimuli, which apparently bind at the initiating cell surface receptor sites, but do not induce extensive formation of phagosomes. Degranulation occurs in this instance to the plasma membrane with release of granular contents into the extracellular medium. This phenomenon is quite useful experimentally because it provides a means for studying neutrophilic reactions without requiring isolation of or recovery from subcellular organelles. Stimuli often used to induce this cellular response include chemotactic peptides and tumor-forming surface-active agents, e.g., phorbol myristate acetate (PMA).

II. NEUTROPHILIC GENERATION OF ACTIVE OXYGEN SPECIES

A. ULTRASTRUCTURAL ORGANIZATION OF THE NADPH OXIDASE

Because the phagosome is formed by invagination of the plasma membrane, it is generally thought to be everted, i.e., with the membrane outer surface becoming the inner surface of the phagosome. This point has not been established experimentally, however, and has, in fact, been challenged by Rikihisa and Mizuno,[22] who used nonpermeant biological markers of both sides of the plasma membrane to examine the cytoplasmic surface of phagosomes formed by ingestion of artificial particles. The markers used were antibodies raised against either surface, influenza virus, which binds to the outer surface of the plasma membrane, and the protein myosin, which is normally found associated with the membrane inner surface. By comparing binding patterns from the four markers, the researchers suggested that the phagosomal membranes were "right side out," rather than everted, so that binding sites on the convex outer surface of the plasma membrane appeared on the convex cytoplasmic surface of the phagosome and so forth. Ambiguities appear to exist in the experimental data, however. The antibodies also bound to granule membranes, so binding sites might have been introduced into the phagosomes from this source during degranulation. Furthermore, since only the outer, or cytoplasmic, side of the phagosomal membrane could be examined by the methods used, much of the evidence was negative in the sense that existence of a specific binding site on the inner surface could only be inferred from the absence of binding to the outer surface. The possibilities could not be dismissed, for instance, that the binding sites were blocked in the phagosome, e.g., by ultrastructural alterations, or were totally absent, e.g., by virtue of their selective exclusion from the nascent phagosomal membrane during particle engulfment. Different binding patterns were observed for each of the three particulate stimuli used, indicating that particle-membrane interactions were sufficiently strong to alter in some way the probe receptor sites. If the negative results are discounted and recognition is made of the potential contribution of granule membrane binding sites of the phagosomal membrane, then the binding patterns observed for the polyacrylamide and opsonized polyacrylamide particles are most consistent with phagocytosis occurring with eversion of the plasma membrane. On the other hand, the patterns for paraffin-oil-bovine

serum albumin emulsions were clearly in accord with a "right side out" phagosome. This last system is considerably less well characterized structurally than the polyacrylamide beads, and there is the possibility that labile emulsion components might cause transitory intramembrane micellarization that could provide the mechanism for transverse diffusion of components, hence, scrambling of marker binding sites on the opposite phagosomal membrane surfaces.[23] In view of these many uncertainties, it is perhaps best to conclude only that *a priori* assumptions regarding the orientation of integral membrane components based upon overall cellular topographies are dangerous, and precedent exists for transverse reorganization of membrane components during phagosome formation in nonphysiological reactions.

The oxygen-reducing site of the NADPH oxidase has generally been regarded as localized at or near the external surface of the plasma membrane because near-stoichiometric formation of superoxide ion from O_2 can be demonstrated[24] in instances where soluble stimuli are used or the cells are treated to minimize endocytosis of particulate stimuli, e.g., with cytochalasin B.[25] Neutrophils contain cytosolic superoxide dismutase[26] (SOD), so the argument has been made[24,26] that if O_2^- were formed at a cytosolic site, a significant fraction would disproportionate before it could diffuse to the external medium and react membrane-impermeable trapping agents, e.g., ferricytochrome *c*.[27] The ratio of O_2^- detected to O_2 consumed is considerably less than unity under conditions of active phagocytosis.[24,28,29] In this instance, it is argued that O_2^- generated by the oxidase within the phagosome is effectively sequestered from the external medium and cannot react with the oxidant. An indication that the activated NADPH oxidase might preferentially accumulate in phagosomal membranes are the observations by Kakinuma and co-workers[30] that two distinct subfractions of plasma membranes can be isolated from disrupted stimulated neutrophils, one of which is disproportionately concentrated in the oxidase, but that the oxidase appears homogeneously distributed before stimulation.

Oxygen reduction by the oxidase is tightly coupled to glucose oxidation via the hexose monophosphate pathway, the glucose ultimately being obtainable from intracellular glycogen stores.[8] Both NADH and NADPH can act as electron donors, although NADPH is generally regarded as the physiological donor based primarily upon its much lower apparent binding constant, K_m. Since the enzymatic system generating NADPH is localized within the cytosol and no evidence has been presented for a functioning NADPH transport system within the plasma membrane, it is generally thought that the reductant binding site is located on the cytosolic side of the plasma membrane. If these inferences are correct, then the NADPH oxidase constitutes a transmembrane electron transport chain similar, in principle, to those described for respiration and photosynthesis.[31] Studies designed to examine this presumed transverse asymmetry, while including elements of ambiguity, have supported the model. Green and co-workers found[28] that respiration by intact neutrophils stimulated with opsonized zymosan (yeast cell wall fragments) was uninfluenced by addition of exogenous NADPH or $NADP^+$, but was enhanced by NADPH and inhibited by $NADP^+$ addition in membranous fragments of the stimulated cells, as expected if a cytosolic reductase site were made accessible by disrupting the cell. The experiments with the disrupted cells were made at pH 5.5 in the presence of Mn^{2+} ion, however. Under these conditions Mn^{2+} can catalyze by a free-radical mechanism the oxidation of NADPH,[32,33] and may also stimulate its aerobic oxidation by MPO.[8] The elementary steps proposed to account for this reaction are depicted in Equations 1 through 4.

$$O_2 + [e^-] \rightarrow O_2^- \qquad O_2^- + H^+ \rightleftharpoons HO_2 \qquad (1)$$

$$H^+ + HO_2 + Mn^{2+} \rightarrow Mn^{3+} + H_2O_2 \qquad (2)$$

$$Mn^{3+} + NADPH \rightarrow Mn^{2+} + NADP + H^+ \quad (3)$$

$$NADP + O_2 \rightarrow NADP^+ + O_2^- \quad (4)$$

with initiation being provided by a source of O_2^- (reaction 1) and any of several reactions involving O_2^- oxidation or reduction (not shown) serving as chain-terminating steps. From the data presented,[28] it seems likely that this reaction contributed extensively to the overall consumption of O_2. Nonetheless, its initiation requires formation of O_2^-, and the observation that $NADP^+$, the reductase product, inhibited the reaction is consistent with the NADPH oxidase being the source of O_2^- and its NADPH binding site being accessible only from the cytosol. In this case, the Mn^{2+} catalyzed reaction would serve only to amplify the enzymatic formation of O_2^-. A further point of contention is whether the $NADP^+$ concentration was sufficiently great under the experimental conditions to completely inhibit the oxidase. The O_2^--forming activity of a partially purified oxidase preparation was not inhibited by 1 m*M* $NADP^+$, the concentration used in the topographic mapping studies.[34] On the other hand, an arylazo derivative of $NADP^+$ was reported to inhibit competitively NADPH activation of the oxidase in membrane fragments,[35] and $NADP^+$ at tenfold lower concentration levels was able to partially protect a purified oxidase from p-mercuribenzoate inhibition,[36] consistent with $NADP^+$ binding at the pyridine nucleotide site. Similar mapping experiments were made by Yamaguchi and Kakinuma[37] using Cibacron Blue, an inhibitor of nucleotide-requiring enzymes. They demonstrated that inhibition of the oxidase in membrane fragments was competitive with NADPH and that Cibacron Blue was not inhibitory in systems using intact neutrophils, presumably because the membrane-impermeable reagent could not gain access to cytosolically localized sites. Babior and co-workers compared the O_2^--forming activity of plasma membrane vesicles made from stimulated neutrophils with phagosomes isolated from neutrophils under a variety of treatments.[38] Unlike phagosomes, the vesicles exhibited an enhanced rate of O_2^- formation upon addition of a vesicle-disrupting surfactant, Triton X-100®, which they attributed to exposure of inaccessible NADPH-binding sites in vesicles for which the normal NADPH orientation was thought to be inverted. Digestion with the protease, trypsin, eliminated the O_2^--forming ability in the absence of Triton X-100®, but did not diminish the magnitude of the incremental response observed with addition of the detergent, suggesting that the oxidase was accessible to tryptic digestion only on the NADPH-binding side of the membrane. The isolated phagosomes, however, were completely susceptible to tryptic inactivation. These results are consistent with the oxidase NADPH-binding site being accessible entirely from the cytosolic side of the phagosomal membrane. One curious result is that about 75% of the NADPH oxidase in the plasma membrane vesicles was inverted, suggesting that the preferred topography of the NADPH oxidase in the vesicles is with the O_2-reductase site oriented toward the convex surface, unlike the phagosomes, where it is apparently directed inwardly.

Cytochemical studies also generally support the conventional view. The initial studies by Briggs and co-workers[39] used Ce^{3+} to localized intracellular sites of H_2O_2 production in stimulated neutrophils. Reaction with peroxide produced precipitates of cerium perhydroxy compounds which were detectable by electron microscopy. The researchers found precipitation was limited to the outer surface of the plasma membrane and to zones in the phagosome between the ingested particles and the inner membrane. A series of control reactions demonstrated that deposition at these sites was a consequence of stimulation of a membrane-bound oxidase, and Ce^{3+} was shown to be slowly taken up by neutrophils by passive diffusion, thereby allowing detection of intracellular H_2O_2-containing sites. Furthermore, by using formaldehyde-prefixed stimulated neutrophils, which presumably were incapable of phagocytosis, they showed that the accumulation of the ceric precipitate in the phagosomes could not be attributed to its prior formation on the plasma membrane and subsequent

ingestion with the particle. One observation not adequately accounted for was the apparent dependence of the precipitate-forming reactions upon the presence of NADH in the external medium. The researchers concluded that the oxidase being probed was an enzyme bound to the external surface of the plasma membrane, i.e., that both pyridine nucleotide and O_2 sites were external. Alternatively, H_2O_2 might have been generated primarily by nonenzymatic catalysis by Ce^{3+} ions of NADH oxidation, e.g., by a mechanism analogous to reactions 2 through 4. If so, it constitutes amplification to detectable levels of oxidase-catalyzed superoxide formation and is not indicative of the pyridine nucleotide binding site. alternative interpretation gains plausibility from experiments demonstrating that of numerous metal ions tested, only Mn^{2+} and Ce^{3+} were capable of catalyzing superoxide-dependent NADPH oxidation.[33] Subsequent research by other workers using similar experimental protocols yielded comparable results, except that the NADH dependence of the reaction was not observed.[40] Recently, Briggs et al. published[41] a cytochemical study of O_2^--forming sites in neutrophils based upon Mn^{2+}-catalyzed oxidation of diaminobenzidene by reactions that are proposed to be analogous to the pyridine nucleotides (reactions 1 to 4). As with the studies on H_2O_2, detectable O_2^- reaction sites were limited to the outer surface of the plasma membrane and intraphagosomal space. Aldehyde prefixation was used to demonstrate that the precipitate found within the phagosomes was attributable to reaction at that site, and enzymatic controls were used to demonstrate that the reactions were O_2^--dependent.

An additional observation of the latter two studies[40,41] was that deposition was confined largely to regions of the plasma membrane that were in contact with the particle stimulus, the invaginating membrane, and the particle-containing phagosome. This asymmetric localization may indicate that particle contact is required for stimulation of the oxidase or that the oxidase tends to be preferentially incorporated into the developing phagosomal membrane, as suggested by the earlier subcellular distribution studies.[30]

The cumulative data strongly support the viewpoint that the O_2^- (and H_2O_2) generating site in neutrophils is located at the external surface of the plasma membrane and is incorporated into phagosomes without changing its orientation, so that O_2 reduction occurs at the inner phagosomal surface. Evidence for the location of pyridine nucleotide binding sites is less compelling, but suggests that they are accessible only from the cytosol at both the plasma and phagosomal membranes. The implication of these results is that the oxidase is transversely oriented across the membrane, which is also supported by recent studies indicating that stimulation by PMA causes depolarization of the plasma membrane attributable to electrogenic transmembrane electron transfer.[42] This particular orientation is conceptually appealing from the perspective of isolating lethal reactions from the cytosolically localized enzymatic processes that are driving them. The neutrophilic cytosol contains enzymes and metabolites,[8] e.g., superoxide dismutase,[26] catalase, and a glutathione-glutathione peroxidase-glutathione reductase cycle that can protect it from respiratory burst products that might escape the phagosomes (see Figure 3), whereas the phagosomal medium, being extracellularly derived, is nearly devoid of these components. Similar protective enzymes could conceivably be introduced into the phagosomal medium by lysis of entrapped bacteria although, as discussed in Section III, these enzymes are probably extensively inactivated in the bacterial cytosol before their cellular envelopes are breached. The organizational illustration (see Figure 3) includes a potentially protective sequence involving conversion of HOCl, a secondary oxidant formed by MPO-catalyzed peroxidation of Cl^- into a less reactive hydrophilic chloramine by reaction with taurine, a sulfonated amine present in the neutrophil cytosol.[43] These reactions are considered in detail in following sections.

B. COMPOSITION OF THE NADPH OXIDASE

The oxidase is generally thought to be composed of several redox centers which are linked to form a transmembrane electron transport chain. This topic has been critically

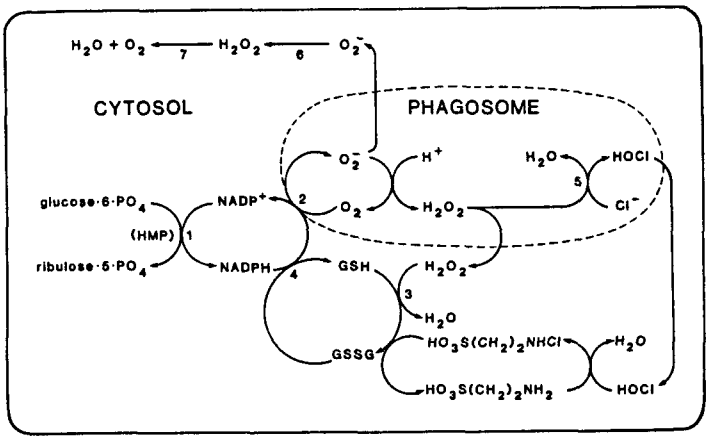

FIGURE 3. Probable topographic arrangement of the NADPH oxidase in the neutrophil and cytosolic protection mechanisms. (1) Dehydrogenases of the hexosemonophosphate shunt (HMP); (2) NADPH oxidase; (3) glutathione peroxidase; (4) glutathione reductase; (5) myeloperoxidase; (6) superoxide dismutase; (7) catalase. (Adapted from Hurst, J. K., *Oxygen Complexes and Oxygen Activation by Transition Metals*, Martell, A. E. and Sawyer, D. T., Eds., Plenum Press, New York, 1988.)

reviewed recently.[44] We present a concise account emphasizing major points of contention and develop a model for the electron transport chain that optimally accommodates the published experimental data.

Evidence has been advanced suggesting the involvement of a unique low-potential cytochrome b, a FAD-containing protein, and/or ubiquinone. Other redox centers which might conceivably be involved, e.g., nonheme iron-sulfur compounds,[45,46] have not been detected. There is disagreement among the various researchers concerning which of the three redox components might comprise the electron transport chain and their relative functional orientations with the chain. Thus, enzyme preparations possessing O_2^-- forming activity have been reported which lack detectable quinone,[45,47-50] FAD,[47,51] and/or cytochrome b,[50] and one preparation appears to be nearly devoid of all of these redox components.[52] The problem arises because, as with most membrane-localized proteins, the oxidase has proven to be exceptionally difficult to isolate and purify.[44] Until very recently, the extractive procedures used gave solubilized enzymes with very low specific activities. Attempts to "reconstruct" the intact oxidase by readdition of essential components that might have been lost during isolation, e.g., FAD,[34,53-55] "structural" phospholipids,[53] frequently have led to increased activity, but one cannot be certain that, e.g., by adding mobile redox components, new nonphysiological redox pathways had not been created.[36] For example, addition of the nonphysiological redox dye, methyl viologen, markedly enhanced rates of reduction of flavin and cytochrome b by NADPH in detergent-solubilized oxidase preparations.[56] With the oxidase, these general difficulties have been compounded by the apparent need to stimulate respiration in the oxidase prior to purification to obtain active oxidase, which was generally unstable and rapidly lost activity, precluding extensive purification procedures.[44] Earlier studies, therefore, tended to use membrane fragments or phagosomal vesicles, rather than isolated oxidase. Quite recently, major breakthroughs in oxidase isolation procedures have been reported, however, which include obtaining a solubilized enzyme with very high O_2^--forming activity[50] and devising means for stimulating the enzyme in cell-free preparations.[21,57-60] As a consequence, we can anticipate that outstanding issues regarding the

composition, organization, and mechanisms of activation of the neutrophil NADPH oxidase will be resolved in the near future.

1. The Neutrophil Cytochrome b

A considerable body of circumstantial evidence has been amassed to indicate participation of a b-type cytochrome in oxidase reactions, including demonstration that its distribution and physical and chemical properties are consistent with oxidase function. Thus, the cytochrome is found in all of the respiring phagocytic cells (neutrophils, eosinophils, monocytes, macrophages), but not in other cell types.[61] In neutrophils, at least, it is localized to the plasma membrane and one component of the granule population[62] (the "specific" granules) and appears in the phagosomal membrane upon inducing phagocytosis;[63] synthesis of the cytochrome has also been shown to occur concurrently with development of NADPH oxidase activity in differentiating monocytes[64] and an HL-60 promyelocytic leukemia cell.[65,66] The protein or proteins comprising the cytochrome are absent in the X-linked form of CGD.[67] The gene thought to be responsible for the congenital defect has been cloned and sequenced;[68] although the corresponding protein sequence predicted is atypical of cytochromes in general, the gene is reported to match the primary structure of a segment of the neutrophilic cytochrome b protein.[69] Furthermore, O_2^--forming capability could be induced by heterologous fusion of monocytes from CGD patients lacking the cytochrome with cells from CGD patients that possessed the cytochrome, but for other reasons were unable to undergo stimulated respiration.[70] Thus, in all of these studies, oxidase activity is associated with the presence of the b-cytochrome. In many preparations, the cytochrome is found to copurify with O_2^--forming activity.[36,47,51,54-56,71-73] However, a recent purification was described by Babior and co-workers for which the enzyme showed unprecedentedly high activity, comparable to that measured in particulate systems, but a marked stoichiometric deficiency of the cytochrome, i.e., only ~0.015 cyt b/oxidase.[50] The isolated protein contained a significant amount of FAD, however (3.0 FAD/oxidase). The researchers suggested that they may have isolated and purified only the pyridine nucleotide dehydrogenase component of a more complex respiratory chain. Gabig and co-workers reported earlier that the oxidase could be fractionated into cytochrome b and a second, FAD-containing, component.[36,74] Unlike the more recent results,[50] this flavoprotein could not catalyze NADPH-dependent O_2^- formation, but could catalyze diaphorase reactions with artificial electron acceptors.[74] Bellavite et al. reported[51] that they obtained an isolated oxidase containing relatively large amounts of cytochrome b over FAD following the preparative methodology of Babior et al., and Doussiere and Vignais reported[52] isolation of a highly resolved oxidase that was nearly devoid of either FAD or cytochrome b. In the latter cases, O_2^--forming activity of the enzymes was low. If confusing, the results underscore the nature of the difficulties besetting researchers in this field.

The cytochrome was originally designated as b-type based upon the position of its ferrous optical absorption spectrum. This assignment has been confirmed by its pyridine hemochromogen spectrum, which identifies the heme as protoporphyrin IX.[75,76] Its signature is an unusually low reduction potential,[45] $E_{m,7.0} = -245$ mV, from which it can be easily distinguished from mitochondrial and microsomal b-cytochromes, and which is sufficient to drive the one-electron reduction of O_2 (see Figure 4).[77] It has correspondingly been designated cytochrome b_{-245}, although the alternative and more rigorous designation of cytochrome b_{558} (less commonly, b_{559}) is also used, based upon the α-band position in the ferroheme optical spectrum. It undergoes reduction coincident with stimulation of neutrophils[78-80] and with NADPH reduction of partially purified oxidase,[45,56,72] and appears to be kinetically competent to participate in electron transfer between NADPH and O_2, at least in the aerobic steady-state.[81,82] The respiratory burst can be reversibly inhibited by strongly heme-binding nitrogenous bases;[83] qualitative correlations of inhibition with cytochrome b spectral changes were observed in these experiments. The cytochrome has been purified by

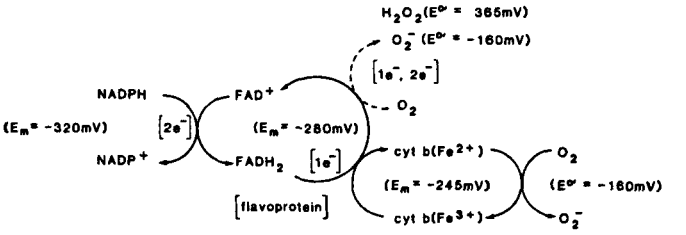

FIGURE 4. Minimal composition and probable organization of the NADPH oxidase respiratory chain. Relevant thermodynamic properties are given parentheses. The dashed arrow represents a minor, possibly artifactual, pathway. (Adapted from Hurst, J. K., *Oxygen Complexes and Oxygen Activation by Transition Metals*, Martell, A. E. and Sawyer, D. T., Eds., Plenum Press, New York, 1988.)

several groups;[68,75,76,84,85] in general, its physiochemical properties are very similar to the cytochrome in the membrane-bound oxidase.[75] The isolated cytochrome was unable to directly catalyze NADPH-reduction of O_2[74,84] or other electron acceptors,[75] however, and was not reduced by NADPH under anaerobic conditions, suggesting minimally that other electron carriers must be involved in the catalytic mechanism.[11] No evidence of N_3^- or CN^- binding to the cytochrome has been reported, either *in situ*[79] or in purified preparations.[75] Weak binding of CO to the ferrous form has repeatedly been detected by optical difference spectroscopy.[71-73,75,76,80,81,86] Complex formation is apparently slow in some preparations, with reaction half-times greater than $t_{1/2} \simeq 10$ min having been reported.[71,76,80] The absorption spectrum of neutrophils measured at 77 K gave no indication of CO adduct formation, even at high pressure, unlike other cells containing CO-binding hemes.[87] Although the researchers concluded that neutrophilic b-cytochrome does not bind CO, the possibility could not be excluded that at the low temperature used, the cytochrome is simply frozen into a particular conformation that will not bind CO, e.g., 6-coordinate ligation. The rate of ferrocytochrome b autoxidation is rapid in stimulated neutrophils,[79] plasma membrane fragments,[81] solubilized oxidase preparations,[72] and the isolated metalloprotein.[74]

The low reduction potential implies strong σ-donation from the cytochrome b axial ligands,[75] thereby stabilizing the relatively electron-deficient higher oxidation state. Comparable potentials have been measured for peroxidases, e.g., for horseradish peroxidase[88] (HRP), $E_{m,7.0} = -271$ mV, and for mono- and bis-imidazole heme model compounds.[89] In these complexes, the reduction potential is thought to be modulated through hydrogen-bonding of the proximal nitrogen atom on the imidazole ligand to nearby bases. With increasing strength of the hydrogen bond, the ligand assumes increasingly the properties of an imidazolate anion, hence, stronger σ-donor capabilities. Experimental evidence documenting these trends has come from comparisons of Fe-N(imidazole)-stretching frequencies[90] and NMR proton chemical shifts[91] of HRP and deoxymyoglobin (deoxyMb), as well as systematic studies of H-bonding effects on the redox electrochemistry of heme model compounds.[89] For the metalloproteins, the deoxyMb reduction potential is about 300 mV more anodic than HRP;[88] its iron-imidazole bond is considerably weaker, as indicated by resonance Raman spectroscopy,[90] and hydrogen-bonding of the axial imidazole to neighboring bases on the protein backbone was too weak to detect by NMR methods, in contrast to HRP, where H-bonding was sufficiently great to impart substantial anionic character to the ligands.[91] A second consequence of strong axial σ-bonding is that CO binding affinities decrease markedly, at least for 6-coordinate complexes.[92] Rates of CO ligation of these complexes would also be expected to be less as a consequence of the enhanced coordinate bond strengths. Recombination rates for CO with 5-coordinate ferroheme-imidazole model complexes are also reported to be sensitive to the hydrogen bond strength at the proximal ring nitrogen

atom, with rate constants decreasing 10^2- to 10^3-fold upon deprotonation.[93] These results have been rationalized as indicating that the stabilizing effect of hydrogen bonding on the 5-coordinate, presumably out-of-plane, geometry exceeds the influence of increased ligand charge donation on lowering the transition state energy. In any event, the neutrophil cytochrome b shows chemical properties compatible with those of a strongly hydrogen-bonded mono- or bis-histidyl hemin.

2. The Flavoprotein Component

Involvement of a flavin in the electron transport chain was suggested from early studies on the partially purified oxidases in which it was found that addition of FAD often enhanced O_2^--forming activity[34,53-55] and that this activity could be inhibited by analogs that were capable of only two-equivalent electron transfers.[54] As with cytochrome b, the issue has become controversial because, as discussed in the preceding section, more highly purified preparations have now been described that possess some oxidase activity, but undetectable levels of flavin.[47,51,52] However, Kakinuma's group has recently reported isolation of an FAD-containing flavoprotein with NADPH-dependent oxidase activity.[94] This enzyme contained no cytochrome b and apparently catalyzed the direct two-electron reduction of O_2 to H_2O_2 under most medium conditions since ferricytochrome *c* reduction was not SOD-inhibitable. Hydrogen peroxide generation was confirmed by its characteristic adduct formation with cytochrome *c* peroxidase. The FAD content was low, comprising only about 10% of the stoichiometric amount based upon the measured oxidase molecule mass. Perhaps the most salient trait of the purified oxidases is that their specific activities tend to parallel, at least qualitatively, their FAD content.[47,50,51,94] This suggests that essential flavin is lost during the lengthier, more intricate, purification sequences, a property that was suspected from the behavior of the earlier, crude preparations.

Kakinuma and co-workers have determined the reduction potential of a flavin localized in the plasma membrane using potentiometric titration coupled to ESR detection of its semiquinone radical ion.[95] The radical signal could be elicited by NADPH only in membranes from stimulated neutrophils and gave a midpoint-potential of $E_{m,7.0} = -280$ mV, appropriate for participation in the oxidase respiratory chain (see Figure 4). This flavin is presumably identical to the isolated FAD-containing protein, but this point has not yet been established. FAD has also been shown to undergo partial reduction by NADPH in an aerobic suspension of the solublized oxidase.[56]

Whether or not it is a flavoprotein, there is good agreement among the various researchers concerning the molecular mass of the protein described as the isolated oxidase, which is 65 to 67 kDa.[50,52,94] This protein may also be the NADPH-binding component. Affinity photolabeling studies on plasma membranes using 2,3-dialdehydo[96] and arylazido derivatives[35] of NADPH have identified a 65- to 66-kDa protein as the site of attachment. Both photolabels acted as competitive inhibitors against NADPH in oxidase reactions in the dark. Other lower molecular mass fragments possessing oxidase activity have also been found in some of the studies.[50]

3. Quinone Controversies

Several laboratories have reported that neutrophils contain nonmitochondrial ubiquinone-10.[36,97,98] A role for this quinone in the oxidase electron transport chain has been postulated based upon the observations that phagosomal membranes appear to accumulate the quinone in parallel with oxidase activation,[99] a resolved flavoprotein component also contains ubiquinone,[36] and addition of short-chain analogs stimulates oxygen consumption and H_2O_2 and O_2^- production in intact and disrupted cells.[98] This activity is lost upon addition of compounds that can act as quinone inhibitors in respiratory chains,[98] and quinone/hydroquinone ratios in the neutrophil and the resolved flavoprotein component vary in response

to substrate addition in a manner consistent with quinone participation in electron transport.[36,98] Schneider and Crawford also believe that the quinone is localized in tertiary granules[99,100] in the resting neutrophil and is translocated to the plasma and/or phagosomal membrane during activation of the respiratory burst.[100] Quantitative aspects of the studies[98] on quinone stimulation of respiration appear anomalous, however. Half-maximal stimulation in disrupted cells occurred at NADPH concentration levels that were at least severalfold greater than its Michaelis constant (K_m) for binding to the oxidase, and stimulation by NADH was nearly equal in magnitude despite being present at a concentration below its oxidase K_m value. This behavior suggests that catalysis may not involve the oxidase and is reminiscent of the nonenzymatic metal-ion-catalyzed autoxidation of pyridine nucleotides described earlier (see Section II.A). Further study of these reactions would seem warranted, particularly in light of the findings that semiquinones directly reduce ferricytochrome *c*, the O_2^--trapping agent usually employed,[101] and that reduction of ubiquinone-5 is mediated by O_2^- in activated macrophages.[102]

Finally, there are conflicting data regarding the ubiquinone-10 content of neutrophils. Studies from several laboratories have been reported indicating that quinone is either absent[46] from neutrophils or present at very low concentration, associated with cellular mitochondria,[48] and is undetectable in neutrophil cytoplasts that are nearly devoid of intracellular granules, but retain an activatable NADPH oxdiase[49] in stimulated plasma membrane fragments[45,95] and in various solubilized oxidase preparations,[47] including the highly active purified enzyme described by Babior and co-workers.[50] The bases for these profound observational differences are not presently understood.

C. FUNCTIONAL ORGANIZATION OF THE OXIDASE

The electron transport chain formed from probable redox components ordered according to increasing reduction potentials is given in Figure 4. This order has been proposed by several groups based upon other considerations.[36,56] The putative respiratory chain is thermodynamically well poised for the one-electron reduction of O_2 by NADPH and possesses several other essential functional elements. According to this scheme, cytochrome b is the terminal oxidase and should, therefore, probably be designated as an o-type cytochrome.[103] The flavin is properly disposed to couple noncomplementary electron transfer between NADPH,[71] which functions as a preferential two-electron (hydride) donor,[104] and the cytochrome b, a one-electron transfer agent.

Ubiquinone has not been included in the scheme because its participation appears unlikely on thermodynamic grounds.[48] The oxidized quinone could conceivably act as a terminal oxidase to generate O_2^- since its one-electron reduction potential is about -200 mV,[101] and electron transfer to O_2 should correspondingly be rapid.[105] However, the cytochrome b is only about 10% reduced in the aerobic steady-state,[80,82] indicating that that portion of the respiratory chain is poised at about -186 mV and that electron transfer to the cytochrome is rate-determining, or nearly so. If ubiquinone were the terminal oxidase, it would therefore be in near equilibrium with cytochrome b. Since the quinone two-electron reduction potential is about $+65$ mV,[106] virtually all of the quinone would be in its reduced, dihydro oxidation state ($[Q]/[QH_2] \simeq 10^{-9}$). One-electron reduction of O_2 by QH_2 is energetically unfavorable under these conditions by about 500 mV. Two-electron transfer to oxygen, yielding H_2O_2 directly, would therefore be expected,[107] contrary to the observation that O_2^- is the predominant product (see Section II.D.) If the quinone was involved as an intermediary in the chain, e.g., the proposed electron shuttle between FAD and cytochrome b, its redox poise would be even more cathodic. Thus, regardless of its position within the electron transport chain, a functioning ubiquinone would be present almost entirely as hydroquinone. In contrast, during aerobic turnover in the stimulated neutrophil and subcellular fractions, only about 6% of the extractable quinone was found to be reduced.[36] The existing experimental data are, therefore, incompatible with a role for ubiquinone in NADPH oxidase respiration.

The ability of the cytochrome to function as a terminal oxidase has been questioned on the basis that CO binding presumably reflects O_2 binding, and a weakly CO-binding cytochrome should not turn over O_2 at a physiologically acceptable rate.[52,76] However, several bacterial cytochromes *o* have been described with similar slow and weak CO-binding characteristics, which also show low sensitivity to respiratory inhibition by CN^- and N_3^-.[103] Several plausible contributing factors can be envisioned to rationalize this behavior. The O_2-binding pocket might be sterically constrained to permit binding of O_2,[81] which favors a bent end-on configuration, but not an axially aligned linear CO molecule.[108,109] An indication that the O_2-binding site is buried is its apparent inaccessbility in membrane vesicles to proteases.[38] The heme might be strongly six-coordinate with electron transfer occurring from its periphery,[83,87] a suggestion that has also been made to explain the rapid reduction of ferricytochrome *c* by O_2^-.[110] Either of these factors could account for the ligand-binding characteristics of the neutrophil cytochrome b. Alternatively, the heme might assume several distinct conformations comprising states of varying catalytic activity, with a ligation site becoming available on the cytochrome only in the active conformers.[82] This behavior has precedent in the conformation-controlled reactivity documented for mitochrondrial cytochrome *c* oxidase.[111] Experimental data consistent with this notion include the observations that NADPH reduction of membrane-bound cytochrome b occurs rapidly only in the aerobic steady-state,[82] recombination of CO with cytochrome b following flash dissociation is rapid, but under other conditions CO ligation is slow,[45] and ESR signals tentatively assigned on the basis of CN^- insensitivity to high-spin, five-coordinate, cytochrome b appear in the oxidase isolated from stimulated, but not resting, neutrophils.[46] The ESR spectra are anomalous in showing a high degree of rhombicity, however. One expects near-axial symmetry for protoporphyrin IX-containing high-spin cytochromes, e.g., cytochrome *o* or cytochrome b_{558} in the terminal oxidase complexes of *Escherichia coli*,[112] so further investigation of the unusual signals in the stimulated membranes is desirable.

D. STOICHIOMETRY OF THE RESPIRATORY BURST

If cytochrome b is indeed the terminal oxidase, then O_2^- is expected to be the sole product of O_2 reduction since ferrocytochrome b is a one-electron donor at these potentials. In general, stoichiometric yields of O_2^- measured by trapping techniques reported for stimulated neutrophils,[29] membrane fragments,[55,113] and solubilized oxidase[34,55,114] have been near the theoretical ratio of O_2^-/NADPH = 2.0. However, Green and co-workers demonstrated in their particulate preparation of the oxidase that the ratio was highly variable,[115] depending upon such conditions as the NADPH and detergent concentration levels, medium pH, and age of the preparation. Furthermore, ferricytochrome c, the commonly used oxidant in O_2^- assay systems, stimulated the oxidase and apparently altered the O_2^-/NADPH stoichiometric ratio.[116] When acetylated ferricytochrome c, which did not increase NADPH oxidase activity, was used to measure O_2^- formation, no more than about 70% of the electron equivalents from NADPH could be accounted for as O_2^-. Green and co-workers concluded that the remainder of the O_2 was reduced directly to H_2O_2 and considered the possibility that the electron transport chain in the oxidase was branched, i.e., that cytochrome b and flavin constituted dual sites of O_2 reductase activity. In other studies, Minakami and coworkers[55] obtained an O_2^-/NADPH ratio of nearly 2.0 from stimulated plasma membrane vesicles using acetylated ferricytochrome c to quantitate O_2^- formation, and Ishimura and collaborators used diacetyldeuteroheme-substituted HRP to demonstrate that O_2^- was the sole detectable reduced product in both intact stimulated neutrophils[29] and an activated cell-free preparation.[114] In the latter study, O_2^- was distinguished from H_2O_2 spectroscopically by its formation of the diacetyldeuteroheme compound III, rather than compound II.

A simple, although presently unsubstantiated, explanation for the variable stoichiometries exhibited by isolated oxidase fractions is that during purification the flavoprotein is partially

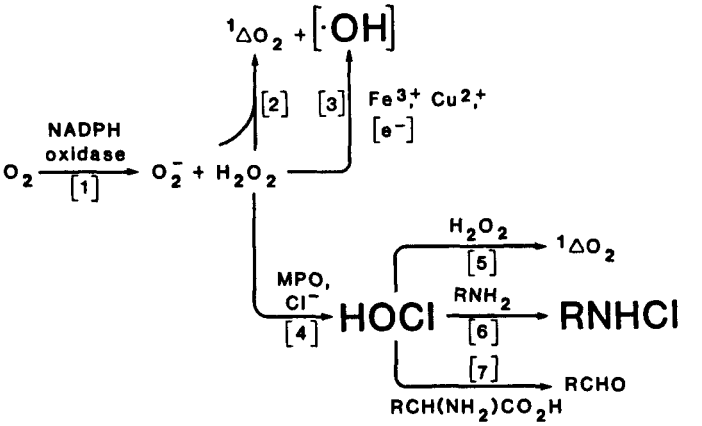

FIGURE 5. Various proposed neutrophil-generated oxidative toxins and their pathways for formation. Most likely ultimate toxins are indicated by the use of larger chemical formulas; [·OH] is intended to represent the product of Fenton-type reactions. (Adapted from Hurst, J. K., *Oxygen Complexes and Oxygen Activation by Transition Metals*, Martell, A. E. and Sawyer, D. T., Eds., Plenum Press, New York, 1988.)

resolved from the cytochrome to a greater or lesser extent, depending upon treatment, with consequent unmasking of a nonphysiological reaction site. By analogy with xanthine oxidase,[115,117,118] the flavin would be capable of one- or two-electron transfers to O_2. As pointed out by Gabig and Lefker,[74] this explanation could also account for observations that purified oxidase fractions exhibit highly varying reactivities toward artificial electron acceptors[34,55,74,119-121] since flavoproteins, in general, exhibit diaphorase activity. Direct electron transfer from the flavin to O_2 is included in Figure 4 as an alternative pathway. The bulk of research suggests that it is not a significant pathway in the *in vivo* NADPH oxidase; in addition to the stoichiometric results, turnover rates of cytochrome b appear to be very nearly equal to rates of O_2^- production.[82] An additional potential contributing factor to the low O_2^- yields, that MPO catalyzes O_2^- dismutation,[122] is considered in Section III.A.2.a.

E. MECHANISMS OF NADPH OXIDASE ACTIVATION

This topic has been the subject of several recent critical reviews[44,123-125] and is not discussed here in any detail. It is clear from recent research that several distinct stimulus-dependent activation mechanisms exist. The two mechanisms most extensively discussed are protein kinase c-catalyzed phosphorylation of integral membrane proteins as the immediate triggering event[124-129] and translocation of essential oxidase components from granule sites with assembly in the plasma membrane consituting activation.[130,131] The latter model is particularly controversial because there exists a body of conflicting data.[130-132] A cytoplasmic protein distinct from protein kinase has recently been isolated that is capable of eliciting the respiratory burst in the presence of other soluble factors.[21] Experiments in which soluble and membranous components of normal and CGD neutrophils were interchanged suggests that one form of CGD arises from the defective cytosolic factor. Because it is relatively simple, this system appears especially amenable to investigation of its molecular mechanism of oxidase activation.

III. OXIDATIVE TOXINS FROM THE RESPIRATORY BURST

Various oxidants that have been proposed as ultimate microbicidal agents formed from the respiratory burst are presented schematically in Figure 5. For purposes of discussion

they can be conveniently divided into two categories comprising chlorinating agents and other active oxygen species.

A. CHLORINATING AGENTS

1. Toxicities

The microbicidal potency of hypochlorous acid and N-chloramines is convincingly demonstrated by their pervasive use in treatment of municipal sewage and other waste waters. Hypochlorous acid, as Dakin's solution,[133] was extensively used in medicine in the treatment of topical wounds until antibiotics became available, and there is continued interest in its possible application as a disinfectant in burn therapy.[134]

A quantitative measure of HOCl toxicity can be had by determining the amount of oxidant required to inhibit replicative ability (as a measure of death) in cellular suspensions. In one study,[135] using flow-mixed solutions of *E. coli* ATCC 25922 and HOCl in phosphate or succinate buffers, the LD_{50} was found to be 4×10^7 and 8×10^7 molecules HOCl per bacterium at pH 5.0 and 7.4, respectively. This is equivalent to 10 to 50 μmol HOCl per g dry weight of cells. Similar values have been obtained for other types of bacteria.[373] The bactericidal effectiveness of NH_2Cl appears to be even greater, based upon direct comparison of viabilities at equivalent oxidant concentrations,[136-138] although Thomas and co-workers have shown in their extensive studies[43,136,139-141] that the bactericidal and cytotoxic properties of N-chloramines vary widely, depending upon their solubility properties, with hydrophilic chloramines being virtually unreactive but lipophilic chloramines being toxic to cells. The potency of these compounds indicates a high selectivity for particular types of biomolecules within cells, among which must exist the lethal lesions.

MPO catalyzes the two-electron oxidation of the halides Cl^-, Br^-, I^- and pseudohalide, SCN^-, by H_2O_2.[1,8] Although the oxidized products of all these reactions are highly toxic to cells, Cl^- is generally regarded as the physiological substrate because it is relatively abundant in intracellular fluids. The microbicidal activity of cell-free MPO-H_2O_2-Cl^- systems has been extensively documented, as have MPO-catalyzed iodination reactions.[1,8] Quantitatively, about 2×10^7 molecules H_2O_2 per bacterium were required to inactivate 50% of a pH 5.0 suspension of *E. coli* 25922 by the cell-free MPO-H_2O_2-Cl^- system, so the enzymatic reaction was twice as effective as adding HOCl in bolus.[135] The peroxidase of monocytes is very similar, if not identical, in catalytic properties to the neutrophil MPO.[142] However, eosinophil peroxidase (EPO) is structurally[143-145] and functionally distinct.[146,147] This enzyme from PMA-stimulated eosinophils preferentially catalyzes oxidation of Br^- in the presence of up to a 4×10^4-fold excess of Cl^-.[147] The absolute concentrations of the ions approximated the physiological milieu, suggesting a possible physiological role for EPO-catalyzed brominations. A similar selectivity was found for SCN^- over Cl^- with EPO, and MPO also preferentially oxidized SCN^- at higher SCN^-/Cl^- concentration ratios, approximating the medium composition of salivary fluids.[148]

Despite the impressive demonstrations of near-universal toxicity of leukocyte peroxidase-generated halogenic oxidants, a primary role for these agents in leukocytic microbicidal processes has not yet been firmly established. Congenital MPO deficiency, characterized by inability of neutrophils and monocytes to synthesize the protein,[10] is thought to occur in roughly 0.02% of the population but, apart from susceptibility expressed by some individuals to infection by the opportunistic yeast *Candida albicans*,[149] this defect is apparently without serious medical consequence. MPO-deficient neutrophils have been used to demonstrate the existence of other oxidative mechanisms,[1-8] in addition to the nonoxidative ones previously mentioned.[18,19] Given the simultaneous presence of at least three types of potentially interacting microbicidal systems within the phagosomes of normal individuals, the task of identifying the contributions of each is formidable. Nonetheless, several points of evidence summarized by Klebanoff[1] suggest that MPO-mediated mechanisms are significant. Briefly,

stimulated neutrophils carry out halogenation reactions, rates of microbial killing of test organisms by MPO-deficient neutrophils are generally slower than in normal neutrophils under identical conditions, the MPO poisons N_3^- and CN^- often severely reduce bactericidal activity in normal neutrophils, and both the rate and extent of the respiratory burst and phagocytosis are enhanced in MPO-deficient neutrophils, which may potentiate killing by other, less efficient, oxidative mechanisms. This last effect is thought to arise, at least in part, as a consequence of absence of MPO-dependent inactivation of neutrophil function and of MPO consumption of H_2O_2 in the deficient cells.

Very recent reports provide additional evidence of the kind listed above, supporting the notion that MPO-dependent mechanisms play an important role in host defense against microbial pathogens. For example, an MPO-dependent oxidative pathway for killing of the fungus *Aspergillus fumigatus* has been suggested in monocytes from comparison of killing rates in normal and MPO-deficient cells and from the protective effects of added reagents that either dissipate H_2O_2 (catalase) or scavenge HOCl by forming nontoxic products (taurine).[150] Aerobic killing of the bacterium *Actinobacillus actinomycetemcomitans* was nearly completely inhibited by addition of CN^- and comprised the predominant mechanism based upon reaction half-times determined from initial killing rates under aerobic and anaerobic conditions.[151] Lymphokine-activated normal neutrophils effectively killed the pathogenic amoeba *Naegleria fowleri*, but this activity could not be elicited from MPO-deficient neutrophils, the amoebae were partially protected by catalase and arginine, another HOCl scavenger, and the cell-free MPO-H_2O_2-Cl^- system was amoebicidal.[152] Protection of *C. albicans* from neutrophilic killing by catalase and HOCl or NH_2Cl traps followed patterns consistent with MPO-generated chlorinating agents[153] and the cytotoxicity of neutrophils towards two species of *Tricophyton*, a dermatophyte fungus, was markedly reduced by N_3^- or catalase added to the reaction medium.[154] Although a complete set of controls excluding alternative mechanisms was not applied in all these studies and, as discussed in subsequent sections, probe methods employing various trapping agents are inherently unreliable, the cumulative results are difficult to rationalize without postulating peroxidase-dependent mechanisms.

2. Leukocyte Peroxidase Structures and Catalytic Mechanisms

a. MPO

The HOCl formed by MPO-catalyzed Cl^- peroxidation is freely diffusible from the enzyme active site.[155] Although in early studies[156] MPO-catalyzed N-chlorination of amino acids exhibited Michaelis-Menten kinetics, more recent investigations have demonstrated an absence of influence of reactive substrates upon chlorination rates.[157-159] The amino acid chlorination dynamics were unusual in showing very little specificity, with K_m and V_{max} values being nearly identical for all the substrates studied. These reactions were measured at pH 5.3, under which conditions the amino acids were predominantly in their unreactive protonated forms.[156] Apparent saturation kinetics probably arose from a change in the rate-limiting step from N-chlorination by HOCl to enzymatic formation of HOCl as the amino acid concentration was increased, rather than as a consequence of amino acid binding to the enzyme.

In those few instances where direct comparisons have been made, the reaction products of oxidation or chlorination by HOCl and the MPO-H_2O_2-Cl^- enzymatic system are almost always identical. One exception to this general observation is that fluorescein derivatives that had been covalently attached to *Saccharomyces cerevisia* cell wall fragments (zymosan) gave a small amount of chlorination at the 2′- and 7′-ring positions in reaction with MPO-H_2O_2-Cl^- in addition to the major 4′-monochloro and 4′,5′-dichloro products obtained from both the enzymatic and HOCl reactions.[160] Fluorescein binding to zymosan is heterogeneous, and MPO adheres strongly to the particles, so that in this instance the differences in product

distribution might be artifactual, arising, e.g., from unusual steric constraints or substrate exposure in the bound polymers. Since there is no evidence of substrate activation by MPO in the great preponderance of studies made, and since reasonable alternative explanations exist in those few instances where activation might be invoked, it seems likely that MPO functions solely to generate HOCl and that its subsequent oxidative reactions are not under enzymatic control, at least in the usual sense. The bactericidal and fungicidal activities of MPO are significantly enhanced by preincubation of enzyme and microbes before addition of H_2O_2, however.[161-163] Adhesion of MPO occurs under these circumstances, apparently driven primarily by electrostatic forces between the cationic protein and anionic cell wall surfaces.[164] As demonstrated for guaiacol and alanine,[161,163] binding may limit access of soluble reactants under these circumstances and favor reaction of HOCl with the bacterial envelope. This sort of selectivity by association could have important consequences in physiological reactions, e.g., by minimizing inconsequential reactions with serum proteins and similar compounds that would otherwise act as sinks for HOCl during phagocytic killing. In addition to this nonspecific effect, it has been recently reported that MPO-binding to specific high-affinity sites on the bacillus *A. actinomycetemcomitans* primes the organism for killing by the MPO-H_2O_2-Cl^- system, but the strongly bound MPO itself is incapable of killing in the presence of H_2O_2 and Cl^-.[163] This remarkable result bears further investigation, particularly with respect to the possible involvement of other granular cationic proteins that can act at higher concentrations as nonoxidative toxins.[6-8,18,19]

The enzyme is an $\alpha_2\beta_2$ dimeric glycoprotein[165,166] possessing green-colored heme prosthetic groups which were the basis for its original name, verdoperoxidase.[167] Its optical properties,[168] including magnetic circular dichroic spectra,[169] are consistent with a chlorin-type heme for which conjugation in one of the pyrrole rings is disrupted by reduction or substitution across the β-carbon double bonds, although the optical data do not exclude the alternative possibility that it is a heme possessing strongly electron-withdrawing substituent groups which red-shift the visible and Soret bands from their usual spectral positions, e.g., as occurs in formyl-containing a-type porphyrins.[103] The isolated heme has, in fact, been assigned as a formyl porphyrin based primarily upon observations that reaction with formyl-reducing chemicals gave products exhibiting normal heme spectra.[170,171] These studies are compromised, however, because the heme is strongly covalently bound to the protein, and the methods required to extract it also elicited chemical transformations.

Several groups[172-175] have now confirmed by resonance Raman (RR) spectroscopy that the prosthetic group in the intact enzyme is a chlorin, the distinguishing features being the large number of bands associated with coupled vibrational modes of the macrocyclic ring (a consequence of the lower symmetry of chlorins vs. porphyrins), and the absence of any bands attributable to formyl or other electron-withdrawing substituents conjugated to the ring. The initial RR studies were made before detailed analyses of chlorin model compounds were available,[176] and there was some indication in the literature that the two hemes in the native enzyme might not be identical.[170,171] Although the RR spectra gave no evidence of inequivalence,[172,173] the data were not compelling, and the remote possibility existed that the numerous vibrational modes might simply be overlapping spectra from the nonidentical hemes. This concern was apparently put to rest with observation of an identical RR spectrum obtained from a green heme protein derived from bovine spleen, which was described as a monomeric protein analogous to the heme-containing heavy subunit of MPO.[173] Subsequent studies differing with this original analysis have appeared, from which it is concluded that the protein isolated from spleen is an $\alpha_2\beta_2$ peroxidase[177] with composition, physical, and kinetic properties nearly identical to MPO.[159] Although these results negate the original arguments "verifying" the interpretation of the RR spectra of MPO, the extensive subsequent work on chlorin model compounds strongly supports the assignment of the heme group as a chlorin.[176] The question of heme inequivalence in the native enzyme also appears to have

been resolved by separation of the dimer into two αβ-protomers, which appear to be identical by various physical measurements.[165] The hemi-MPO exhibits catalytic activity,[178] including Cl^- peroxidation, inactivation of *E. coli*, and degradation to peroxidase compound II, that is nearly equal to the rates of corresponding reactions of the native dimer, suggesting that the heme-containing catalytic centers act independently.

The RR spectra of MPO also indicate that the chlorin is six-coordinate, high-spin in its ferric oxidation state and five-coordinate, high-spin in its ferrous state.[172,173] The high-spin ferric electronic configuration has been repeatedly demonstrated as well by EPR spectroscopy.[169,179-181] The NO complex of ferrous MPO exhibits hyperfine splitting, indicating that the fifth ligand binds iron through a nitrogen atom, most likely from a proximal histidine imidazole group.[181] Consistent with this assignment, a band at 248 cm^{-1} ascribable to the Fe-N imidazole stretching frequency is observed in the ferrous MPO RR spectrum[173] and a single proton resonance at 76 ppm in the ferrous NMR spectrum of spleen MPO,[182] comparable to the N_1-H signal displayed by other imidazole-coordinated hemes. The six-coordinate position of ferric MPO must be occupied by a weak-field ligand since the heme is high-spin. A water molecule has been suggested from the magnitude of paramagnetic relaxation rates measured for solvent water protons in the presence of MPO.[182] Alternatively, a carboxylate ligand has been proposed,[183] based upon the observation that Cl^- binding at a spectroscopically detectable site is controlled by an ionizable group with an apparent pK_a value of 4, the assumption being that protonation of this group disrupts its coordinate bond to the heme iron, allowing ligation of the other anion. This latter suggestion is less appealing on the basis of other evidence discussed next. Other anions can displace the endogenous sixth ligand. Fluoride ion binds to ferric MPO to give a complex whose EPR spectrum shows decreased rhombicity with nuclear hyperfine splitting of its $g_{\|}$ component.[179] Binding of N_3^- or CN^- causes conversion to the low-spin electronic state, as expected for strong-field ligands.[179] One infers from these effects that the anions are axially bound to the heme. Added Cl^- is capable of competitively reversing these spectral changes and, in excess, gives a ferric MPO EPR spectrum distinct from the resting native enzyme, with slightly altered rhombic character.[179,180] Changes in the MPO RR spectrum upon Cl^- addition,[174] its effects upon the ferrous-ferric midpoint potential,[180] and the bulk water proton relaxation rate[182] have also been interpreted as indicating heme binding by this anion. As mentioned previously, chloride ion binding is associated with protonation of an enzyme functional group with apparent $pK_a \simeq 4$;[183-185] for MPO isolated from spleen, chloride binding has been shown to require prior protonation of the group.[185] Its enthalpy of ionization was determined to be $\Delta H \simeq 8$ kcal/mol, consistent with involvement of a histidine imidazole, but not a carboxylate group.[185] Based upon this result and an interpretation of the pH dependence observed for NMR chemical shifts of heme methyl substituent protons,[182] Ikeda-Saito and Inubushi have proposed that the ionizable group is a distal imidazole that is hydrogen-bonded to a water molecule occupying the sixth coordinate position. Wever and associates had previously suggested involvement of a distal histidine in MPO catalysis of SCN^- oxidation, although in this instance they measured a group pK_a value of 6.[158]

The catalytic mechanism has been studied by several groups,[157,158,184,186-189] but is not yet completely understood. Complications arise because MPO exhibits catalatic activity towards H_2O_2,[190] compound I undergoes relatively rapid degradation[191] to unreactive compound II which accumulates during peroxidase turnover,[189,192] and inactivation by heme bleaching occurs at higher H_2O_2 concentrations.[157-159,187] Compound I, the ferryl π-cation formed by two-equivalent oxidation of ferric MPO by H_2O_2,[193] is assumed to be the immediate two-electron acceptor from Cl^- (alternatively, oxonium ion donor to Cl^-) because compound II, the one-equivalent oxidized ferryl ion, is unreactive towards Cl^-,[187] ascorbic acid stimulates MPO chlorinating activity by preventing accumulation of compound II,[192] and HOCl can react rapidly with ferric MPO to give compound I under certain conditions,[187] which is

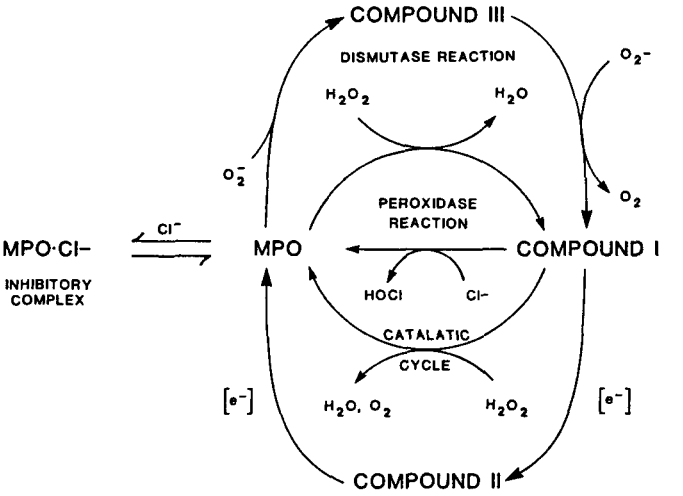

FIGURE 6. Kinetic scheme for reactions of neutrophil myeloperoxiase. The endogenous one-electron reductants of Compounds I and II are unknown and are represented by [e⁻].

ostensibly the reverse of the Cl^- peroxidation reaction. Chloride or SCN^- have been shown to competitively inhibit the reaction with respect to H_2O_2,[157,184,187,188] and H_2O_2 appears capable of competitive inhibition of the reaction with respect to Cl^-.[157,189] The kinetic data can be accommodated into reaction sequences featuring either shuttle-type mechanisms involving ferric MPO and compound I as the alternate enzymatic forms[157,159,187,189] or ternary complexation,[188] where both substrates bind simultaneously to the ferric enzyme. The substrate inhibition patterns demand the existence of two distinct binding loci for Cl^-, but the apparent H_2O_2 inhibition may be a consequence of two-electron reduction of compound I as part of the MPO catalatic cycle,[190] rather than reversible binding at an inhibitory site. A plausible reaction scheme is given in Figure 6. Inhibition by Cl^- almost certainly involves heme coordination[184,187] because compound I, the Cl^--reactive form of MPO, has a ferryl oxygen occupying the six-coordinate position and possesses no available Cl^- binding sites, and anation at this site in ferric MPO would competitively block reaction with H_2O_2. Therefore, according to this scheme, the spectroscopically detectable[157,159,169,174,179-185,187] chloroheme is the inhibitory complex, and the catalytic site for Cl^- peroxidation is located elsewhere, most likely either within the heme pocket in the vicinity of the ferryl oxygen or at the heme periphery. One major unresolved problem with these assignments is that the thermodynamic constants (K_d) determined for Cl^- binding should equal the inhibition constants (K_i) determined from steady-state kinetics. This has generally not been the case, and measured values of K_d have been reported to be 10- to 10^2-fold less than the kinetically determined K_i values.[157,183,184] However, Harrison was able to adequately fit steady-state kinetic data for the (Cl^-)-dependence of chlorination of monochlorodimedone to a shuttle mechanism using K_d as the inhibitory constant and reasonable estimates for H_2O_2-binding and rate constants for the elementary reaction steps.[187] In contrast, from results of an early study equating K_d and K_m, the Michaelis constant for Cl^- binding, it was suggested that axial ligation of Cl^- by the heme constituted substrate activation,[183] with HOCl formation presumably occurring by direct attack of H_2O_2 on the heme-bound Cl^-. This mechanism provides no readily discernible means for Cl^- inhibition, however.

MPO has recently been shown to avidly bind O_2^-, giving the superoxo-adduct, compound III.[122] This reaction also appears to occur in phagocytosing neutrophils based upon optical difference spectra[194] and was probably also observed in earlier studies on reactions catalyzed

by NADPH oxidase in membrane fragments from stimulated neutrophils,[194] although the researchers did not identify the chromophore at that time. In the latter study, the spectral changes could not be elicited by NADPH in plasma membrane fractions from resting normal or stimulated CGD neutrophils, establishing a circumstantial link with products from the respiratory burst. Very similar spectral changes occurred upon addition of durohydroquinone ($E'_{0,7}$ [DQ/DQH$_2$] = 50 mV)[196] to either stimulated or resting membrane fragments from normal neutrophils.[195] Under these conditions, MPO compound III may have formed by hydroquinone reduction of ferric MPO ($E'_{m,7} \simeq 20$ mV)[170,180] followed by O_2 addition. Wever and associates have reported that compound III reacts with O_2^- to give compound I.[122] MPO, therefore, appears capable of using either H_2O_2 or O_2^- as halide oxidants. This interpretation of physiological function seems intuitively preferable to the alternative suggestions that these reactions serve to protect phagosomal enzymes from the degradative action of the oxidizing respiratory burst products[194] or that MPO acts as the terminal redox component of the NADPH oxidase.[195] The overall reaction of ferric MPO with O_2^- also constitutes superoxide-dismutase activity. Furthermore, compound I appears to be capable of rapidly oxidizing ferrocytochrome *c* to its ferric oxidation state.[122] These reactions may have contributed to the low stoichiometric yield of O_2^- formation noted by Green and coworkers[115,116] in their careful studies on membranous NADPH oxidase activity.

b. EPO

The developmental biology and physiology of eosinophils roughly parallels that of neutrophils. Eosinophils develop from precursor bone marrow cells and, when mature, are released into the bloodstream as nondividing cells containing primarily storage granules and glycogen. Upon stimulation they undergo a respiratory burst and degranulate and are capable of phagocytosing bacteria and other small particles. The granule composition differs from that of the neutrophil; EPO is a major component,[197] proportionately severalfold higher in concentration than the MPO in neutrophils.[143] Stimulated eosinophils and cell-free EPO-H_2O_2-halide systems exhibit wide-ranging cytotoxicity. Although eosinophils are bactericidal, their primary microbial targets are thought to be parasitic worms because infestation gives rise to increased numbers of eosinophils in the bloodstream of the host, and eosinophils adhere strongly to and damage or destroy their larvae in *in vitro* experiments. As with MPO, the microbicidal potency of EPO is significantly increased by binding to its cellular target. These aspects of eosinophil function and EPO toxicity have recently been reviewed by Klebanoff[1] and associates.[146]

EPO is an $\alpha\beta$ glycoprotein, similar in structure to hemi-MPO.[197,198] The prostetic group has generally been regarded as protoheme[143-145] because its physical properties closely resemble the heme in lactoperoxidase (LPO), the antibactericidal peroxidase found in milk, saliva, and similar fluids.[199] For both enzymes, vibrational bands attributable to vinyl substituents have been identified in RR vibrational spectra, and other features are also consistent with protoporphyrin IX structure.[145,200,201] Optical spectra of the pyridine hemochromes of the solubilized heme from LPO[202] and detergent-denatured EPO[143] are also indistinguishable from protoheme and the magnetic circular dichroic (MCD) spectrum of LPO is very similar to other protoporphyrin IX-containing metalloproteins.[203] From recent mass spectrometric and proton NMR characterization of the porphyrin obtained by reductive cleavage of LPO, it has been suggested that the heme in LPO is a modified protoporphyrin IX containing an unusual thiomethyl β-substituent on its C- or D-pyrrole ring, which is presumably covalently bound to the enzyme by disulfide linkage to a cysteine thiol residue.[204] This type of functional derivatization would be difficult to detect by the other physical techniques employed and could account for the difficulties historically encountered in isolating the heme from LPO. It also poses the intriguing possibility that structural differences between LPO (and EPO) and MPO might simply be a consequence of the nature of covalent binding to protein

sulfhydryl groups at a common site. The optical spectrum of MPO resembles that of sulfhemoglobins and sulfmyoglobins,[168] for which sulfur addition is thought to occur across protoporphyrin IX β-pyrrolic double bonds to form a chlorin-type heme.[205,206]

The EPO and LPO ferrihemes are six-coordinate high-spin, as indicated by their RR spectra.[145,200,201] The electronic spin state has been confirmed by EPR spectroscopy.[143] Hyperfine splitting of the EPR signal from the ferrous nitrosyl derivatives indicate that nitrogen is the coordinating atom of the proximal axial ligand.[181] In LPO, the ligand has been assigned as a histidine imidazole, based upon MCD comparisons with metalloporphyrins of known structure and model complexes[203] and upon detection of a resonance-enhanced Raman band at 258 cm^{-1} assignable as the histidine iron-nitrogen stretching frequency.[200] The sixth, distal ligand was suggested to be a carboxyl group based upon MCD comparisons,[203] although confirmation by other methods is desirable. The RR spectrum of native EPO also shows a band at 258 cm^{-1},[145] and the ligand field parameters for low-spin derivatives of EPO and LPO determined by EPR spectroscopy are very similar,[143] suggesting that the EPO proximal ligand is also histidine. Therefore, at least five of the six iron ligation sites may be structurally isomorphous in the two enzymes. The positions recorded for the LPO and EPO Fe-N(his) stretching frequencies are comparable to values measured for other peroxidases,[90,207] including MPO,[173] and indicate considerable imidazolate character in the proximal ligand. This interpretation is supported by ^{15}N NMR studies on monocyano complexes of several peroxidases, including LPO, the chemical shifts of which were far upfield of those of met-cyanohemoglobin or myoblobin.[208] The differences in chemical shifts for the two classes of heme proteins paralleled changes observed upon deprotonation of a heme-imidazole model complex, suggesting that the origin of the upfield shift is strong σ-donation from the *trans*-ligand. As discussed in Section B.1, the strongly σ-donating character of the proximal imidazole in peroxidases also probably accounts for their low $Fe^{III/II}$ reduction potentials.[89]

c. *Some Mechanistic Puzzles*

Eosinophil peroxidase and LPO differ functionally in that EPO can catalyze H_2O_2-dependent chlorination reactions,[146,209] but LPO cannot.[210] Both enzymes can catalyze peroxidation of Br^-, I^-, and SCN^-. Given the apparent structural similarities of their active sites revealed in their physical properties, a reasonable expectation is that the differences are thermodynamic. Subtle structural variations arising from interactions with the proteins might therefore account for these reactivity differences. The skeletal deformation modes in the RR spectra of protoporphyrins have been shown to correlate directly with the ring π-electron density and inversely with the porphyrin core size.[211] By this criterion, the π-ring electron density is greater in EPO, and the iron is more in-plane since these modes are uniformly at lower energies than in LPO. These differences are consistent with a lower $Fe^{III/II}$ reduction potential for EPO than LPO and, if extrapolated to the corresponding compounds I, poorer oxidizing capability in EPO, contrary to expectations. The identical positions of the proximal Fe-N(his) stretching frequencies are also incompatible with a higher reduction potential for EPO.

The inadequacies of thermodynamic rationalizations of the relative catalytic activities of peroxidases towards Cl^- are even more apparent in comparison of EPO with MPO. As discussed above, both enzymes are six-coordinate, high-spin, with axial ligands that include, most probably, a histidine imidazole and a second weak-field group. Ring reduction in forming the chlorin would be expected to decrease the compound I reduction potential by about 200 mV.[212,213] On thermodynamic grounds, therefore, chlorins should be less effective catalysts of halide peroxidation than the analogous porphyrins. Although EPO is capable of Cl^- peroxidation,[209] its selectivity for this ion is substantially less than exhibited by MPO.[146,147] The relative reactivities, therefore, do not correlate with projected thermodynamic driving

TABLE 1
Thermodynamics of Hydrogen Peroxide Oxidation by Chlorine

Reaction	$\Delta G°$(kcal/mol)[a]	$-\Delta H°$(kcal/mol)
$Cl_2 + H_2O_2 \rightarrow O_2 + 2H^+ + 2Cl^-$	32.8	28.3
$HOCl + H_2O_2 \rightarrow O_2 + H^+ + Cl^- + H_2O$	37.4	34.8
$OCl^- + H_2O_2 \rightarrow O_2 + Cl^- + H_2O$	47.6	38.0
$OCl^- + HO_2^- \rightarrow O_2 + Cl^- + OH^-$	44.4	32.9
$HOCl + HO_2^- \rightarrow O_2 + Cl^- + H_2O$	53.3	43.1

[a] At 25°C.

From Held, A. M., Halko, D. J., and Hurst, J. K., *J. Am. Chem. Soc.*, 100, 5732, 1978. With permission.

forces. Two possible alternative explanations[145,172] for the enhanced reactivities of chlorins are (1) disruption of π-conjugation introduces flexibility to the porphyrin ring, facilitating axial ligation of weak-field ligands such as chloride,[214] and (2) charge asymmetries inherent in hydroporphyrin rings favor oxidation of nucleophiles such as chloride at compound I ring peripheral sites. The first alternative seems unlikely since the axial MPO chloride-binding site is thought to be inhibitory.[184,187] However, the second alternative is attractive on several counts. The predominant reaction between Cl^- and metalloporphyrin[215] or other aromatic π-cations[216] is electron transfer, rather than ring chlorination by nucleophilic addition. As discussed in the next section, HOCl reactivities are highly dependent upon the nucleophilic character of the reactant partner.[217,218] If two-electron Cl^- oxidation proceeds by way of an analogous reaction coordinate, i.e., if Cl^- oxidation to form HOCl is essentially the reverse of HOCl reduction, then reactivity will be controlled by the electrophilic character of the oxidant electron acceptor site. Ferryl π-cations derived from chlorins are calculated to be relatively electron-deficient at the methine positions remote from the dihydropyrrole group.[219] These electron-deficient positions arising from the chlorin electronic asymmetry might correspondingly provide uniquely reactive sites for halide oxidation. Prospects for this type of mechanism are heightened by recent research on other peroxidases, indicating that one-equivalent substrate oxidation probably occurs via electron transfer at the heme periphery.[220]

3. General Reactivity Characteristics of HOCl

Although compounds containing chlorine with a formal valency of +1 are powerful oxidants, their reactivities towards reductants vary widely. This behavior is well illustrated by their oxidative behavior towards H_2O_2.[217,218] Standard free energies of formation for reaction with Cl_2, HOCl, and OCl^- are given in Table 1. The reactions are all highly exothermic. Nonetheless, the reactant pairs $Cl_2 + H_2O_2$ and $OCl^- + H_2O_2$ rapidly form O_2, but direct reaction between $HOCl + H_2O_2$ and $OCl^- + HO_2^-$ is undetectably slow.[217] Furthermore, by replacing the HOCl proton with a *tert*-butyl group, it was demonstrated that the true reactants under alkaline conditions are $HOCl + HO_2^-$, i.e., not the predominant species in solution.[218] These reactivity patterns can be rationalized in terms of electrophile-nucleophile interactions between the electron-deficient chlorine atom and electron-rich sites on the reductant, an appropriate transition-state structure for which is given in Figure 7. From its reactivity with organic compounds, HO_2^- is known to be a powerful nucleophile, whereas the protonated form, H_2O_2, is only very weakly nucleophilic.[221] Interaction of HO_2^- with chlorine oxidants possessing suitable electron-withdrawing groups, X, is viewed as occurring with incipient bond formation, followed by two-electron transfer from oxygen to chlorine. These reactions are akin to atom transfer reactions and may involve transitory formation of ClOOH[222], which subsequently rapidly decomposes to O_2 and HCl. Relative

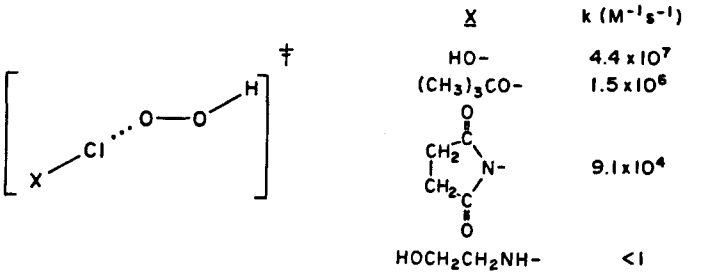

FIGURE 7. Hypothetical transition-state structure and kinetic data for reaction of the hydroperoxide anion with chlorine (+1) compounds. All reactions obey the rate law, $R = k[X-Cl][HO_2^-]$. (Adapted from Hurst, J. K., Carr, P. A. G., Hovis, F. E., and Richardson, R. J., *Inorg. Chem.*, 20, 2435, 1981.)

rates can be expected to be extremely sensitive to magnitudes of the initial associative interactions because the reactions are highly exergonic. Hydroperoxide protonation will severely diminish these interactions, as will replacement of OH with less electron-withdrawing substituents on the chlorine atom. Consistent with these expectations, relative rate constants for reaction with HO_2^- were found to vary proportionately with the electron-withdrawing character of X.[218] Replacement of oxygen by the less electronegative nitrogen atom, in particular, causes marked diminution in rate, although the reaction is still observable if electron-withdrawing carbonyl groups are attached, as in N-chlorosuccinimide. The unreactivity of OCl^- towards HO_2^- can be ascribed primarily to the instability of the unprotonated oxide ion leaving group. Other aspects of the reaction are also easily rationalized by this transition-state geometry. Both atoms of O_2 derive from H_2O_2, as observed,[223] and, at least for HOCl + HO_2^-, the oxygen product is entirely in its $^1\Delta$ electronic state as required by conservation of electronic spin.[217] Alternative mechanisms involving stepwise one-electron transfer between Cl_2 and H_2O_2, forming $H_2O_2^+$ and Cl_2^- intermediates,[224] can be excluded on energetic groups,[217] although radical intermediates could be formed in reactions catalyzed by transition metal ions.[224] Primary and secondary amines and α-amino acids appear to react by an analogous mechanism,[225] except that the chlorine atom transferred displaces a proton to form relatively stable N-chloramine and N-chloroamino acid products (see Figure 5). Consistent with the electrophile-nucleophile character of these reactions, rate constants have been found to vary proportionately with nitrogen basicities.[226]

Hypochlorous acid, which is relatively unreactive towards H_2O_2, can apparently be activated by association with Cl^- and a proton, forming a species identified from the rate law as H_2OCl_2.[217] This species, kinetically distinct from Cl_2, is thought to have an electronic structure analogous to Cl_3^-, in which the central chlorine atom is electron-deficient and, hence, more reactive than HOCl toward H_2O_2. Formation of H_2OCl_2 is rate-limiting, as is evident from the independence of reaction rates upon H_2O_2 concentration. This pathway may make a major contribution to biological chlorination processes with the phagosome.[227]

The leukocyte peroxidase-generated oxidant has frequently been designated as the chlorinium ion, Cl^+. Although, in general, this assignment is casual and only intended to indicate +1 valency on chlorine, the question of Cl^+ as a discrete species in organic chlorination reactions has stimulated considerable investigation. Originally proposed to account for substrate-independent terms such as k[HOCl] and k'[HOCl][H^+] in rate laws for aromatic chlorination reactions, its existence in any kinetically significant concentration is unlikely on thermodynamic groups.[228] Moreover, a reevaluation on selected reactions failed to reproduce the rate law of the early kinetic studies and provided no evidence for Cl^+ involvement.[229] A structurally distinct species, the hypochlorous acidium ion, H_2OCl^+, may contribute

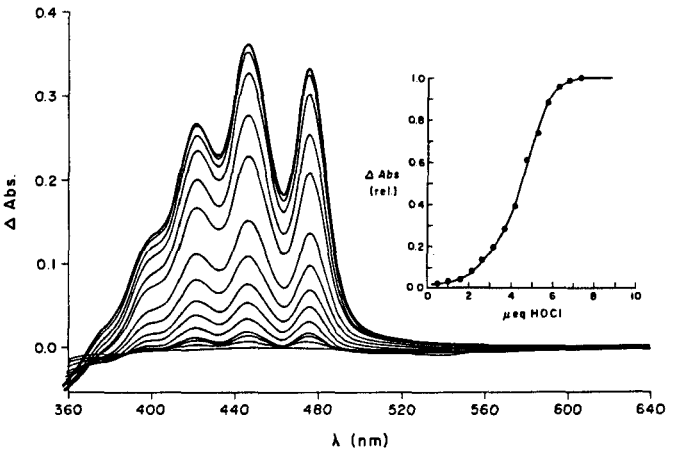

FIGURE 8. Difference spectral titration of *S. lutea* with HOCl. The difference curves obtained are identical to carotene absorption spectra. The inset gives the titrimetric change in absorbance at 447 nm corrected for dilution by titrant. (From Albrich, J. M., McCarthy, C. A., and Hurst, J. K., *Proc. Natl. Acad. Sci. U.S.A.*, 78, 210, 1981.)

to aromatic chlorination.[229] Consistent with the electrophile-nucleophile character of these reactions, protonation of HOCl on oxygen would increase the electrophilicity of the chlorine atom, but the acid dissociation constant is estimated to be $pK_a \simeq 10$.[230] Under physiological conditions the H_2OCl^+/HOCl concentration ratio would be about 10^{-17}, rendering it impossible for H_2OCl^+ to contribute to biological oxidation or chlorination reactions. We found no evidence for H_2OCl^+ involvement in our thorough study of the HOCl-H_2O_2 reaction.[217]

One predicts from these general reactivity characteristics that HOCl should exhibit high selectivity for sites within biological molecules that are relatively electron-rich. This is, in fact, the experimentally observed behavior.[231] As classes, the reactive biomolecules include nitrogen bases of amines and amino acids, sulfhydryl groups and ferredoxin-type iron-sulfur clusters, and a variety of compounds containing extended π-unsaturation, including conjugated polyenes, the nitrogen heterocycles of nucleotide bases, and heme prosthetic groups. However, nucleophiles that are strong bases exist at physiological pH values primarily in their protonated, conjugate acid forms, which tend to be unreactive towards HOCl. Thus, although both HO_2^- and the free base forms of amines and amino acids react with HOCl with rate constants approaching diffusion-controlled limits, their overall rates are considerably attenuated at neutral pH, allowing competitive reaction with other biological nucleophilic sites. On the other hand, biomolecules not possessing nucleophilic sites, including many of the structural components of bacterial cell envelopes, are unreactive towards HOCl.

This same high selectivity for nucleophilic sites is observed in the reactions of HOCl and MPO-H_2O_2-Cl^- cell-free systems with intact bacteria. Oxidation of accessible sulfhydryl groups[136,139] and N-chlorination[16,136,139,232] have been shown to be extensive in MPO-mediated chlorination reactions directed against *E. coli*. Similarly, bleaching of specific chromophores such as b-cytochromes in *E. coli* and β-carotene in *Sarcina lutea* by bactericidal concentration levels of HOCl or MPO-H_2O_2-Cl^- has been demonstrated by optical difference spectroscopy,[231] as well as loss of iron-sulfur centers in the succinate dehydrogenase complex of *E. coli* by cryogenic EPR studies on isolated membrane fragments.[374] Changes in *S. lutea* pigmentation accompanying titrimetric addition of HOCl are illustrated in Figure 8.

4. Intraphagosomal Chlorination

Various points of evidence have been advanced suggesting that, in addition to their proposed microbicidal function, leukocyte peroxidases secreted into the medium play major

roles in diverse extracellular processes, including activation, inactivation, or modulation of their own granular enzymes, regulation of other secretory cells and modulation of chemotactic signals, the respiratory burst, and host inflammatory response, as well as engaging in tumoricidal activity and tissue cell damage. Many of these specific funtions have been reviewed recently[1,3,146,233-240] and are not discussed further here. Secretion occurs by degranulation during the normal course of forming the phagosome, by degranulation induced by soluble endogenous stimuli or when the target particle is too large to be engulfed, and possibly also from unsealed phagosomal vacuoles[241,242] and by cytolysis of stimulated leukocytes at the end of their life cycles. Recognition that leukocyte peroxidases have numerous potential alternative functions weakens teleological arguments advanced in support of their involvement in microbicidal mechanisms. In fact, the counterproposal has recently been made that because of differing medium conditions arising from microcompartmentation, MPO-catalyzed reactions play only a minor role in the microbicidal function of neutrophils, but remain effective in extracellular regulation and cytotoxicity.[194] The argument is based upon recognition that the pH optimum for MPO-catalyzed halide peroxidation decreases with increasing $[H_2O_2]/[Cl^-]$ ratios,[157,158,186] that the intraphagosomal pH is neutral or slightly alkaline over the time frame of bacterial killing,[13,160,241] and upon the supposition that H_2O_2 concentrations can reach levels as high as 100 m*M* in the phagocyte vacuole, a value estimated from the O_2 consumption rate in the respiratory burst. Under these conditions, MPO catalysis of Cl^- peroxidation was projected to be largely suppressed in favor of catalytic decomposition of H_2O_2 via compound III formation.[194] However, H_2O_2 will be lost from the phagosome both by passive diffusion and by enzymatic catalase and peroxidase reactions, particularly when bacteria that might contribute catabolic enzymatic pathways are present. Also compound III reaction with O_2^- has in one study[122] been determined to yield compound I, the enzymic form reactive with Cl^-, and MPO-catalyzed chlorination has repeatedly been observed to occur at appreciable rates *in vitro* at millimolar concentration levels of H_2O_2 and other conditions approximating the phagosomal milieu. These conflicting views underscore the importance of assessing chlorinating activity within the neutrophil phagosomes.

Azide-inhibitable incorporation of $^{36}Cl^-$ into protein was observed upon phagocytosis of serum-opsonized *Staphylococcus epidermis* by neutrophils; the extent of chlorination continued to increase well beyond completion of phagocytosis, indicating that the reactions occurred at least partly within the phagocytic vacuole.[243] N-chlorination of hydrophilc amines has also been noted[244] under phagocytosing conditions, as has ring chlorination of lipophilic trimethoxybenzene.[245] In these latter studies it was not possible to ascertain the relative amounts of chlorination occurring within the intact phagosome, as opposed to secreted MPO. Intraphagosomal chlorination has been inferred from measurements of fluorescence changes accompanying phagocytosis of opsonized zymosan containing covalently attached fluorescein.[160] Reactions of this dye with HOCl or MPO-H_2O_2-Cl^- yield predominantly 4-monochloro and 4′,5′-dichloro derivatives, the fluorescence yields of which are diminished by 25 and 55%, respectively. Neutrophils stimulated with PMA in the presence of unopsonized fluoresceinated zymosan exhibited a rapid loss in fluorescence intensity initiated at the onset of the respiratory burst that exhibited the proper response to controls to implicate MPO-catalyzed reactions. Thus, fluorescence loss could be totally inhibited by addition of N_3^- or catalase and did not occur with CGD or MPO-deficient neutrophils, although the reaction could be elicited by addition of MPO to the system containing MPO-deficient neutrophils. Fluorescence intensity changes could not be attributed to pH changes since the medium was buffered. Furthermore, partial recovery of the dye from hydrolyzed particles gave fluoresceins which cochromatographed with authentic mono- and dichloro derivatives. Analogous changes in the fluorescence spectrum were observed when the opsonized fluorescein-zymosan conjugate was reacted with normal neutrophils, although no change was observed with MPO-deficient cells, and CGD cells exhibited a slow progressive decline in intensity (see Figure

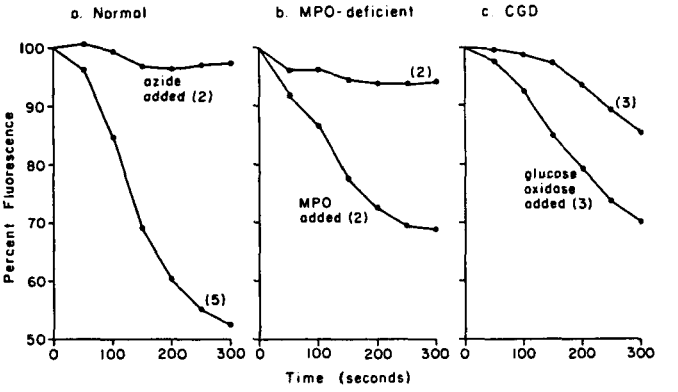

FIGURE 9. Fluorescence changes upon ingestion of opsonized fluoresceinated zymosan by normal and deficient neutrophils. Fluorescence intensity changes are expressed as percent of initial values. (From Hurst, J. K., Albrich, J. M., Green, T. R., Rosen, H., and Klebanoff, S. J., *J. Biol. Chem.*, 259, 4812, 1984. With permission.)

9).[160] Under these conditions nearly all the fluorescent particles were ingested, and temporal changes in fluorescence coincided with respiratory activity.[241] As in the extracellular reactions, fluorescence loss was inhibited by N_3^- in normal neutrophils and could be induced in MPO-deficient neutrophils by including MPO in the assay medium. Fluorescence losses were also enhanced in CGD neutrophils by introducing an enzymatic H_2O_2-generating system.[160] The extent of optical changes in these reactions was consistent with nearly complete monoclorination and extensive dichlorination of the probe, although structural confirmation could not be obtained by its recovery and analysis. Comparable losses in fluorescence intensity were noted in an independent study of neutrophil phagocytosis of fluoresceinated zymosan, although the researchers did not investigate the underlying reactions.[241] Finally, evidence has recently been obtained suggesting that MPO-mediated reactions participate extensively in postmortem degradation of bacterial proteins.[15] The cell-free MPO-H_2O_2-Cl^- system gives predominantly products with peptides that are N-chlorinated on terminal amino groups.[246,247] These compounds, presumably formed during the respiratory burst by reaction of accessible bacterial proteins or subsequently by chloramine exchange reactions, are generally unstable and in some reactions undergo slow peptide bond cleavage with loss of CO_2 and other small molecules.[246,247] The several studies that address the point therefore suggest that the chlorinating capacity of neutrophils remains high during and immediately following formation of the phagosome.

A second matter of concern is the intraphagosomal acidity, which is not only intimately linked with expression of enzymatic activities of the peroxidase,[157-159,187] NADPH oxidase,[248] and digestive proteins,[13] but also can control relative reactivities at various susceptible microbial sites. For example, rates of HOCl bleaching of heterocyclic compounds such as hemes and nucleotide bases generally increase with acidity in neutral to weakly acidic solutions (although the H^+ dependence is complex),[231] but amine N-chlorination is inversely dependent upon the H^+ concentration.[225] At pH 5.4, selectivity for the heterocycles over amines will be 10^3 to 10^4 greater than at normal cytosolic pH values of 7.4. This varying reaction site selectivity undoubtedly accounts for the pH dependence of lethal doses of HOCl towards *E. coli* that were previously mentioned[135] (see Section III.A.1). Since Metchnikoff's first studies, researchers have repeatedly used dye particles or dye-stained microbes to visually determine intraphagosomal acidities with the general concurrence that, to a greater or lesser extent, the phagosomes acidify.[160] These conclusions have been supported by direct measurement of proton release from stimulated neutrophils into unbuffered media[249-251] and

the intracellular accumulation of the weak base 5,5-dimethyl-2,4-oxazolidinedione (DMO) during phagocytosis.[252] However, the oxidative products of most of the dyes used in these studies, particularly the sulfonphthaleins, are visually indistinguishable from their yellow, acidic forms and N-chlorination of DMO[375] could provide a mechanism for its intracellular accumulation. These indirect methods therefore do not give, even qualitatively, an indication of phagosomal pH values. When examined by ^{31}P-NMR spectroscopy 10 min after stimulation with PMA, neutrophils gave signals attributable to inorganic phosphate at pH 5.9 to 6.4. As noted by the researchers, this technique does not identify the subcellular location of the acidic phosphate ions.[253] However, electroanalytical measurements have indicated that phosphate is not excreted by PMA-stimulated cells.[250] The results from the two physical methods are, therefore, not in accord since the NMR data imply acidification of the neutrophil cytosol, whereas direct pH measurements indicate acidification of the extracellular medium and, inferentially, the neutrophil phagosome.

More recent studies using fluorescein-labeled particles have focused on changes occurring within the first few minutes following recognition and binding, which is the time frame of phagocytosis, enhanced respiration, and microbicidal action. Segal and co-workers, using fluorescent *S. aureus*, first reported[13] that the phagosomal environment increased slightly in alkalinity for several minutes following phagocytosis, then gradually fell to 6.0 to 6.5 after several hours, but that phagosomal pH values in CGD neutrophils immediately fell below 6.5, ultimately reaching pH ~5.5. These effects were fully confirmed by Cech and Lehrer in an elegant, careful study[241] of spectral changes accompanying ingestion of fluorescent zymosan in which quantitative account was taken of oxidative losses in fluorescence and the presence of unsealed phagosomal vacuoles, the major sources of error in the analyses. Again, shortly after phagocytosis, sealed vacuoles had become slightly alkaline, and then slowly acidified, reaching an average pH of ~5.7 after 1 h. The data given in Figure 9 are also consistent with this behavior; thus, normal neutrophils containing N_3^--inhibited MPO and MPO-deficient neutrophils show no fluorescence intensity losses that would occur upon protonation of the chromophore, although acidification is immediately detectable upon stimulation of CGD neutrophils.

The mechanisms underlying the dynamic changes in phagosomal acidities are not well understood. In particular, the seemingly conflicting results that protons are released to the extracellular medium after only a short lag period following stimulation[249,250] and that phagosomes do not acidify until stimulated respiration is nearly terminated[13,241] require resolution. Reduction of $NADP^+$ by the hexosemonophosphate shunt during the respiratory burst has been shown to occur with complete oxidation of glucose-6-phosphate to CO_2.[249] Oxygen reduction to H_2O_2 by glucose via the NADPH oxidase and hexosemonophosphate shunt therefore occurs by the following net reaction:

$$C_6H_{12}O_6 + 12H_2O + 12O_2 \rightarrow 6HCO_3^- + 6H^+ + 12H_2O_2$$

for which 0.5 protons are released per O_2 consumed. The overall proton stoichiometry depends upon the fate of the H_2O_2 produced. If H_2O_2 disproportionates or is reduced by the glutathione cycle (see Figure 3), then the overall reaction stoichiometry becomes

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6HCO_3^- + 6H^+$$

and the $[H^+]/[O_2]$ ratio is 1.0. If the H_2O_2 is consumed as substrate in peroxidative reactions, then the overall stoichiometry is dependent upon the identity of the ultimate reaction end-products. For example, MPO-catalyzed HOCl formation, followed by its reaction with compounds that release protons upon oxidation or chlorination, such as thiols or protonated amines, gives an overall stoichiometry of

$$C_6H_{12}O_6 + 12O_2 + 24RSH \rightarrow 12RSSR + 12H_2O + 6HCO_3^- + 6H^+$$

with $[H^+]/[O_2] = 0.5$. Here one-electron oxidation of the sulfhydryls has been assumed; if oxidation proceeded completely to cysteic acid, then $[H^+]/[O_2] \simeq 0.83$. If HOCl reacts without net proton release, as in heterocyclic ring chlorination or peptide N-chlorination, then the appropriate reaction stoichiometry is

$$C_6H_{12}O_6 + 12O_2 + 6H^+ + 12Cl^- + 12AH \rightarrow 6HCO_3^- + 12H_2O + 12ACl$$

for which 0.5 protons are *consumed* per oxygen molecule reduced. Therefore, the proton stoichiometry is, in principle, highly variable and dependent upon the distribution of oxidizing equivalents in complex systems. Because the measured $[H^+]/[O_2]$ ratio was very near unity in the experiments measuring extracellular release of protons,[249,250] oxidase-generated H_2O_2 in PMA-stimulated cells is suggested to decompose primarily by disproportionation. Reaction within the phagosome, where MPO is relatively concentrated, might include greater participation of peroxidative reactions and thereby lower the extent of acidification.

The transverse organization of the NADPH oxidase (see Figure 4) suggests that the acid is formed initially in the neutrophil cytosol. Oxygen reduction could lead maximally to uptake of two protons within the phagosome and release of 2.5 protons in the cytosol. However, the electron transport chain is electrogenic[42] and requires transmembrane movement of ions to maintain electroneutrality. Even if this were accomplished by proton cotransport with the electron,[42] CO_2 hydration would be expected to acidify the cytosol. Equilibration with the extracellular medium is rapid because proton release has been shown to follow very closely oxygen consumption.[249,250] It is not clear whether the observed proton flux could be sustained by CO_2 diffusion across the plasma membrane, but an electronneutral Na^+/H^+ antiporter has recently been described[254] that facilitates H^+ efflux by exchange with extracellular Na^+. If this and similar porters were preferentially excluded from the phagosomal membrane, then the observed temporal response within the vacuole might arise by proton consumption from oxygen reduction with only slow intercompartmental equilibration of proton gradients by passive diffusion. Alternatively, these gradients could be maintained by proton pumping. An ATPase located in the neutrophil granules has been described that was suggested to act in this capacity upon fusing with the plasma membrane,[251] and O_2^- formation from NADPH is exergonic by about 160 mV, so that proton pumping by the neutrophil oxidase is thermodynamically feasible. However, the notion of proton pumping is incompatible with experimental observations that addition of lipid-soluble proton carriers to PMA-stimulated neutrophils did not affect proton translocation to the extracellular medium,[250] indicating intercompartmental pH differences were not nonequilibrium distributions maintained by active transport. It is also incompatible with evidence suggesting the existence of NADPH oxidase-associated proton channels in the neutrophil plasma membrane.[42]

In summary, evidence for intraphagosomal chlorination on the timescale of microbial killing is found in the spectroscopic behavior of HOCl-reactive dyes; the reaction medium appears to be approximately neutral, despite a gradual acidification of the environment arising from glucose oxidation.

5. Microbicidal Mechanisms

Quench-flow experiments have demonstrated that *E. coli* is killed within 100 ms of exposure to HOCl at concentration levels just sufficient to sterilize the suspension.[135] These results establish that the lethal reactions must involve biological molecules that are highly reactive towards HOCl, i.e., good nucleophiles. The loci of attack are almost certainly within the bacterial envelope. Several studies have demonstrated bacterial cell wall protein degradation,[16,232] loss of titratable sulfhydryl groups,[136,139] release of N-chlorinated peptides

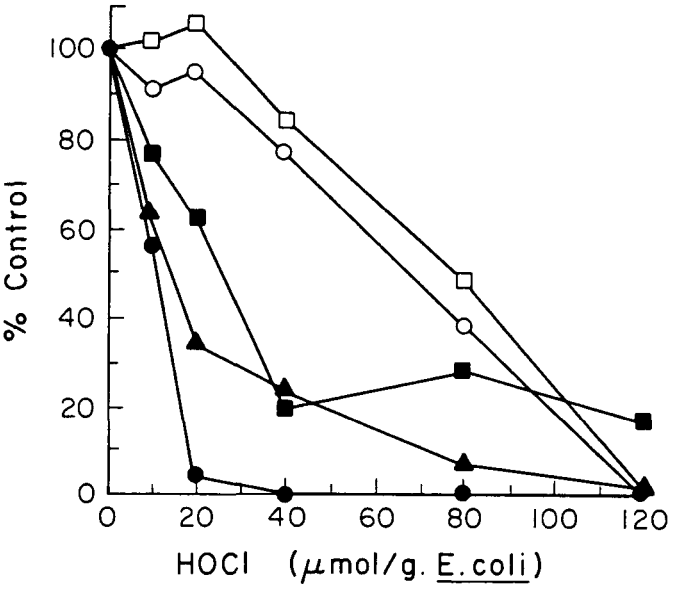

FIGURE 10. Comparison of HOCl-promoted titrimetric loss of viability with respiratory function in *E. coli*. (●) = cell viability, expressed as colony-forming units; (■) = O_2 respiratory rate; (▲) = succinate dehydrogenase activity in membrane vesicles; (○ and □) = relative amplitudes of esr signals from S_1 and S_3 FeS centers, respectively, of succinate dehydrogenase in the membrane vesicles. (From Hurst, J. K., *Oxygen Complexes and Oxygen Activation by Transition Metals*, Martell, A. E. and Sawyer, D. T., Eds., Plenum Press, New York, 1988.)

which appear to be cytoplasmic in origin,[139] loss of metabolite transport,[8,137,255,256] and inactivation of specific membrane-localized enzymes[257-259] and other chromophores,[231,259] as well as physical effects such as alteration of cellular surface charge,[255] upon exposure of *E. coli* to HOCl and/or the cell-free MPO-H_2O_2-Cl^- system. In contrast, cytoplasmically localized biomolecules that are highly susceptible to oxidative degradation by HOCl, such as the enzymes aldolase[231] or β-galactosidase[137,260] and nucleotide bases,[138] are not affected until HOCl concentrations severalfold in excess of that required for killing are added.[137,138,256,260]

An approach that has proven useful in distinguishing which of the numerous cellular reactions of HOCl might be cytotoxic is to compare directly loss of specific metabolic function or reaction at specific sites with the extent of killing following incremental addition of the oxidant. The data are often plotted in the form of titration curves (e.g., Figure 8). They differ from true titration curves in the sense that many other reactions proceed simultaneously. For example, about 10 HOCl/carotene are required to bleach the molecule in sodium dodecylsulfate-micellar suspension;[231] cellular bleaching requires about 10^3 to 10^4 HOCl/carotene, based upon the spectrophotometric changes observed in *S. lutea*, so that only 0.1 to 1% of the added oxidizing equivalents is directed against this pigment. Furthermore, the pronounced lag observed before the onset of bleaching (see Figure 8) is not observed in reactions of the micelle-solubilized carotene, indicating that other sites exist within the bacterium that react preferentially. Correlation between titrimetric curves for a particular reaction and cellular death, therefore, will not establish a causal relationship, but if a particular metabolic process or biological component remains unaffected at lethal levels of oxidant, its participation in the microbicidal processes is unlikely. A second example illustrating this approach is given in Figure 10, where cell viabilities of *E. coli*, measured by their ability to form colonies, are compared to respiratory rates of cell suspensions and

succinate dehydrogenase activities and iron-sulfur signal intensities in respiratory membrane fragments derived from the cells. The titration curve was constructed from a single bacterial culture, with all of the measurements at a given oxidant concentration being made on the same sample. From the data, one infers that the respiratory chain is vulnerable to oxidative inactivation by HOCl and that loss of respiration may involve succinate dehydrogenase (SDH), although SDH inactivation does not appear to be a consequence of oxidation of its iron-sulfur clusters. Because respiratory loss lags somewhat behind loss in cell viabilities, the data also suggest other metabolic dysfunctions are associated with death. These general relationships have been repeated countless times in our laboratory and are independent of bacterial strains employed, their growth conditions, and experimental methodologies.

Using this general approach, Thomas first showed that oxidative killing by HOCl or MPO-H_2O_2-Cl^- coincided with loss of titratable bacterial sulfhydryl groups, but not with amine N-chlorination.[136,139] Subsequently, Sips and Hamers reported that cellular death in *E. coli* ML-35 induced by HOCl, MPO-H_2O_2-Cl^-, or phagocytosis by neutrophils was associated with increased permeability of the bacterial plasma membrane towards small molecules.[260] This conclusion was based upon observation of enhanced intracellular hydrolysis of ortho-nitrophenylgalactoside (ONPG) by the oxidatively inactivated cells. The bacterium is a mutant which lacks a functional lactose permease, but synthesizes constitutively the cytoplasmic enzyme, β-galactosidase. Since the substrate is not taken up by undamaged bacteria, the researchers reasoned that enhanced reactivity could only arise from oxidative degradation of the cellular permeability barrier by HOCl. A linear correlation between *E. coli* inactivation and increased β-galactosidase activity was noted; at the point of complete killing essentially all of the intracellular β-galactosidase was reported to be accessible to added ONPG. In subsequent work,[261] these researchers and collaborators have found that the extent of exposure of *E. coli* ML-35 β-galactosidase by neutrophils is substantially less, reaching maximally about 20% of the total bacterial β-galactosidase over the course of the phagocytic reaction. Furthermore, β-galactosidase activity reached twofold higher levels when MPO-deficient neutrophils were used, indicating that enzyme envelope "perforation" is not obligatorily associated with MPO reactions. The absence of effect upon phagocytosis by CGD neutrophils established that the reactions were oxidative in character. The decreased maximal β-galactosidase activity in *E. coli* isolated from normal neutrophils was attributed to MPO-specific oxidative inactivation of the enzyme accompanying disruption of the membrane permeability barrier, and quantitative differences in the two studies were attributed to the use of opsonized vs. unopsonized bacteria. The more recent studies are reported to be sufficiently reproducible to form the basis for clinical assays of neutrophil function, provided bacterial growth conditions and opsonizing serum levels are carefully controlled.

We have also examined the effects of HOCl upon β-galactosidase activity of *E. coli*, including both ML-35 and a strain containing an active lactose permease.[137] As with normal neutrophils, maximally 10 to 20% of the total bacterial β-galactosidase became accessible to ONPG at the titrimetric point where 99% of the cells had been killed. Unlike the earlier reports, enzymatic activity increased progressively with time over a period of several hours after bacterial oxidation from initial levels that were barely detectable, and 25 to 40% of the activity that developed was extracellular. We attribute these reactions to HOCl-induced lysis of a small fraction of the bacterial cells. In the same study, we found by direct measurement that the rates of transmembrane diffusion of glycerol and protons were unchanged in *E. coli* oxidized with HOCl at concentration levels several-fold in excess of lethal doses. Subsequent research has shown that the cells are also capable of maintaining their proton motive force at this level of oxidation,[138] confirming that the bacterial membranes retain their intrinsic impermeabilities to small molecules and ions. The bactericidal mechanism of HOCl, therefore, does not seem to be associated with nonspecific degradation of the membrane barrier.

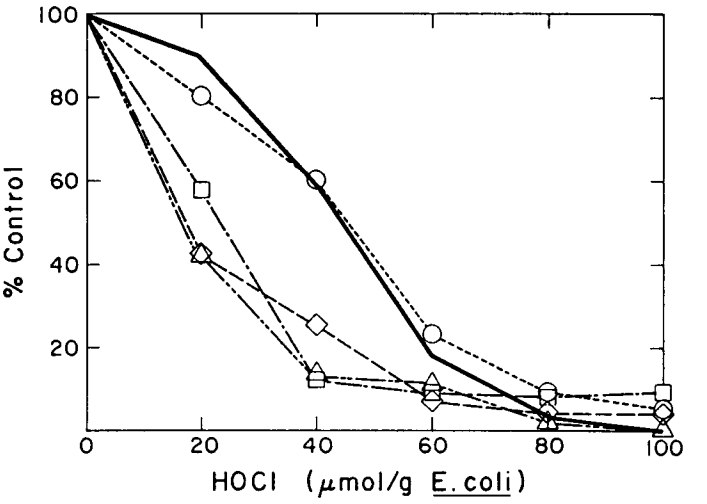

FIGURE 11. ^{14}C-labeled metabolite uptake by HOCl-treated *E. coli*. Solid line = cell viability as colony-forming units; (○) = thiomethylgalactoside uptake; (□) = leucine uptake; (◇) = glutamine uptake; and (△) = proline uptake. (From Albrich, J. M., Gilbaugh, J. H., Callahan, K. G., and Hurst, J. K., *J. Clin. Invest.*, 78, 177, 1986.)

Rosen and collaborators have undertaken a detailed study of MPO-inflicted damage on *E. coli* in relation to viabilities. Initial experiments demonstrated loss of radiolabeled iron[262] and labile sulfur[263] from *E. coli* exposed to MPO and halide in the presence of an H_2O_2-generating system (glucose/glucose oxidase), suggesting involvement of components of the bacterial respiratory chain. Iron release lagged well behind the time course of inactivation, but could be promoted by the addition of chelating agents; however, the loss of labile sulfide from the bacteria actually slightly preceded the killing rate. More recently, the membrane-localized b-type cytochromes, which include respiratory chain components, have been shown not to undergo oxidative degradation during killing, although succinate-linked respiratory and cytochrome b reduction rates by the intact cells and SDH activity in electron-transport particles were lost over the same time frame as viability.[258] The loss of these functions was attributed to SDH inhibition, not loss of succinate transport to the cytosol since direct measurement of uptake of radiolabeled substrate indicated that transport was impaired by only about 50% in the dead cells. In general, these observations correspond to our titrimetric results using HOCl as the oxidant (see Figure 10). The parallel losses of labile sulfur and viability[263] and the high intrinsic reactivity of ferredoxin FeS centers toward HOCl[231] suggest that SDH inactivation by MPO-H_2O_2-Cl might possibly involve its iron-sulfur clusters. However, with HOCl as titrant, EPR evidence (Figure 10) indicates that the centers are not oxidatively damaged at bactericidal concentrations of oxidant. These latter studies have also included power saturation studies to probe the EPR-"invisible" site in SDH[264,265] (Hurst and Barrette, unpublished obervations). One notable difference in the studies is that transport of succinate was partially maintained in MPO-inactivated cells, whereas transport of metabolites, including succinate, is completely blocked in HOCl-inactivated cells.[137] Representative data for HOCl are given in Figure 11, which includes examples of transport driven by proton symport and by ATP hydrolysis. We have also now determined that the third major bacterial nutrient transport system (see Figure 12), the sugar:phosphoenolpyruvate (PEP) phosphotransferase system, is also oxidatively inactivated in *E. coli* by bactericidal concentration levels of HOCl.[373] Massive hydrolysis of ATP was found to accompany transport loss, although basal respiratory levels appeared sufficient to maintain a normal

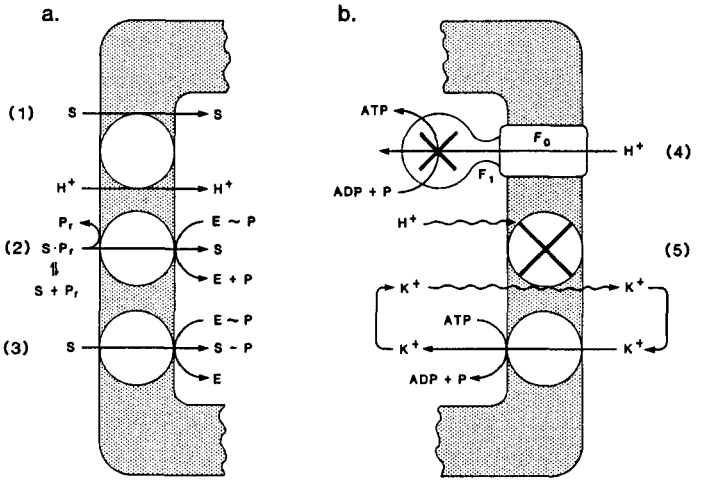

FIGURE 12. (Panel a) Bacterial transport mechanisms; (Panel b) plausible mechanisms for HOCl-induced phosphoanhydride bond hydrolysis. Panel a: (1) substrate (S) uptake coupled to proton translocation; (2) substrate uptake coupled to hydrolysis of a phosphoryl donor metabolically derived from ATP; Pr is a substrate-specific periplasmic binding protein; (3) substrate uptake by group translocation driven by hydrolysis of phosphoenolpyruvate. Panel b: (4) HOCl inhibition of the ATP-hydrolyzing F_1 subunit of ATP synthase blocks chemiosmotically coupled ATP synthesis; (5) futile cycle caused by HOCl-induced leak in a K^+-specific proton symporter with attempted compensation by a K^+-translocating ATPase. (From Hurst, J. K., *Oxygen Complexes and Oxygen Activation by Transition Metals*, Martell, A. E. and Sawyer, D. T., Eds., Plenum Press, New York, 1988.)

proton motive force.[138] The latter observation suggests that the proton symporters are inactivated by direct oxidative attack, which is consistent with evidence that they contain essential,[266] and presumably vulnerable,[139,231] sulfhydryl groups. Loss of ATP-dependent transport might occur by oxidation of membrane-localized porters, loss of thermodynamic driving force,[31] or possibly N-chlorination of periplasmic-binding proteins.[139] The molecular basis for inhibition of PEP-dependent sugar uptake has not been explored.

The inability of HOCl-inactivated *E. coli* to maintain proper ATP levels may be a manifestation of the bactericidal mechanism since the inability to store metabolic energy renders the cell incapable of undertaking biosynthetic functions essential to repair and growth.[267] Net loss of ATP could arise directly by inactivation of cellular ATP-generating systems or indirectly by accelerated utilization (see Figure 12). Inability to assimilate sugar and inactivation of the membrane-localized proton-translocating ATP synthase would effectively block cellular ATP synthesis.[31] We have now found that the hydrolytic activity of the ATP synthase F_1 complex[31] declines in parallel with loss of viability in *E. coli*.[373] The ATP-synthesizing capabilities are almost certainly also lost, although this remains to be established. A hypothetical example of the second type of mechanism is loss of control in an ATP-independent reaction, e.g., unregulated efflux of cytosolic ions (K^+, PO_4^{3-}) arising from damage to the transporter gating mechanism (see Figure 12). Other, ATP-dependent ion transport systems could become engaged in a "futile cycle" to attempt to compensate for the leak and thereby rapidly dissipate intracellular ATP reserves.[268] Preliminary results suggest that futile cycles are less important mechanisms because net hydrolysis rates following *E. coli* inactivation are not markedly dependent upon oxidant dose levels and are nearly identical to rates observed when respiration and phosphorylation are uncoupled with protonophores. The notion that inhibition of ATP synthesis and metabolite transport con-

stitutes the bactericidal mechanisms of HOCl is appealing because loss of these metabolic capabilities is certainly lethal to all cells and for prokaryotes, at least, the topographic location of these components within the plasma membrane renders them vulnerable to extracellular oxidants. Consistent with the "universal" character of this mechanism, we have observed analogous nutrient transport inhibition and ATP hydrolysis for the obligate aerobe, *Pseudomonas aeruginosa*, and the strict anaerobe, *Streptococcus lactis*. Respiratory inhibition is also an early metabolic dysfunction in the sequence of events attending progressive HOCl oxidation of the aerobic bacteria[135,138] that may contribute to limitation of their energy-transducing capabilities.[269]

The mechanisms of chemical reactions of N-chloramines are most likely analogous to HOCl.[218] The microbicidal mechanisms are not necessarily identical, however, because the N-Cl bond is substantially less reactive towards potential biological nucleophiles than HOCl, and differing solubilities might affect the distribution of oxidants over various microdomains within the bacterial cell, hence the accessibility of potentially lethal sites. The dramatic differences in cytotoxic potential of hydrophilic and hydrophobic amines[136,140] and the differing reactivities of chloramines and HOCl towards H_2O_2[217,218] are previously cited examples of these effects. Analytical schemes based upon reactivity differences towards a common set of reductants have also been developed to distinguish among HOCl and several N-chlorinated amines in solution.[270] Thomas and co-workers estimated the effective concentrations of primary amino groups in serum and the neutrophil cytosol are 0.05 and 0.15 *M*, respectively,[270] and, on this basis, have suggested that the cytotoxic reactions of MPO-generated HOCl *in vivo* must be mediated by lipophilic chloramines (see Figure 5, reaction 6).[271] In support of this proposal, they have shown that stimulated neutrophils in suspension oxidize erythrocyte oxyhemoglobin in an MPO-dependent reaction to its ferri, or met, form without prior cell lysis, a reactivity pattern consistent with oxidation by NH_2Cl, but not HOCl.[141,272] Addition of NH_4^+ or lipophilic amines also potentiates slightly the bactericidal effects of the MPO-H_2O_2-Cl^- system,[136-139] and chloramines have been shown to accumulate in suspensions of stimulated neutrophils.[43,273] The differences in succinate uptake in HOCl-oxidized and MPO-H_2O_2-Cl^--inactivated *E. coli* noted previously[138,258] may prove to be a second manifestation of these reactivity differences since, in general, lipophilic chloramines at bactericidal levels are less effective at inhibiting nutrient transport than HOCl.[138] However, there is some counter-evidence that indicates that chloramines are not necessarily the ultimate oxidants in phagocytic chlorination reactions. In the erythrocyte model, others have reported that if cell contact with neutrophils were forced by cocentrifugation of the suspensions, relatively high concentrations of NH_4^+ were required to retard erythrocyte lysis,[274] and the preferential lysis of erythrocytes to neutrophils was consistent with oxidation by HOCl, but not NH_2Cl.[275] These results suggest that binding of MPO to the target surface may effectively sequester the reaction site from potential amine reactants, a suggestion which is also supported by studies demonstrating that neutrophils on subendothelial matrices can protect secreted elastase from inactivation by the α-1-proteinase inhibitor peptide in solution.[238] It is also apparent from reaction kinetics that a variety of nucleophilic compounds undergoing oxidation in the bacterial envelope, including sulfhydryl groups, react directly with HOCl since many of these compounds react considerably more slowly with lipophilic chloramines than is observed in MPO-catalzyed reactions.[231] The kinetic competency of chloramines in the physiological reactions is itself open to question because quenching experiments indicate that bacteria must be exposed to chloramines for several minutes before they are killed,[138] which borders on the upper limit of estimated intraphagosomal rates.[12,13,15] Careful delineation of reactivity patterns and rates in neutrophil or cell-free MPO-catalyzed reactions in comparison to the chemical oxidants should provide a basis for distinguishing between HOCl and chloramines as oxidants. In this context, it might be noted that bactericidal concentration levels of NH_2Cl, like HOCl, cause nearly complete hydrolysis of ATP phosphoanhydride

bonds[138] and essentially complete inhibition of the sugar:PEP phosphotransferase system in *E. coli*, *P. aeruginosa*, and *S. lactis*,[373] suggesting a common bactericidal mechanism for the two oxidants.

Endogenous aldehydes formed by decomposition of N-chloramine acids (see Figure 5, reaction 7) had also been proposed as microbicidal toxins,[232] although they can be excluded on the grounds that their formation rates[16,247,276] are far slower than intraphagosomal killing[12,13,15] and that the toxicities of aldehydes are relatively low.[8]

The reactions of monopositive chlorine species have been discussed in terms of two-electron oxidation or atom transfer processes which would not be expected to generate free radical products. Recently, low levels of aromatic free radicals or radical cations have been detected when the parent compounds are exposed to HOCl, taurine chloramines, or chloramines produced in neutrophil-stimulated reactions.[277] The researchers suggested that these reactions might be important in drug toxicity or chemical carcinogenesis; they might also contribute to the mutagenic activity of chloramines.[278,279]

B. REACTIVE OXYGEN INTERMEDIATES

1. Superoxide Anion and Hydrogen Peroxide

The immediate products of the respiratory burst, per se, are not generally regarded as major contributors to leukocytic cytotoxicities, although there is considerable discussion that the products of metal-catalyzed superoxide reduction of H_2O_2 may constitute an important microbicidal mechanism (see Figure 5, reaction 2). The redox chemistry of superoxide is strongly dependent upon its reaction environment.[280] Although it behaves as a weak acid in aqueous solution, the anionic form is strongly hydrogen-bonded. The aqueous superoxide one-electron reduction potential is relatively low, presumably because the product peroxide dianion must be stabilized by proton acquisition; consistent with this interpretation, perhydroxyl (HO_2) is a stronger oxidant than O_2^- by about 130 mV.[281] In aprotic media, O_2^- is stable with respect to both reduction and disproportionation, again, a consequence of the instability of the product O_2^{2-} ion.[280] In this environment O_2^- is a powerful nucleophile and can effect net oxidation of substrates by complex mechanisms that appear to involve proton abstraction, disproportionation of HO_2, and oxidation of the substrate anion by O_2. Superoxide in aprotic media is also a good reductant, another property attributable to the absence of anion-stabilizing strong solvation. Because it is strongly hydrated, aqueous O_2^- is a poorer nucleophile, but will oxidize bases containing abstractable protons, including various biological compounds.[1] Superoxide will also undergo rapid one-electron oxidation and/or reduction with metal ions possessing appropriate potentials, including iron, copper, and manganese; these reactions form the basis for O_2^- disproportionation by SOD metalloproteines, which appear to have evolved carefully tuned potentials for simultaneous optimization of the two half-reactions.[282]

The aqueous chemistry of O_2^- is, therefore, limited, particularly since other reactions must compete with rapid spontaneous dismutation of the ion. In the microheterogeneous media of which cells are made, the strongly solvating O_2^- should partition almost exclusively in aqueous microdomains so that opportunity for aprotic reactions will be minimized. This expectation is realized in the neutrophil, at least, since (near)-stoichiometric conversion of O_2 to O_2^- is measured in cells activated with soluble stimuli (see Section II.D). The observation that an endogenous O_2^--forming diaphorase reaction confers autotoxicity upon the bacterium *Streptococcus sanguis* in inverse relation to its intracellular manganous SOD content suggests that O_2^- may not be innocuous under all conditions.[283] Direct involvement of O_2^- in the bactericidal reactions was inferred from the lack of protection by infused dimethyl sulfoxide, a hydroxyl radical scavenger. In view of the possible sequestration of ·OH at reactive target sites (see Section III.B.3.d), additional evidence would seem desirable.

Hydrogen peroxide is a commonly used disinfectant and is toxic to bacteria in high

concentrations. Nonetheless, they are often tolerant of lower levels of H_2O_2, presumably because endogenous catalases and peroxidases dissipate the oxidant. This tolerance is most evident in aerobic microorganisms that produce H_2O_2 as their respiratory endproduct and in qualitative correlations of the level of intracellular activities of H_2O_2-scavenging reactions with susceptibilities to inactivation by H_2O_2[8] or with virulence of pathogens.[284] One also infers from the enormous potentiation of bacterial toxicity by MPO or trace metal ions capable of catalyzing Fenton-type reactions (see Figure 5, reaction 3) that the concentrations of phagocyte-generated H_2O_2 are sublethal in most instances. However, parasitic worms and certain tissue cells appear highly susceptible to H_2O_2, based upon the protective effects of catalase on phagocytic killing. Additional discussion of O_2^- and H_2O_2 toxicities can be found in the reviews by Klebanoff[1] and Klebanoff and Clark.[8] One point to emphasize is that demonstration of participation of H_2O_2 in cytotoxic reactions does not establish its microbicidal role. Hydrogen peroxide taken up by cells could either react directly with endogenous substrates or serve as an intermediary species, e.g., in Fenton-type reactions with endogenous reductants.

2. Singlet Oxygen

As with H_2O_2 oxidation by chlorine (+1) compounds (see Table 1), several reactions of oxygen species of intermediate valency are sufficiently exothermic to form O_2 in its $^1\Sigma$ and/or $^1\Delta$ excited electronic states.[285] These include the O_2^- and H_2O_2 disproportionation reactions,

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (6)$$

and the one-electron reduction of H_2O_2 by HO_2, the acidic form of superoxide (see Figure 5, reaction 2):

$$HO_2 + H_2O_2 \rightarrow H_2O + \cdot OH + O_2 \quad (7)$$

Reduction by the basic form, O_2^-, is not sufficiently energetic at pH 7 to produce O_2 in an electronically excited state.[281] Furthermore, reaction 7 is slow[286,287] unless catalyzed by metal ions,[288,289] which are thought to function in the cycle,

$$HO_2 + M^{n+} \rightarrow M^{n-1} + O_2 + H^+ \quad (8)$$

$$H^+ + M^{n-1} + H_2O_2 \rightarrow M^{n+} + H_2O + \cdot OH \quad (9)$$

commonly called the metal-catalyzed Haber-Weiss reaction or the superoxide-driven Fenton meachanism. In the absence of chain-propagating steps involving O_2^- or ·OH, catalysis will be efficient only if the metal ion has a reduction potential between those of O_2 and H_2O_2, i.e., $-0.16\ V < E^{\circ\prime} < 0.46\ V$ at pH 7, assuming metal ion reduction by HO_2. Since the thermodynamic potential for the uncatalyzed reaction is barely sufficient to form $^1\Delta O_2$ ($\Delta G^{\circ\prime} \simeq 21$ kcal/mol, $\Delta E(^3\Sigma \rightarrow {}^1\Delta) \simeq 22$ kcal/mol) and the thermodynamic constraints imposed by the need for rapid cycling of the metal ion are such that only oxidants less powerful than H_2O_2 will be effective, the overall driving force for metal ion reduction by HO_2 (see reaction 8) will generally be insufficient to form electronically excited O_2. Only when the catalyst reduction potential is very close to the H_2O_2 one-electron potential will this be possible, and then the reaction requires high selectivity for HO_2 over O_2^-, which is in 10^3-fold excess at physiological pH values. As these circumstances are improbable, it is unlikely that the catalyzed reaction will yield $^1\Delta O_2$.

The $^1\Sigma O_2$ excited state is rapidly deactivated to the $^1\Delta$ state in aqueous solution at rates that preclude any substantive bimolecular chemical reaction,[290] but $^1\Delta O_2$ is more long-lived, with decay half-time of the order of a few microseconds.[291] $^1\Delta O_2$ is toxic to microbial cells, at least when exposure is maintained by continuous excitation, e.g., as in dye-sensitized energy transfer processes.[292] It is conceivable, therefore, that intraphagosomal reactions, including H_2O_2 formation in the respiratory burst (see reaction 5), catalatic inactivation of H_2O_2 (see reaction 6), a sequence involving MPO-catalyzed peroxidation of Cl^- followed by nonenzymatic oxidation of H_2O_2 (see Figure 5, reactions $1 \rightarrow 4 \rightarrow 5$), or metal-catalyzed H_2O_2 oxidation of HO_2 (see reactions 8 and 9) could contribute to the microbicidal action of neutrophils by serving as sources for $^1\Delta O_2$.

The very recent development[293,294] of near-infrared-sensitive spectrophotometers for detecting photons emitted during radiative decay of $^1\Delta O_2$ to its $^3\Sigma$ electronic ground state has aided considerably in determining yields of excited oxygen in these reactions. The $(0,0)^1\Delta \rightarrow {}^3\Sigma$ transition gives a line at 1270 nm that is diagnostic for $^1\Delta O_2$ and considerably more intense than the red dimol emission bands previously used in chemiluminescence studies, particularly at low concentrations since the intensity of the latter depends upon the square of the $^1\Delta O_2$ concentration.[295] Using infrared spectrophotometers, it has been shown that the yields of $^1\Delta O_2$ formed by spontaneous dismutation of O_2^-[296] and catalase-catalyzed disproportionation of H_2O_2[297] are negligible and that $^1\Delta O_2$ formed in MPO-H_2O_2-halide enzymatic systems are halide- and pH-dependent.[298] Singlet oxygen formation with Br^- approached the theoretical maximum at pH 5, but was only about 20% at pH 4 and negligible at pH 7 with Cl^- as substrate.[298,299] The results for the disproportionation reactions confirm results from earlier studies[280,300,301] using primarily chemical trapping and dimol luminescence measurements that $^1\Delta O_2$ was not formed in these reactions. These methods have also been used to establish that SOD-catalyzed O_2^- dismutation does not produce $^1\Delta O_2$.[302] The results for MPO-H_2O_2-Cl^- differ from reactions of HOCl with H_2O_2 in that the reaction between the reactive pair, HOCl and HO_2^-, which predominates above pH 7, gives $^1\Delta O_2$ in 100% yield.[217,303] Using the near-infrared spectrophotometer, it has also been established that $^1\Delta O_2$ yields are large in more acidic media where other reaction pathways predominate.[298] The basis for the difference in MPO-mediated H_2O_2 oxidation and chemical reaction with HOCl is not understood; one possibility is that the MPO chlorin provides heavy-atom quenching in the system.

Even if MPO-catalyzed reactions leading to O_2 formation gave exclusively the singlet excited molecule, this reaction is not expected on first principles to be important in the phagosomal milieu because HOCl is relatively unreactive towards H_2O_2.[227,231] For example, at pH 7 the relative reactivity of HOCl for H_2O_2 and primary amines is given from the reaction rate laws by the ratio, $k[H_2O_2]/k'[RNH_3^+]$ (although these are not the true reactants, i.e., HO_2^- and RNH_2, in this form the ratio k/k' takes proper account of their differing basicities). The appropriate rate constants k and k′ are $3 \times 10^3\ M^{-1}\ s^{-1}$ [217] and about $10^7\ M^{-1}\ s^{-1}$,[225] respectively. Accepting Thomas et al.'s estimation[271] of 0.05 *M* for the effective amine concentration in serum and assuming that maximal accumulation of H_2O_2 is about 1 m*M*, the calculated ratio is about 10^{-5}. Thus, H_2O_2 should not be competitive for MPO-generated HOCl with other endogenous substrates, here represented by primary amines, and the opportunity for formation of significant quantities of $^1\Delta O_2$ by this reaction probably does not arise in the phagosome. Consistent with these relative reactivities, it has recently been found that taurine chlorination by HOCl is quantitative even in the presence of equimolar H_2O_2.[304]

Phagosomal formation of $^1\Delta O_2$ has been probed using the near-infrared spectrophotometer[299] and with phagocytosable particles containing cholesterol, which gives a hydroxylated product distinct from the products formed by radical autoxidation.[305] No enhanced luminescence was observed at 1270 nm, and the isolated cholesterol oxidation

product distribution was consistent with reaction with HOCl, but gave no compounds that would arise from $^1\Delta O_2$ trapping. These studies and the results with the individual enzymatic reactions comprising primary oxidative processes in stimulated neutrophils convincingly establish that $^1\Delta O_2$ is not an important phagosomal microbicidal agent, despite occasional strong assertions to the contrary.[306]

Studies designed to gain information on the possible involvement of short-lived or highly reactive oxygen intermediates such as $^1\Delta O_2$ and OH have relied heavily on the use of enzymatic and chemical scavengers and/or chemical trapping agents. From the patterns of system response upon adding these compounds, one infers the extent of participation of oxygen intermediates that are reactive with the probe. For example, the inhibition of bactericidal action in the presence of either SOD or catalase is often taken as evidence for toxicity conferred by OH or similar oxidants formed in Fenton-type reactions with O_2^- as the reductant (see Figure 5, reaction 2). Similarly, inhibition by catalase, but not SOD, is taken as evidence for either direct participation of H_2O_2 or peroxidase-catalyzed reactions. As discussed by G. Rosen and collaborators,[307] it is also possible for SOD to accelerate ·OH formation in Fenton-type reactions under conditions where H_2O_2 formation by the system is rate-limiting, thereby introducing ambiguity into interpretation of the reactivity patterns. Chemical perturbation experiments are problematic on at least two additional counts. First, much of the evidence is negative, and the absence of reaction in physiological systems has alternative explanations, e.g., sequestration of the reaction environment from the probe. Second, particularly with chemical probes, reaction specificity is often low, so that the identity of the reactant is not unambiguously determined. This is obviously not a great problem if one is attempting to distinguish chlorinating compounds from oxygen-based oxidants, and the probe gives stable chlorinated products, e.g., fluorescein, but several probes originally thought to give unique products with particular oxygen intermediates have subsequently been found to react with others, making them considerably less useful as diagnostic tools. The interpretative difficulties are well illustrated by the reaction of diphenylfuran (DPF) with MPO-H_2O_2-Cl^-. Formation of the product *cis*-dibenzoylethylene (DBE) was inhibited by a number of compounds capable of physically quenching $^1\Delta O_2$, and the DBE yield increased when the reaction was run in D_2O.[308] DBE is formed upon Diels-Alder addition of $^1\Delta O_2$ to DPF, and the enhanced rate in D_2O is anticipated on the basis of the significantly greater $^1\Delta O_2$ lifetime in this solvent.[301] However, HOCl[309] and oxy radicals[310] also oxidize DPF to DBE. Aromatic chlorination by HOCl occurs with an inverse solvent isotope effect,[311] presumably because the reaction involves a preequilibrium protonation step, and the same property that makes the protective agents good quenchers of $^1\Delta O_2$, i.e., their nucleophilicity, also makes them highly reactive towards HOCl[227,231,312] and other chemical oxidants. The two alternative mechanisms that can account for the data, physical quenching of $^1\Delta O_2$ or chemical reaction of the probes with HOCl, are distinguishable in this instance by the stoichiometric amount of O_2 produced in the reaction. Quantitative determination of O_2 yields has established that the protective action of the probes was, in fact, due to chemical scavenging of HOCl produced by the MPO-H_2O_2-Cl^- system. Although chemical and enzymatic trapping studies can provide valuable information on reactive intermediates, it should be recognized that the data interpretation is rife with pitfalls.[189]

3. Hydroxyl Radical and Related Species

a. Nature of the Reactive Intermediates

Transition metal ions often enhance oxidations by H_2O_2 in reactions that are thought to involve the intermediacy of hydroxyl radical or, alternatively, reactive metal higher oxidation states.[313] In physiological systems, these reactions must be catalytic with respect to the metal ion since the free ion concentrations in biological fluids are small.[314] One possible source of ·OH is the metal-catalyzed Haber-Weiss reaction (see reactions 8 and 9), for which O_2^-

is the ultimate electron donor to H_2O_2.[288,289] Analogous cycles can be written in which H_2O_2 or endogenous reductants, e.g., ascorbate,[314] replace O_2^- as the one-electron donor. The catalyzed simultaneous one-electron oxidation and reduction of H_2O_2 constitutes a mechanism for its disproportionation that involves the intermediacy of O_2^- and OH.[313] As discussed in Section III.B.2, efficient catalysis by this simple mechanism requires that the metal ion reduction potential lie between that of the reductant and the H_2O_2/OH couple.[281] However, if the reactive intermediates initiate chain reactions with substrates, the range of acceptable potentials is extended somewhat since an energetically unfavorable (hence, slow) initiation step can lead to appreciable net fluxes if the chain length is long.[315] Alternative schemes that do not lead to ·OH formation involve peroxo ligation or two-electron oxidation of the metal ion, e.g.,[313]

$$Fe^{2+} + H_2O_2 \rightarrow \begin{cases} FeOOH^+ + H^+ \\ \text{or} \\ FeO^{2+} + H_2O \end{cases}$$

The ferryl ion, formed by heterolytic cleavage of H_2O_2, is analogous to compound II intermediates in peroxidase-catalyzed reactions. Iron and copper are metals that, as biological complexes, could engage in cellular reactions of these types. One-electron reduction potentials for cupric complexes are typically 0.1 to 0.2 V,[281] which is appropriate for the Haber-Weiss reaction. The standard reduction potential for aqueous Fe^{3+} is 0.77 V which, if retained at pH 7, would not support ·OH production by Fe^{2+} reduction of H_2O_2. However, to prevent Fe^{3+} precipitation as a hydroxy polymer, it is necessary that it be coordinated to strongly Fe^{3+}-binding ligands, which also lower its reduction potential. For polyaminocarboxylato complexes, the potentials are shifted within the catalytically active range,[281] e.g., for Fe(EDTA), $E^{\circ\prime} = 0.12$ V. Lowered reduction potentials are also expected upon deprotonation of bound H_2O or phosphate ligation.[316] Thus, although two-electron oxidation of Fe^{2+} and Cu^+ by H_2O_2 to their formal (IV) and (III) valencies, respectively, may be energetically less demanding[317] than one-electron oxidation yielding ·OH, there appears to be no *a priori* reason to exclude the latter reaction on thermodynamic grounds.

Several kinetic approaches have been tried in an attempt to distinguish between ·OH and the alternative reactive intermediates. Winterbourn[318] and Winterbourn Sutton[319,320] found from competition experiments that the relative reactivities of nominally OH-reactive compounds in Fe(EDTA)-catalyzed Haber-Weiss reactions were identical to values predicted from measured rate constants for their reaction with OH; other complexes, including presumably phosphate-bound ions, gave different product ratios, implicating other reactive intermediates. One study suggested that a potentially biologically significant iron complex formed with xanthine oxidase, which was proposed to react directly with oxidizable substrates at the iron-binding site.[320] Catalysis of H_2O_2 oxidation by xanthine oxidase-bound iron had also been suggested from earlier studies.[321] The possibility was also discussed[320] that failure to recognize this reaction might account for discrepancies in the literature concerning the relative effectiveness of various ferrous complexes as Haber-Weiss catalysts since xanthine oxidase-catalyzed reactions have frequently been used as a source of O_2^- and H_2O_2 in these studies. On the other hand, stopped-flow kinetic analyses suggest that the reactions of H_2O_2 with Fe(EDTA)[322] and other ferrous polyaminocarboxylato ions[323] may be complex and involve formation of both ·OH and other intermediates with clearly distinguishable reactivities, suggested to be ferryl complexes. Similarly, formation of Cu^{3+} rather than ·OH was suggested in studies of radiolytically generated Cu^+ with H_2O_2 from the uncharacteristically short chain length for subsequent methanol oxidation to formate,[324] and the kinetics of decay of Cu(*o*-phen)$_2^+$ in the presence of H_2O_2 and oxidizable compounds were interpreted to

indicate initial formation of a copper(I)-peroxo complex, followed by decomposition by two competing pathways with formation of either ·OH or Cu(III).[325]

b. Trapping Studies

The spin trap 5,5′-dimethylpyrroline-N-oxide (DMPO) has been used by several groups to detect hydroxyl radical formation by stimulated neutrophils.[307,326,327] This compound reacts with both ·OH and O_2^- to give nitroxides that are distinguishable on the basis of magnitudes of the hyperfine coupling constant of the α-carbon proton.[307] Nearly exclusive formation of the DMPO-OH was observed upon particulate stimulation of neutrophils in all of the reported studies. The reaction was completely inhibited by SOD, but catalase had little effect upon trapping yields. The soluble stimulant PMA elicited formation of both DMPO-OH and DMPO-O_2H.[307,326] The peroxidase- and catalase-inhibitory anions, CN^- and N_3^-, enhanced yields,[307] and the hydroxyl radical scavenger mannitol decreased yields. Adduct formation was greater for stimulated MPO-deficient than normal neutrophils and was unobserved for CGD neutrophils.[327] Based upon these patterns, two groups inferred that ·OH was formed during the respiratory burst.[326,327] However, it was subsequently shown that the DMPO-O_2H adduct is easily reduced to DMPO-OH, and decomposition occurs with partial release of ·OH into the medium.[307] The data are therefore interpretable by mechanisms in which DMPO-O_2H is an obligatory precursor to DMPO-OH, which provides a convenient explanation for the absence of inhibition of trapping by catalase. Presumably, then, enhanced trapping yields obtained with MPO-deficient neutrophils are a consequence of the prolonged respiratory activity of these cells. In any event, any conclusions that might be drawn from these studies are compromised by the instability of the DMPO-O_2H adduct. The inability to directly trap ·OH, as concluded by Rosen and associates,[307] should not be taken as evidence against ·OH formation since the possibility remains that it could form and react at sites sequestered from the trapping agent. Also, indirect evidence has been given for ·OH formation by stimulated neutrophils in the presence of added Fe^{3+} and DMSO. Detection in this instance involves reaction of DMSO-derived $CH_3^{\cdot}$ with DMPO.[307]

Chemical scavengers of ·OH have also been extensively used in probing for its involvement in reactions of stimulated leukocytes, although nearly all of these compounds are now recognized as nonselective with respect to their reactivities toward other neutrophil-generated oxidants. In addition to the previously mentioned singlet oxygen- and HOCl-reactive compounds (Section II.B.2), thiourea, dimethylthiourea, dimethylsulfoxide, oxypurinol, the ethylene-producing 2-oxo-4-thiomethylbutyric acid (DMB), and benzoic acid have all been shown to react with HOCl or MPO-H_2O_2-Cl^- assay systems;[328-331] 3-methylthiopropanal (methional) is reactive toward a variety of alkoxy radicals;[332] and KMB also releases ethylene upon reaction with $^1\Delta O_2$.[330] Of the commonly used chemical scavengers, only sugars[331,333] and the simple alcohols appear to be selective for products of metal-catalyzed H_2O_2 reduction.

The difficulties in interpreting chemical trapping studies are illustrated in the two following examples. In one study,[334] neutrophil killing of serum-opsonized *S. aureus* was partially inhibited by thiourea, dimethylthiourea, and DMSO, but not mannitol, urea, dimethylurea, SOD, or catalase. These results were interpreted in terms of ·OH involvement but, based upon our current understanding of probe reactivities, are more consistent with scavenging of HOCl and the simultaneous expression of nonoxidative bactericidal mechanisms (to account for the lack of SOD or catalase protection). Klebanoff and Rosen clearly demonstrated[330] a MPO dependence for ethylene formation from KMB by comparing reactivities of normal and MPO-deficient neutrophils, which gave <10% of the product from the normal cells. Comparable yields could be obtained by adding MPO to the deficient cells. The reactions required addition of EDTA for expression of optimal yields, suggesting possible involvement of metal-catalyzed Haber-Weiss reactions. Ethylene formation in the cell-free MPO-H_2O_2-Cl^- system was also enhanced by EDTA. Although the presence of trace amounts

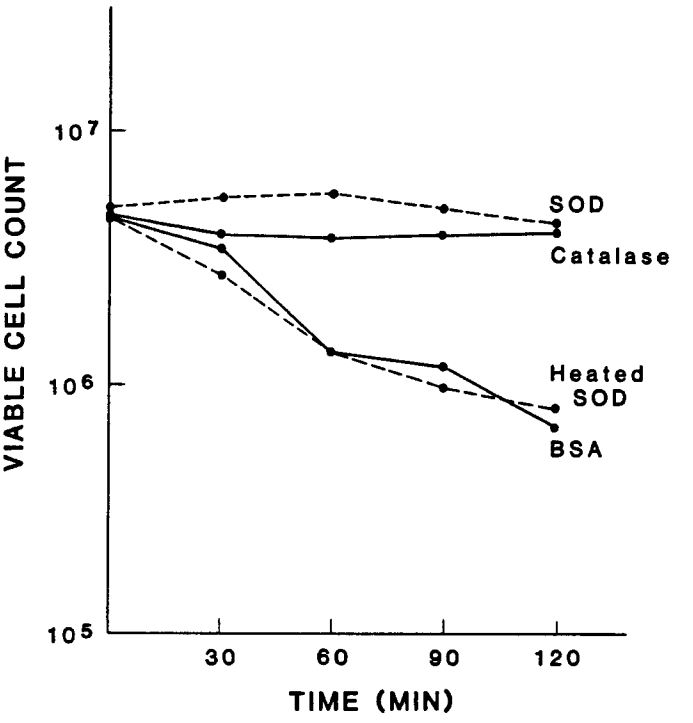

FIGURE 13. Phagocytic killing of *S. aureus* by human neutrophils. The effect of cophagocytosis of latex particles containing bound enzymes is shown. (From Hurst, J. K., *Oxygen Complexes and Oxygen Activation by Transition Metals*, Martell, A. E. and Sawyer, D. T., Eds., Plenum Press, New York, 1988.)

of Fe^{3+} could not be excluded, the patterns of inhibition of the DMB reaction by added chemical traps were consistent with oxidation primarily by HOCl. However, a most striking observation was that the model reaction could be nearly totally inhibited by SOD. Since SOD should not inhibit HOCl formation, this result defies simple interpretation in terms of parallel reactions of noninteracting oxidants and therefore portends mechanistic complications.

These examples should not be taken to indicate that chemical trapping experiments always give inconsistent results. For example, Henderson and Klebanoff[335] have reported that leukotriene degradation by normal neutrophils stimulated by the calcium ionophore A23187 was inhibited by catalase and N_3^-, but not SOD, mannitol, or ethanol, although degradation by stimulated MPO-deficient neutrophils was inhibited by all these reagents. These patterns are consistent with respiratory burst-generated H_2O_2 consumption primarily by peroxidase-catalyzed pathways in normal neutrophils, but by the metal-catalyzed Haber-Weiss reaction in the absence of peroxidase. Control experiments established that leukotrienes were susceptible to oxidation by both the MPO-H_2O_2-Cl^- and acetaldehyde-xanthine oxidase-Fe^{3+} assay systems. The data support a commonly held viewpoint that MPO-dependent killing is predominant in normal neutrophils but that other, possibly redundant, oxidative mechanisms come into play in the absence of the peroxidase.[1,8] However, results from an early, careful study by Johnston and co-workers[336] suggest that the MPO-dependent mechanisms are less important in intraphagosomal killing. These researchers induced co-phagocytosis of bacteria and enzyme-coated latex spheres and monitored rates of subsequent loss of viability. Typical results for *S. aureus* are shown in Figure 13. Latex-adsorbed SOD or catalase nearly completely protected the bacteria, whereas heat-denatured SOD or a non-

catalytic serum protein did not influence killing rates. Some protection was also afforded when mannitol was included in the reaction medium. Protection by SOD calls to question the primacy of MPO-mediated bactericidal mechanism since, as noted previously, catalysis of H_2O_2 formation should not inhibit the chloride peroxidation reaction. Thus, a paradoxical situation arises in which considerable evidence indicates intraphagosomal generation of the potent microbicide, HOCl, but its toxicity is apparently not expressed. Simultaneous activation of both oxidative pathways in PMA-stimulated normal neutrophils is suggested from observations that mannitol partially inhibits benzoate decarboxylation.[331]

c. Role of Lactoferrin

The reduction potential of serum transferrin has been calculated at $E^{\circ\prime} = -(140$ to $320)$ mV from estimates of the Fe^{3+} and Fe^{2+} ion binding constants.[337] Direct measurement[338] by equilibration with methyl viologen radical cation gave $E^{\circ\prime}_{7.3} = -400$ mV at 25°C with $[HCO_3^-] = 21$ m*M*. The discrepancy can probably be attributed[338] primarily to use of the $Fe^{3+/2+}$ standard couple in the calculations, rather than an undoubtedly lower, but unknown, value appropriate to physiological conditions. A very similar $E^{\circ\prime}$ is anticipated for lactoferrin, which binds Fe^{3+} even more strongly than do transferrins. As such, ferric lactoferrin would not be reduced by O_2^- or, for that matter, any physiological reductants and, therefore, would not be expected to catalyze the one-electron reduction of H_2O_2.

Results from model studies using various reductants and H_2O_2 are in general concurrence that native lactoferrin, which as isolated is primarily demetalated, inhibits iron-catalyzed ·OH formation, presumably by scavenging contaminant (or added) Fe^{3+} ion. Iron-saturated lactoferrin has been reported to stimulate ·OH formation,[339] although this observation has not always been confirmed in subsequent studies. In fact, the majority opinion among actively involved researchers is that iron-saturated lactoferrin is without effect upon these reactions in neutral solutions. The paper by Aruoma and Halliwell[340] provides a thorough current review of this controversy.

The question of biological sources of iron to saturate apolactoferrin has also been addressed. Stimulated neutrophils have been shown[341] to release iron from ferritin storage granules by O_2^- reduction of encapsulated Fe^{3+}, and H_2O_2 and other organic peroxides appear capable of degrading hemoglobin with release of iron.[342] As previously mentioned, products of the MPO-catalyzed chloride peroxidation reactions release bacterial iron.[262] Studies with a model system in which stimulated neutrophils were used as a source of O_2^-, H_2O_2, and lactoferrin and exogenous Fe^{3+} was added as its diethylenetriaminepentaacetate complex ion to catalyze ·OH formation gave evidence only for lactoferrin inhibition of ·OH formation. Continued ·OH production was detected only upon removal of lactoferrin by conjugation with antibody or overwhelming its binding capacity with excess Fe^{3+} ion.[307]

Neutrophil lysis of eukaryotic cells[343,344] and neutrophil[345] and macrophage[346] killing of microbes have been reported to be enhanced by lactoferrin. Part of this effect is apparently due to increased microbial phagocytosis[346] or adhesion of the neutrophil and target cells[345,346] since lactoferrin is cationic and binds strongly to the negatively charged cell surfaces. Apolactoferrin and the iron-bound form appear equally effective in this capacity. However, cell lysis or microbial killing was potentiated only when the lactoferrin contained iron.[343,344,346] This effect was lactoferrin-specific since addition of Fe^{3+} alone to lactoferrin-deficient systems did not enhance the lytic or cidal reactions.[343,344] The results suggest that membrane-associated lactoferrin may behave quite differently from the solubilized metalloprotein. It has been proposed that bound ferric lactoferrin targets specific membrane sites for H_2O_2-mediated damage.[343,344] This would seem possible if membrane association reduced the Fe^{3+} binding affinity by some means, e.g., localized acidification within the sequestered site and/or loss of coordinated HCO_3^-, thereby increasing its reduction potential to allow reaction with endogenous reductants.

d. Biological Reactivity

Hydroxyl radical is a powerful oxidant that reacts with virtually all biomolecules at rates approaching encounter-controlled limits;[333,347] ·OH therefore exhibits little selectivity toward its reaction partner. This circumstance presents a conceptual problem because one of the tenets for effective microbicidal action is that the toxins are selective for vulnerable cellular sites. Uncontrolled ·OH formation in the aqueous milieu of the phagosome would not allow the oxidizing potential to be directed efficiently against susceptible sites on captive bacteria. Recognition of this problem has led to the very appealing proposal[348] that the reactions are *de facto* site-specific because the redox metal ions that are obligatory catalysts for ·OH formation from H_2O_2 are often located at biological sites involved with essential cellular functions. Accordingly, ·OH generated at these sites is then directed against targets in the immediate vicinity with consequent loss of function. Thus, it is envisioned that H_2O_2 and O_2 or endogenous one-electron reducing agents are the true reactants and selectivity is conferred upon the system by the location of the catalyst. Since H_2O_2 is permeable to cells, the reaction sites could be intracellular. The reduction potential of ferryl ion may be as much as 1 V less oxidizing than ·OH.[317] It might therefore exhibit greater selectivity in its oxidative reactions, but since biological iron is predominantly macromolecularly associated, reactions involving ferryl or ferric peroxy intermediates should also be site-directing.

Experimental evidence advanced in support of this concept generally includes observations that efficient reaction in metal-catalyzed H_2O_2 oxidations coincides with Fe^{3+} or Cu^{2+} binding and that chemical scavengers of ·OH and/or enzymatic scavengers of its precursors are less effective than expected from their behavior in homogeneous systems. Recent demonstration of these effects include enhanced toxicities toward viruses[348] and bacteria[349,350] containing bound Fe^{3+} or Cu^{2+}, degradation of DNA containing intercalated $Cu(o\text{-phen})_2^{2+}$,[351] and inactivation of several enzymes upon nonspecific binding of Cu^{2+} or Fe^{3+}.[352-355] A quantitative kinetic study of chemical quenching of Fe^{2+}-promoted O_2 oxidation of deoxyribose also suggested site-directed reaction,[355] although the effects of various ligands upon Fe^{3+}-catalyzed damage to phospholipid membranes by H_2O_2 and O_2^- was not entirely in accord with a simple mechanism equating reactivity with binding.[357]

e. Reactions with Microbes

The few studies undertaken to probe intracellular killing by Fenton-type mechanisms have not given entirely self-consistent patterns of reactivity. Neutrophil oxidation of linoleyl alcohol adsorbed to phagocytosable latex beads was detectable only if the beads also contained adsorbed Fe^{3+} ion,[358] suggesting that phagosomal ·OH formation requires exogenous, presumably bacterial, sources of iron. Preincubating *S. aureus* in iron-rich media increased both intracellular Fe^{3+} concentration levels and susceptibility to killing by H_2O_2.[359] The data suggest intracellular sites for the lethal reactions, which is supported by other studies with several strains of *E. coli* implicating DNA as the target site.[360] Specifically, at H_2O_2 concentration levels roughly comparable to those achievable in the phagosome, mutant *E. coli*-lacking enzymes required for DNA recombinational repair were more susceptible to killing than wild type, and exposure of wild-type organisms to sublethal levels of H_2O_2 induced synthesis of both DNA repair enzymes and catalase, with subsequent protection of the bacteria at otherwise lethal H_2O_2 concentration levels. On the other hand, neither were iron-loaded *S. aureus* more susceptible[361] nor were *E. coli* induced to synthesize increased cytosolic concentration levels of catalase and SOD less susceptible[362] to killing by neutrophils than normally grown cells. In the latter studies, addition of catalase or SOD to the external medium inhibited neutrophil inactivation of the bacteria, consistent with the earlier observations of Johnston and co-workers[336] and confirming a microbicidal role for metal-catalyzed Haber-Weiss reactions. The results also imply that the lethal reactions occur on the bacterial surface.

Two studies suggest that the target sites in Cu^{2+}-promoted killing are located within the bacterial cytoplasmic membrane and hence are probably also extracellular in origin. In a preliminary account of Cu^{2+}-mediated paraquat toxicity to *E. coli*, Chevion and co-workers reported that the ability to retain K^+ and leucine was lost and the ATP content "diminished" roughly in parallel with viability, but little damage beyond single-strand breaks was found to the DNA.[363] Earlier, inhibition of *Paracoccus denitrificans* growth attending addition of Cu^{2+} or several Cu^{2+} complexes with bidentate nitrogen heterocycles to the medium was attributed to inhibition of microbial respiration.[364] Specifically, selective inhibition of DNA, RNA, or protein synthesis did not occur, nor was the H^+/O phosphorylation ratio altered, indicating maintenance of respiration-driven energy transduction. However, the extent of growth inhibition coincided with loss in respiratory rates using either O_2 or nitrate as terminal electron acceptors. Inactivation was suggested to occur before cytochrome b, which is the branchpoint for the aerobic and anaerobic pathways, although reaction at multiple sites could not be excluded.

C. INTERCONVERSIONS BETWEEN REACTIVE OXYGEN SPECIES AND CHLORINATING AGENTS

Hill and Okolow-Zubkowska proposed[365] that ·OH can be generated by one-electron reduction of HOCl by O_2^-, i.e.,

$$HOCl + O_2^- \rightarrow \cdot OH + O_2 + Cl^-$$

the plausibility of which has been established in kinetic studies[366] for which a bimolecular rate constant of $k = 7 \times 10^6\ M^{-1}\ s^{-1}$ was measured at 23°C. This or similar reactions might account for some of the anomalies observed in trapping experiments, e.g., the inhibition by SOD of MPO-dependent ethylene formation from KMB.[330] However, Winterbourn has presented evidence[367] that MPO strongly inhibits ·OH formation in a cell-free xanthine oxidase-hypoxanthine-Fe(EDTA) model reaction, indicating that MPO competes effectively with the ferrous complex for H_2O_2, and possibly with the ferric complex for O_2^-,[122,194] and that HOCl formed by enzymatic peroxidation reacts preferentially with substrates other than O_2^-. Control experiments established that MPO did not scavenge ·OH at the concentrations used.

Hypochlorous acid has been proposed as an intermediate in copper ion-mediated spermicidal reactions[368] and in H_2O_2 bleaching of cuprous complexes of 2,9-dimethyl-1,10-phenanthroline.[369] Although the data presented were not conclusive, the studies raise interesting questions regarding nonenzymatic catalysis of Cl^- peroxidation. The reaction

$$H^+ + H_2O_2 + Cl^- \rightarrow HOCl + H_2O$$

is exergonic by 0.25 V at pH 7, reflecting the close balance between the H_2O_2/H_2O and $HOCl/Cl^-$ couples, with $E^{\circ\prime} = 1.32$ and 1.07 V, respectively.[281] To be an effective catalyst, a metal ion must have a two-electron reduction potential lying within this relatively narrow range. This condition probably excludes most peroxidases, e.g., for HRP, $E^{\circ\prime}$ (compound I/Fe^{3+}) $\simeq$ 0.95 V.[370] The reaction of Cl^- with ·OH, i.e.,

$$2 \cdot OH + H^+ + Cl^- \rightarrow HOCl + H_2O$$

is highly exergonic, with $\Delta E^{\circ\prime} = 1.24$ V (based upon $E^{\circ\prime}[OH/H_2O] = 2.31$ V). The reaction must proceed in one-electron steps, however, involving the intermediacy of chlorine radicals. In addition to ·Cl, the species $HOCl^-$ and Cl_2^- have been described with relative energies governed by the equilibria:[372]

$$\cdot OH + Cl^- \rightleftharpoons \cdot HOCl^- \quad (10)$$

$$\cdot HOCl^- + H^+ \rightleftharpoons \cdot Cl + H_2O \quad (11)$$

$$\cdot Cl + Cl^- \rightleftharpoons \cdot Cl_2^- \quad (12)$$

where $K_1 = 0.7\ M^{-1}$, $K_2 = 1.6 \times 10^7$, and $K^3 = 2 \times 10^5\ M^{-1}$, and all of the forward reactions are nearly diffusion-controlled. The rate of ·Cl formation (see reaction 11) is proton-dependent, so that at neutral pH values, the pseudo-first order rate constant by this pathway will be only about $k_2' = 10^3\ s^{-1}$. The rate constants from which the above equilibria were determined were measured in weakly acidic media and so are not totally appropriate to physiological conditions. Nonetheless, barring the existence of a pathway involving unassisted dissociation of OH^- from $\cdot HOCl^-$ that makes a major contribution at neutral pH, decomposition of $\cdot HOCl^-$ to form ·Cl will be relatively slow. Formation of the relatively stable $\cdot Cl_2^-$ will therefore be "blocked" because any ·OH generated from respiratory burst products will react rapidly with biological components in the phagosome. The species ·OH and $\cdot HOCl^-$ might be at near-equilibrium but, at physiological Cl^- concentration levels, $\cdot HOCl^-$ can achieve no more than 10% of the ·OH steady-state concentration level. Therefore, chlorine radicals should not comprise major pathways for ·OH-mediated oxidation reactions, a conclusion in agreement with previous analyses by Koppenol and Butler.[281] Hydroxyl radical oxidation of Cl^- to HOCl, if it occurs, would seem to require the intermediacy of metal ions capable of two-equivalent oxidation of Cl^-, i.e., with $E^{\circ\prime} > 1.07$ V, to avoid formation of the high-energy chlorine radicals.

IV. CONCLUDING COMMENTS

Major advances are presently being made in our understanding of the oxidative biochemistry of phagocytic cells. The composition and ultrastructural organization of the respiratory chain known as the NADPH oxidase has been outlined and an intensive effort is underway to identify the mechanisms of activation of the respiratory burst. The list of potential oxidative toxins has been shortened by elimination of some "red herrings" and the microbicidal reactions of MPO-generated chlorine compounds are coming into focus. The role of H_2O_2-mediated processes is more problematic, and may benefit from new approaches directed at identifying metabolic dysfunction attending cellular inactivation in model systems. Considerable progress is also being made in understanding nonoxidative microbicidal mechanisms and the role of secreted granule components in chemotaxis, leukocyte regulation, and the host inflammatory response, topics that were not reviewed here. There is also the hint that the multiple, seemingly redundant, neutrophil microbicidal components may act synergistically in combating infection. Clearly, numerous surprises still await researchers in this field for, as a sagacious man once wrote, "Nature continues to display more imagination than any observers."[372]

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