Oxidative inactivation of *Escherichia coli* by hypochlorous acid

Rates and differentiation of respiratory from other reaction sites

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1. INTRODUCTION

The enzyme myeloperoxidase (MPOase) catalyzes peroxidation of Cl^- to form hypochlorous acid, which is potently microbicidal [1]. Oxidation by hypochlorous acid is markedly selective for nucleophilic compounds [2,3]. Among the most reactive biological substrates are the electron-rich iron–sulfur proteins, carotenes, porphyrins and heme proteins, nucleotides and proteins containing active sulfhydryl groups [4]. These types of compounds are rapidly bleached by both HOCl and cell-free MPOase-catalyzed chloride peroxidation systems [4–6]. From these observations and the demonstration that bacterial cells exposed to HOCl undergo irreversible oxidation of cytochrome *b*, carotene and adenine nucleotides, we have inferred that the microbicidal action of HOCl arises from interruption of energy-linked cellular respiration and, because phagocytizing leukocytes that contain MPOase are capable of generating HOCl [1], that leukocytic disinfection can occur by analogous mechanisms [4].

The proposed general mechanisms predict several consequences that are amenable to experimentation. If HOCl is selective for vulnerable cellular sites, then the rate of inactivation must be rapid since selectivity is the expression of relative rates. Furthermore, if cellular respiratory systems are the targets for oxidative disinfection by HOCl, then loss of respiration in bacterial cells should correlate with other indicators of cellular death, e.g., loss of ability to replicate. This relationship has been examined briefly in [7–9]. From these data there appears to be rough correspondence between the 2 phenomena, but the reports differ in quantitative detail, i.e., loss of respiration is either more [8] or less [7,9] sensitive than *E. coli* survival to chlorine oxidation. It is important to resolve this difference since loss of viability preceding respiratory loss would indicate the existence of other HOCl disinfection mechanisms, whereas parallel loss would indicate that aerobic respiration is crucial to survival.

We report here the results of experiments which demonstrate that *E. coli* is inactivated within milliseconds of exposure to HOCl and that the initially oxidized sites responsible for killing are not respiratory components.

2. MATERIALS AND METHODS

2.1. *Reagents*

HOCl was prepared by vacuum distilling Chlorox (NaOCl) after adjusting the acidity to pH 7–8 with dilute sulfuric acid; concentrations were determined by spectrophotometric analysis (ε_{235} = 100 $M^{-1} \cdot cm^{-1}$). Other chemicals and biochemicals were best-available quality and were used as obtained from commercial suppliers. *Escherichia coli* ATCC 25922 was treated as in [4]. Bacterial cell concentrations were determined by phase-contrast microscopy using a hemocytometer. Myeloperoxidase from canine uterine pus was a gift from Dr Seymour Klebanoff (University of Washington, Seattle). The enzyme was assayed immediately before use by measuring oxidation of 0.3 mM *o*-dianisidine by 0.9 mM H_2O_2 in 0.01 M phosphate buffer (pH 6.0); one unit of activity is defined as an

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absorbancy change at 460 nm of $0.01 \cdot ml^{-1} \cdot min^{-1}$ at 25°C.

2.2. *Cellular oxidations*

Rates of HOCl-promoted inactivation of *E. coli* were examined using a quench-flow system comprising 3 mechanically-driven syringes coupled by 2 tangential jet mixing chambers (fig.1). Bacteria and HOCl were mixed in the first chamber, then reaction was quenched by mixing with sodium thiosulfate or dithiothreitol solution in the second chamber. The times of exposure to the oxidizing agent were varied by changing flow rates and/or flow pathlengths between mixing chambers. HOCl reduction by the quenching agents was shown to be completed within the time of mixing (< 10 ms) under the experimental conditions by direct optical measurement of HOCl absorbance in a Gibson-type stopped flow instrument. Rates of oxygen uptake by *E. coli* in quenched solutions were measured using a Clark-type polarographic electrode [2]. The number of cells capable of supporting colonial growth was determined by pour-plate analysis. Appropriately diluted aliquots were mixed with 5% tryptic soy agar, incubated overnight at 37°C, and the colonies formed counted. The presence of quenching agent in the incubation medium had no effect upon the cell count.

Myeloperoxidase-catalyzed oxidations were studied directly in the oxygen cell. In preliminary studies we observed that MPOase binds to the glass walls of the oxygen electrode chamber. Since MPOase also binds strongly to bacterial cell walls [10,11], this effect could be prevented by preincubating the enzyme with suspensions of *E. coli* for a few minutes before addition to the electrode chamber. Reaction was initiated by adding hydrogen peroxide to the MPOase–*E. coli* suspensions in buffers containing 100 mM Cl^- at the point on uptake curves where oxygen tensions in solution corresponded to atmospheric concentrations. Inhibition was determined as the change in rate of uptake after recovery of the electrode from the physical effects of addition (< 1 min). Oxygen consumption was linear over the time course of the measurements (~5 min). Viable cells remaining after reaction were determined by pour-plate analysis.

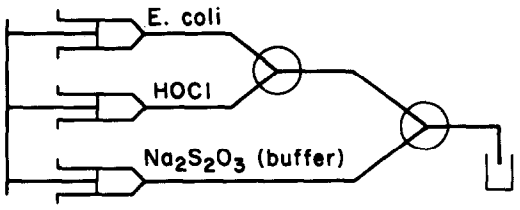

Fig.1. Schematic diagram of quench-flow apparatus. Bacterial suspensions were flowed through two 12-jet tangential mixers placed in tandem.

3. RESULTS

3.1. *Rate of E. coli inactivation by HOCl*

Parallel quench-flow measurements were made in which *E. coli* was mixed with varying concentrations of HOCl, then with either buffer or excess sodium thiosulfate solution. A wide range of medium conditions was investigated using 25 mM succinate-phosphate buffers (pH 5.0–7.0) containing 0–100 mM Cl^- and 0.4–1.0 mM thiosulfate quench concentrations. Exposure times in the quenching runs were varied from 0.1–1.0 s between addition of HOCl and the quenching agent. Under no conditions was there found any systematic difference in counts of surviving bacteria in quenched

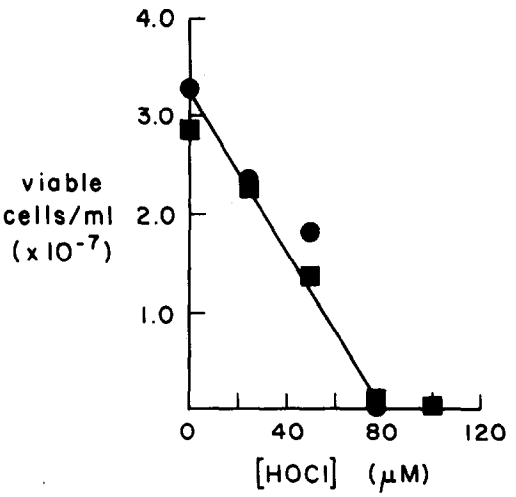

Fig.2. Quench-flow studies of *E. coli* inactivation rates. Viable cell count in presence (▪) and absence (•) of 1.0 mM sodium thiosulfate; 0.55 s exposure time between mixing *E. coli* with HOCl and quench. Conditions: 25 mM succinate buffer (pH 5.0), 100 mM NaCl, 23°C.

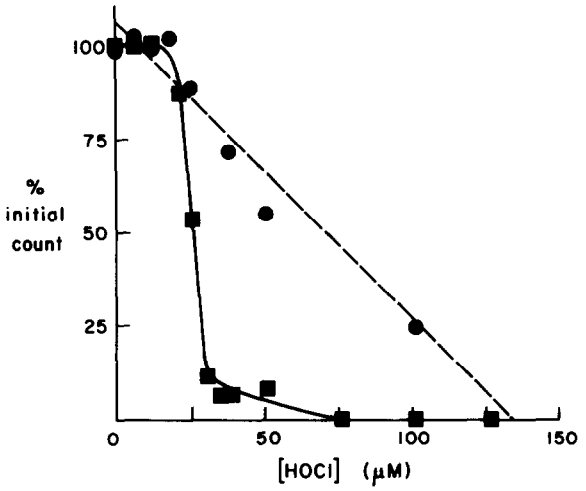

Fig.3. *E. coli* cellular inactivation and respiratory loss by HOCl. Conditions: 5.0×10^8 cells *E. coli*/ml in 25 mM succinate buffer (pH 5.0), 100 mM NaCl, 23°C; (•) % initial rate of oxygen uptake; (▪) % initial viable cell count by pour-plate analysis.

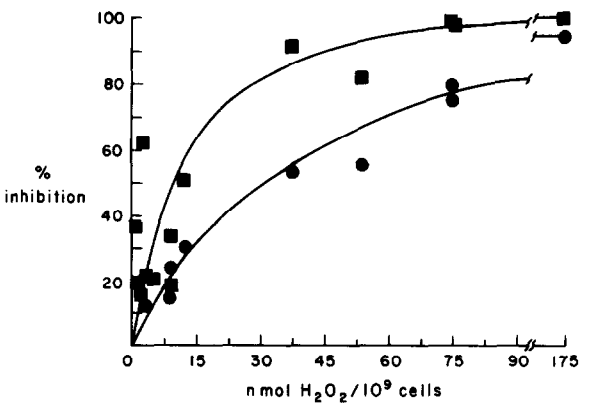

Fig.4. *E. coli* cellular inactivation and respiratory loss by MPOase–H_2O_2–Cl^-. Conditions: 1.7×10^9 cells *E. coli*/ml in 25 mM succinate buffer (pH 5.0), 100 mM NaCl, 23°C; (•) decrease in rate of oxygen uptake; (▪) decrease in viable cell count by pour-plate analysis. Data are the accumulated results of 3 runs with MPOase at 2, 6, 16 units/ml.

and unquenched solutions. Representative data are given in fig.2. We conclude that the reactions leading to cellular death occur within the time frame of mixing oxidant and quench, i.e., in < 100 ms, and that thiosulfate and dithiothreitol are unable to reverse the effects of oxidative inactivation.

3.2. *Correlation of respiration with cellular viability*

Results from simultaneous determination of respiratory loss and cellular inactivation accompanying *E. coli* oxidation by HOCl are given in fig.3 for reaction at pH 5.0. Very similar results are obtained at pH 7.4 in 25 mM phosphate buffer containing 25 mM succinate and 100 mM Cl^-, with the exception that ~2–4-fold greater concentrations of HOCl are required to reach comparable levels of inhibition of respiration and viability for the same number of organisms at the higher pH. The most striking observation is that loss of viability occurs well before the onset of appreciable respiratory loss. Thus, it is possible to prepare oxidized organisms with nearly unimpaired respiratory function that are unable to divide. The titrimetric results indicate that $\sim 4 \times 10^7$ molecules/bacterium are required to inhibit *E. coli* division and 2×10^8 to inhibit respiration at pH 5.0 and $\sim 8 \times 10^7$ and 8×10^8 to inhibit division and respiration, respectively, at pH 7.4.

Oxidative inactivation of *E. coli* by the MPOase–H_2O_2–Cl^- system also exhibits differentiation between viability and respiratory losses (fig.4). Results are independent of MPOase concentration levels over the measured range (2–16 units/ml). Addition of MPOase in the absence of H_2O_2 caused no change within experimental error in either *E. coli* respiration or colonial growth. No systematic variation in the colony counts was observed when oxidized cells were sampled at 0.5–20 min after H_2O_2 addition; aliquots were therefore routinely taken a few minutes after initiation of reaction. The titration data indicate loss of viability at 2×10^7 H_2O_2 molecules/bacterium, with respiratory inhibition requiring $\sim 1 \times 10^8$ H_2O_2/bacterium in pH 5.0 buffer.

4. DISCUSSION

Phagocytosis involves activation of diverse and complex sets of enzymes involved with leukocytic respiration and isolation, inactivation and digestion of the microbe [1]. Without some guiding principle, it is nearly impossible to identify the actual leukocyte-generated toxins and their microbicidal reac-

tions within the broad spectrum of biological reactions occurring in stimulated leukocytes. The problem is exacerbated by the likelihood that there is redundancy in the disinfection process, i.e., several alternative mechanisms can cause cellular death [1].

We have assumed that the lethal reactions are among the first that occur during phagocytosis [4], in part because lysozyme-insensitive microbial cells lose their ability to replicate well before the onset of cellular degradation [11–15]. For hypochlorous acid, this premise is clearly substantiated by the quench-flow studies, where its toxic character and selectivity are demonstrated by *E. coli* inactivation occurring within milliseconds of exposure to the oxidant. The result provides an essential kinetic criterion for the discrete reactions involved with the HOCl disinfection process, i.e., reactions proposed to be part of the microbicidal event must occur within the same time frame.

Cytochromes and iron–sulfur proteins are among the most reactive biological compounds with HOCl identified under phagosomal conditions [4]. Irreversible bleaching of *b*-type cytochromes coincides quantitatively with respiratory loss in *E. coli* [4]. Nonetheless, these reactions involve only a few % of the total added HOCl [16] and the observation that viability loss precedes respiratory loss, at least under the experimental conditions of these studies, suggests that other, more vulnerable HOCl oxidation sites exist in *E. coli*. Amine and amino acid groups of bacterial cell wall structural proteins have been shown to react with HOCl and the MPOase–H_2O_2–Cl^- system [14,17]; rates are sufficiently rapid under the reaction conditions [14] that amine or amino acid *N*-chlorination can compete with cytochrome oxidation. However, because the products of biological *N*-chlorination, chloramines and aldehydes, are not particularly toxic to bacteria [7,14,17,18] and the extent of formation of endogenous chloramines bears no simple relationship to cellular death [7,17], we believe that the role of these reactions in physiological disinfection processes is negligible. This conclusion is supported by our titrimetric results which show that HOCl is a more effective toxin in weakly acidic than in neutral solutions. Protonated amines are unreactive towards HOCl [19], hence chlorination rates decrease with increasing acidity. If endogenous chloramine formation is a competitive side reaction that does not lead to *E. coli* inactivation, then we would expect its protective action to be proportionately greater at higher pH, as is observed. Cytochrome oxidation rates increase with increasing acidity [4]; loss of respiration with lower quantities of added HOCl in acidic media can similarly be rationalized.

Loss of capacity for 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB) reduction, which has been attributed to reaction of accessible sulfhydryl groups, also occurs upon bacterial oxidation. The extent of the loss correlates directly with HOCl or MPOase–H_2O_2–Cl^- promoted loss of cell viability [7,17]. It should be noted, however, that studies of both bacterial amine and sulfhydryl reactions have invariably involved relatively lengthy periods of incubation of organisms with oxidants so that, by the kinetic criterion, the relationship between these observations and the actual microbicidal reactions is uncertain. Sulfhydryl oxidation at the bacterial plasma membrane could lead to inactivation by loss of transport of metabolites, since several porters are known to contain active site sulfhydryl groups [20,21]. Alternatively, entirely different types of reactions may be involved. For example, the *E. coli* adenine nucleotide pool is also rapidly oxidized by HOCl [4], so that inactivation might be a consequence of metabolic dysfunction caused by its loss. Ultimately, identification of mechanisms of oxidative inactivation will require use of the methods and criteria outlined above.

Our studies using flow-mixing of oxidant and bacteria differ from earlier reports [7–9] equating (approximately) respiratory loss and cellular death of *E. coli*. This discrepancy must relate to the very rapid rate of inactivation. In all previous work, HOCl was added directly so that high local concentrations of oxidant 'swamp' oxidation sites on nearby organisms, reducing the inherent reaction selectivity. The great practical advantage of flow-mixing is that it allows preparation of large numbers of inactivated organisms that can be examined for loss of essential metabolic function, hence the molecular mechanisms of disinfection, with minimum interference from following degradative reactions.

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