

# Biological reactivity of hypochlorous acid: Implications for microbicidal mechanisms of leukocyte myeloperoxidase

(bactericide/biological oxidation/neutrophil/respiration/toxin)

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**ABSTRACT** Oxidative degradation of biological substrates by hypochlorous acid has been examined under reaction conditions similar to those found in active phagosomes. Iron sulfur proteins are bleached extremely rapidly, followed in decreasing order by  $\beta$ -carotene, nucleotides, porphyrins, and heme proteins. Enzymes containing essential cysteine molecules are inactivated with an effectiveness that roughly parallels the nucleophilic reactivities of their sulfhydryl groups. Other compounds, including glucosamines, quinones, riboflavin, and, except for N-chlorination, phospholipids, are unreactive. Rapid irreversible oxidation of cytochromes, adenine nucleotides, and carotene pigments occurs when bacterial cells are exposed to exogenous hypochlorous acid; with *Escherichia coli*, titrimetric oxidation of cytochrome was found to coincide with loss of aerobic respiration. The occurrence of these cellular reactions implicates hypochlorous acid as a primary microbicide in myeloperoxidase-containing leukocytes; the reactivity patterns observed are consistent with the view that bactericidal action results primarily from loss of energy-linked respiration due to destruction of cellular electron transport chains and the adenine nucleotide pool.

Bacteria commonly lose their ability to divide within minutes of encountering phagocytosing leukocytes (1). Loss of cell viability often occurs well before the onset of cellular digestion as determined by physiological changes (2), macromolecular degradation (3, 4), or loss of macromolecular biosynthesis (5). The specific reactions giving rise to cellular death have not yet been identified, at least in part because leukocytes are capable of initiating a diverse set of processes which are potentially lethal (6, 7). Given the above observations, however, the microbicidal reactions must be among the first that attend interaction with the leukocyte.

Reactions catalyzed by myeloperoxidase (MPOase) appear to make major contributions to the microbicidal action of polymorphonuclear leukocytes (PMNs) (6, 7). The cell-free MPOase- $H_2O_2$ - $Cl^-$  system is potentially microbicidal; chlorination of bacteria by the cell-free system (8, 9) and of macromolecular fractions during PMN digestion of bacteria (9) suggests that the toxic mechanisms may involve direct reaction with hypochlorous acid (HOCl), the oxidized product of MPOase catalysis. Because HOCl is freely diffusible from the enzyme (10) and the gross features of cellular reactions with phagocytosing PMNs, the MPOase- $H_2O_2$ - $Cl^-$  system, and exogenous HOCl are similar (4, 8, 11–13), it is likely that the actual microbicidal reactions are uncatalyzed. If so, minimal requirements for toxicity would seem to be that HOCl show high selectivity in its reactions with biological substrates and that its action be directed against susceptible sites—e.g., specific energy-generating or biosynthetic systems.

In general, rates of oxidation and chlorination by various monovalent chlorine compounds can be understood in terms of

electrophile–nucleophile interactions involving association of electropositive chlorine with electron-rich centers on the substrate; reaction pathways are correspondingly highly dependent upon medium conditions, particularly  $H^+$  and  $Cl^-$  concentrations (14). With the exception of amines and amino acids (11, 12, 15), the reactions of biological compounds with HOCl are not understood (16).

We report here the results of a survey of simple biological compounds which can be taken as prototypic of various components of bacterial cells. The data demonstrate that HOCl is strongly selective toward nucleotides and compounds that are models for certain components of respiratory redox chains. This selectivity is shown to extend to bacterial cells.

## MATERIALS AND METHODS

**Reagents.** HOCl was prepared by vacuum distilling Chlorox (NaOCl) after adjusting the pH to 7–8 with dilute sulfuric acid; concentrations were determined by spectrophotometric [ $\epsilon_{235} = 100$  (17)] or iodometric analyses. Ethanolchloramine was prepared by reaction of HOCl with a 3-fold excess of ethanolamine in 0.1 M phosphate buffer at pH 5.0. Aldolase (EC 4.1.2.13) was prepared from brewer's yeast (Standard Brands) by ammonium sulfate precipitation (18) in the presence of proteolysis inhibitors (19); the purified enzyme had a specific activity of 64 units/mg of protein. Other enzymes and reagent-grade biological compounds were used as obtained from commercial suppliers. *Escherichia coli* (ATCC 25922) were grown on media 2 (20) plus succinate (2 g/liter) at pH 6.5 and harvested in stationary phase. The bacteria were centrifuged, washed, and resuspended in buffer immediately before use. *Sarcina lutea* (*Micrococcus luteus*, generously provided by S. J. Klebanoff, University of Washington Medical School, Seattle) were grown on 5% tryptic soy agar plates (Prepared Media Laboratories); suspensions were made by carefully scraping the cells from the plates and agitating them in buffer. Bacterial cell concentrations were determined by phase-contrast microscopy with a hemocytometer.

**HOCl Oxidations.** Reaction with biological compounds was monitored by using either a Cary 16 spectrophotometer or Gibson-type stopped-flow apparatus (21). Rate laws were determined from initial rates of bleaching; rate constants for the compounds listed in Table 1 were determined from loss of their characteristic absorption bands when HOCl was in large excess. The reactions are generally complex, involving numerous oxidized intermediates. Any rapid initial degradative steps that did not give rise to large spectral changes (e.g., heterocyclic ring chlorination) would go undetected. Consequently, the rate constants reported would be lower limits for these steps. Enzyme inactivation was studied by adding portions of HOCl to solutions

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Abbreviations: MPOase, myeloperoxidase; PMN, polymorphonuclear leukocyte.

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containing freshly dialyzed enzyme and then measuring, by appropriate standard assay procedures, the residual activity of aliquots taken from the reaction solution. In the enzyme and cellular oxidation studies, HOCl was added as a bolus to rapidly stirred reactant solutions. HOCl-promoted loss of bacterial respiration was measured with a Clark electrode and recorder assembly (14).

Optical difference spectra of bacterial suspensions were recorded on an Aminco DW-2 spectrophotometer. Cellular nucleotide concentrations were determined, by using an Aminco Chem-Glow photometer, from the integrated light intensity of the luciferin-luciferase assay (22) taken over the first 3 sec of reaction. In the nucleotide studies, reactions after exposure to HOCl were quenched by rapidly withdrawing samples into a spring-loaded syringe containing perchloric acid. Intervals between mixing and quenching (10–300 sec) were chosen so that reaction with HOCl was completed and subsequent intracellular reactions were minimized. In several experiments, the reaction was quenched with sodium thiosulfate before acidification to ensure that oxidation was not due to molecular chlorine formed from residual HOCl. The results were identical to those for runs in which thiosulfate quenching was omitted. Mass spectra were recorded on a Hitachi-Perkin-Elmer RMU-7 instrument operated in the field desorption mode.

## RESULTS

**Oxidation of Biological Substrates.** Degradative oxidation of various biological compounds that contain electron-rich functional substituents was rapid. In each instance the susceptible site comprised a  $\pi$ -conjugated or similar electron delocalized center. Examples from each reaction type are given in Table 1.

Ferredoxin oxidation occurred at rates too fast to measure by using stopped-flow methods, even at submillimolar reactant concentrations. The rate constants listed for these reactions were calculated by assuming that the rate law is first-order in each reactant; no  $\text{Cl}^-$  dependence was observed over the range investigated (1–100 mM, pH 5). With spinach ferredoxin, oxidation occurred with complete loss of the iron-sulfur chromophore at stoichiometric ratios of about 10 HOCl/ $\text{Fe}_2\text{S}_2$  unit; the product spectrum was identical to that of apoprotein (23), suggesting oxidation of sulfur with probable loss of iron. Chromophoric bleaching of ferredoxins initiated by MPOase-catalyzed peroxidation of  $\text{Cl}^-$  ion is also rapid (unpublished data).

Oxidation of  $\beta$ -carotene is first-order in both reactants and first-order in  $\text{Cl}^-$  concentration; rates increased only slightly with  $\text{H}^+$  concentration in the weakly acidic region (pH 4–7). Complete bleaching occurred at a ratio of HOCl to carotene of

$\approx 12:1$ . Immediate reaction products had masses that increased in units of  $32 \pm 1$  atomic mass units, suggesting epoxidation through intermediary formation of chlorohydrins. Because retinol oxidation by HOCl gives products with masses increasing by  $16 \pm 1$  atomic mass units, the opposite ends of the carotene molecule may be reacting largely independently.  $\beta$ -Carotene is also bleached by the  $\text{MPO-H}_2\text{O}_2\text{-Cl}^-$  system (24).

Oxidation of numerous porphyrins, hemes, and hemoproteins, including protoporphyrin IX, mesoporphyrin IX dimethyl ester, 2-formyl-4-vinyl-deuteroporphyrin dimethyl ester, 2,4-diformyldeuteroporphyrin dimethyl ester, ferriprotoporphyrin IX (hemin) and its dimethyl ester, ferridiacetyldeuteroporphyrin, cobalt(III) mesoporphyrin IX dimethyl ester, myoglobin, microperoxidase, and cytochrome *c*, also occurred rapidly. Porphyrin and heme solutions contained 1% NaDodSO<sub>4</sub> to solubilize the macrocycle. Cytochrome *c* oxidation by the cell-free  $\text{MPO-H}_2\text{O}_2\text{-Cl}^-$  system is also rapid (unpublished data).

Of the porphyrin compounds examined in detail (protoporphyrin IX, mesoporphyrin IX dimethyl ester, hemin, and cytochrome *c*), all except protoporphyrin IX exhibited bleaching rates that increased with increasing  $\text{H}^+$  concentration in the pH region 4–7, although the dependency was complex and less than first-order; protoporphyrin IX rates were inversely dependent upon acidity in this region. Reactions of hemin and cytochrome *c* were first order in both reactants. Oxidation of metal-centered porphyrin compounds was  $\text{Cl}^-$  independent; that of metal-free porphyrins was linearly dependent upon  $\text{Cl}^-$  concentration. Bleaching stoichiometries increased from HOCl/porphyrin  $\approx 4, 10, 12,$  and  $24$  for the compounds myoglobin, hemin, microperoxidase, and cytochrome *c*, respectively. The progressive increase in stoichiometry through the series was associated roughly with decreasing accessibility of the porphyrin ring to solvent. Addition of excess HOCl to solutions containing cytochrome *c* gave sequential bleaching and precipitation, indicating that reaction with HOCl is selective for the porphyrin ring, at least to the extent that reaction at other sites is insufficient to cause extensive protein denaturation. Reaction of hemes and hemoproteins with hydrogen peroxide and ethanolchloramine was found to occur at rates no more than  $1/1000$ th those with HOCl.

Oxidation of adenine nucleotides was first order in substrate, HOCl, and  $\text{Cl}^-$ ; the  $\text{H}^+$  dependence was complex, with rates increasing with acidity and exhibiting less than first-order behavior. The initial reactions appeared to involve ring chlorination, as suggested by the bathochromic shift of the UV absorbance maximum (25). Subsequent loss of the UV chromophore, which was rapid (Table 1), indicates oxidative destruction of the heterocyclic ring. Complete bleaching required addition of HOCl to a ratio of HOCl/AMP  $\approx 10$ .

Introduction of electron-withdrawing substituents onto aromatic or heterocyclic rings was found to decrease their reactivity toward HOCl. GMP, CDP, and IDP reacted at rates comparable to the rate for ADP; UDP was less rapid. Riboflavin and a quinone (menadione, ubiquinone-10 in 1% NaDodSO<sub>4</sub>) were totally unreactive under the experimental conditions (Table 1). Hydroquinone was oxidized only to the level of benzoquinone; no chlorine substitution or other evidence of oxidative degradation was found by spectrophotometric or mass spectrometric analyses. Oxidation rates were rapid; measured rate constants were comparable to those for cytochrome and nucleotide bleaching.

Compounds that can be taken to represent cell wall components (*N*-acetylglucosamine, *N*-acetylmuramic acid, glucose, and mannose) and, with the exception of ethanolamine which forms ethanolchloramine, those representing structural cell

Table 1. Rate constants for oxidative bleaching of biological substrates by HOCl

Compound	$\lambda$ , nm	$k$ , ( $\text{M}^{-1}\text{s}^{-1}$ ) $\times 10^{-3}$
Spinach ferredoxin	400	$\geq 100^*$
Clostridial ferredoxin	420	40
$\beta$ -Carotene in 1% NaDodSO <sub>4</sub> <sup>†</sup>	459	23
Cytochrome <i>c</i>	409	2
Hemin-Cl in 1% NaDodSO <sub>4</sub> <sup>†</sup>	394	2
Adenosine monophosphate	280	2
Amines and amino acids	—	0.1–4 <sup>‡</sup>
Hydrogen peroxide	—	§

Reactions were in 0.05 M phosphate at pH 4.5;  $\text{Cl}^- = 0.10$  M; temperature = 23°C.

\* Too fast to measure.

<sup>†</sup> NaDodSO<sub>4</sub> is unreactive.

<sup>‡</sup> Calculated from data in ref. 15.

<sup>§</sup>  $t_{1/2} = 7$  sec (14).

membrane components (phosphatidycholine, choline, and glycerol) were unreactive over periods of up to several hours after mixing with HOCl.

**Enzyme Inactivation.** Reaction of HOCl with cysteine apparently occurs with preferential side-chain oxidation; no chloramine formation was noted in studies in which sulfhydryl oxidation was carried to the level of sulfonic acid (12). Loss of catalytic function resulting from enzyme sulfhydryl oxidation has been suggested to be the microbicidal event, both from studies on enzyme inactivation (26) and from studies correlating loss of cell viability with apparent loss of titrable sulfhydryl groups in *E. coli* (8, 13).

To examine these putative relationships more closely, we measured HOCl inactivation of several enzymes with different sulfhydryl functions. Of the enzymes studied, papain (EC 3.4.4.10), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), and alcohol dehydrogenase (EC 1.1.1.1) have exceptionally reactive active-site cysteine groups (27–29), yeast aldolase (EC 4.1.2.6) and lactate dehydrogenase (EC 1.1.1.27) have essential cysteines which are not the most reactive in the protein (30, 31), and muscle aldolase contains catalytically active cysteine groups classed as having only auxiliary function (32). The enzymes and HOCl were allowed to react in 0.05–0.10 M phosphate buffer containing 0.10 M Cl<sup>-</sup> at pH 7 and in weakly acidic media. The pH range was limited by irreversible loss of activity which occurred at more acidic conditions. In general, pH values of 4–5 were accessible, although for alcohol dehydrogenase the limiting pH was 6.1. Over the ranges investigated, inactivation kinetics appeared to be independent of pH. In general, loss of activity was not linearly proportional to the amount of added HOCl; HOCl became less effective with increasing extent of the reaction. For this reason, stoichiometries to 50% total inactivation have been reported (Table 2). Sensitivity to HOCl inactivation followed nucleophilic reactivity of catalytically active sulfhydryl groups, providing some circumstantial evidence for their involvement. It is clear from the data, however, that HOCl oxidation of side-chain groups is fairly nonselective. The relatively high HOCl/enzyme inactivation ratios, nonlinear titration behavior, and absence of any discrimination between reactivities in essential and nonessential cysteine groups in the titration curves—e.g., for yeast aldolase (30)—all support this contention.

**Oxidation of Bacterial Cells.** In accordance with others (13, 26, 33) we found that HOCl caused rapid loss of oxygen uptake by respiring bacteria. The spectra of *E. coli* to which incremental additions of HOCl were made are compared with those of untreated bacteria in Fig. 1. Under the experimental conditions the cytochromic components of resting cells exist almost completely in their reduced oxidation states; addition of dithionite to either cuvette in the absence of HOCl gave no change in the baseline difference spectrum. The spectral peaks appearing at

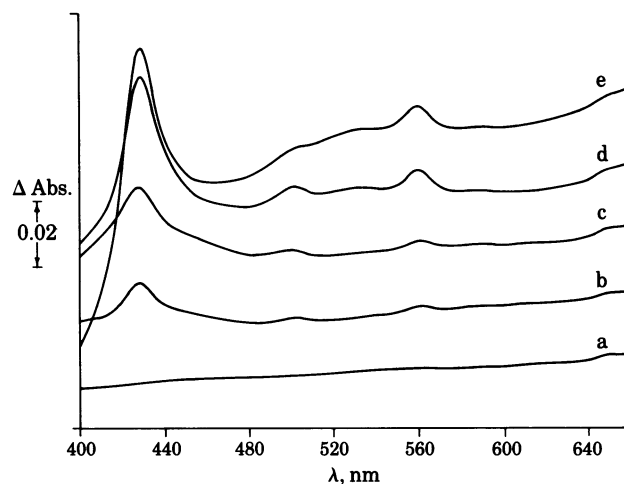


FIG. 1. HOCl oxidation of *E. coli*. Conditions:  $5 \times 10^9$  cells per ml suspended in 0.1 M phosphate buffer at pH 5.0; Cl<sup>-</sup> = 0.1 M; temperature = 23°C. Curve a: difference spectrum, resting cells vs. respiring cells; curves b–e, incremental additions of 50  $\mu$ l of 60 mM HOCl to the reference cell, with an equal volume of buffer added to the sample cell. Respiration ceased with addition of 150  $\mu$ l of HOCl (curve d). The titration was carried to 500  $\mu$ l total added HOCl (not shown) without further change in difference spectrum intensities.

429, 529, and 558 nm with HOCl addition are indicative of oxidation of *b*-type cytochromes (34–36). Reaction was complete within the time interval between mixing and recording of spectra (1–2 min). Amplitudes of the difference peaks increased proportionately with HOCl addition to the point of complete inhibition of respiration. Continued addition of HOCl caused no further change in the visible region.

It was not possible to distinguish between simple oxidation to the ferric state and oxidative destruction of the hemo-porphyrin from the spectral data because the cytochrome spectrum is obscured by larger optical changes occurring below 420 nm, presumably attributable to changes in light-scattering properties of the cells accompanying oxidation. However, dithionite was incapable of reversing the effects of reaction with HOCl, suggesting that cytochrome bleaching had occurred. In contrast, oxidation by ethanolchloramine or H<sub>2</sub>O<sub>2</sub> gave a nearly identical

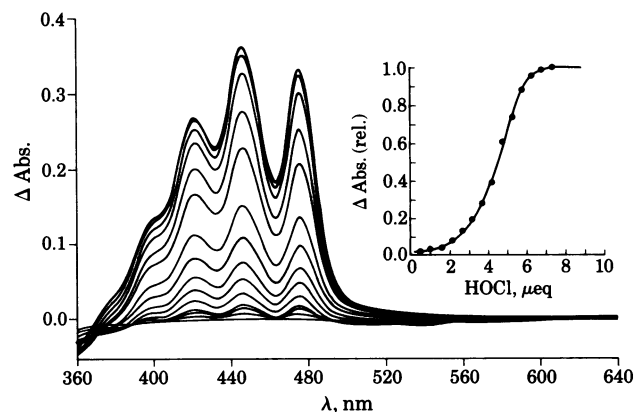


FIG. 2. Difference spectrum titration of *S. lutea* with HOCl. Conditions: 0.05 M phosphate at pH 4.45; Cl<sup>-</sup> = 0.1 M; temperature = 23°C. Sequential additions of 30  $\mu$ l of 17.5 mM HOCl to the reference cell containing 3.0-ml suspension of  $9 \times 10^8$  coccal pairs of *S. lutea* per ml; an equal volume of buffer was added to the sample compartment. Difference curves are identical to carotene absorption spectra. (Inset) Titrimetric behavior at 447 nm; optical changes have been corrected for dilution by titrant.

Table 2. Enzyme inactivation by HOCl

Enzyme*	SH groups, no./catalytic unit		HOCl/catalytic unit to 50% inactivation, mol/mol
	Essential	Total	
Papain	1	1	1.0
ADH	2	8–9	≈1
LDH	1	4	≈2.5
GAPDH	1	3	≈3
Aldolase (class II)	2	4	≈6
Aldolase (class I)	0	8	≈23

\* ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 3. Adenine nucleotide levels in HOCl-treated *E. coli*

Reaction time, sec	Integrated intensity (relative)*			Nucleotide concentrations (relative)	Adenylate energy charge
	ATP	ATP & ADP	ATP & ADP & AMP		
0 (control)	19	31	43	1.0	0.58
10	6	8	17	0.40	0.41
35	8	12	19	0.44	0.53
300	6	8	16	0.37	0.43

$5 \times 10^8$  cells per ml; HOCl = 90  $\mu$ M in 0.05 M phosphate buffer at pH 4.5; Cl<sup>-</sup> = 0.10 M; temperature = 23°C. HOCl concentration was just sufficient to inhibit respiration.

\* Luciferin-luciferase assay as described in text (22).

difference spectrum which was lost upon dithionite addition, indicating fully reversible oxidation with these reagents.

Exposure of *S. lutea* to HOCl gave rise to oxidative bleaching of its carotenoid components (Fig. 2); reaction was instantaneous and led to total loss of pigmentation.

Adenine nucleotide oxidation also occurred within seconds of mixing *E. coli* with HOCl. Approximately two-thirds of the total nucleotide concentration was lost upon addition of a quantity of HOCl just sufficient to inhibit respiration; the reaction was complete within 10 sec after mixing (Table 3).

## DISCUSSION

Phagocytosis stimulates glycolysis-linked respiration, generating hydrogen peroxide in neutrophils. Bacterial engulfment is accompanied by migration of leukocytic granules, containing among their constituents the enzyme MPOase, to the site of the forming phagosome. During subsequent fusion of granule and phagosomal cell walls, the granule proteins are injected into the phagosome. As degranulation proceeds, the phagosome becomes progressively more acidic. The entire process from the point of encounter of bacterium with PMN to completion requires only several minutes and leaves the bacterium isolated within a vacuole containing MPOase and an enzymatic H<sub>2</sub>O<sub>2</sub>-generating system in a medium containing 0.1 M Cl<sup>-</sup> at pH 4–6 (6, 7); these conditions are optimal for MPOase-catalyzed generation of HOCl. As noted above, the time frame for phagocytosis is the same as that required for loss of cell viability. Nonetheless, bacterial digestion within the leukocyte continues for several hours.

The reactivity patterns found here indicate that cytochromes, iron-sulfur proteins, and nucleotides are highly vulnerable to oxidative degradation by HOCl. Because these types of compounds appear essential to virtually all aerobic and anaerobic electron transport systems (37, 38), their loss undoubtedly would be lethal. The cell envelope appears to afford little protection against oxidation by exogenous HOCl because, except for amines, other structural cell wall and membrane components are unreactive. It is noteworthy that oxidation of metalloporphyrins and ferredoxins is chloride-independent; reaction of similar redox centers bound within the lipophilic membrane interior is unlikely to be limited by the low levels of Cl<sup>-</sup> at those sites. Furthermore, the increase in phagosomal acidity that accompanies PMN degranulation serves to enhance selectivity for these compounds over amines. Nucleotide, heme, and hemo-protein bleaching rates increase with acidity, whereas chloramine formation rates decrease, so that in neutral to weakly acidic media the former are more rapid (Table 1). HOCl oxidation of H<sub>2</sub>O<sub>2</sub>, which is relatively slow (14), is also minimized in weakly acidic solution. The reactive compounds listed in Table 1 are therefore representative members of the only biological classes yet identified which undergo rapid degradative oxidation by HOCl under conditions similar to those existing within the PMN phagosome.

These types of reactions also appear to take place during oxidation of bacterial cells. Respiratory loss in *E. coli* that occurs as a consequence of oxidation by HOCl (13, 26, 33) or the MPO–H<sub>2</sub>O<sub>2</sub>–Cl<sup>-</sup> system (6) has been shown to coincide, more or less, with loss of cell viability (13, 26, 33, 39). Irreversible oxidation of bacterial cytochromes also coincides with respiratory loss (Fig. 1). Although the exact sites of reaction remain to be determined, the correlations suggest a probable link between loss of electron transport function and cellular death. Our contention that bacterial cell walls do not impose any substantial barrier to attack by HOCl is supported by the direct observations of reactant bleaching in the cell membrane (carotene, Fig. 2) and possibly the cytosol<sup>†</sup> (adenine nucleotides, Table 3). Loss of ATP resulting from HOCl oxidation of *E. coli* has also been reported by others (33), although it was not determined whether phosphate ester hydrolysis or purine oxidation had occurred. Our studies show that, when sufficient HOCl is added to just inhibit respiration, the predominant effect is oxidative degradation, not loss of adenylate energy charge—e.g., that might occur as a consequence of impaired capacity for oxidative phosphorylation.

The biological compounds we have studied are generally markedly less reactive toward other types of oxidants formed in phagocytosing leukocytes. In a cursory survey, we find no appreciable reaction of hemes, carotene, or AMP with H<sub>2</sub>O<sub>2</sub> or ethanolchloramine. Spinach ferredoxin is oxidized only slowly by H<sub>2</sub>O<sub>2</sub> but is rapidly bleached by the chloramine. The possibility exists, therefore, that HOCl oxidation of bacterial iron-sulfur centers might be mediated by formation of endogenous chloramines (8, 13). Hemes are unreactive toward oxidative degradation by superoxide ion (41, 42); from consideration of its chemical reactivity (42), superoxide ion can be anticipated to be a poor oxidant for the other compounds as well.

To summarize briefly, the character of the biological oxidations described here is appropriate for effective inactivation of cells, and reactions occur on time scales associated with cellular death; other leukocytic oxidizing agents appear generally not to meet these criteria. We therefore propose that HOCl is the microbicidal agent resulting from MPOase-catalyzed reactions and it exerts its toxic action by oxidizing or oxidatively destroying electron transport chains and the adenine nucleotide pool (ATP, ADP, and AMP). The vulnerability of these systems to HOCl suggests a universal mechanism of leukocyte toxicity toward fungi and mammalian cells [particularly tumor cells (43)] as well as bacteria.

Alternative proposals for killing mechanisms have invoked various consequences of sulfhydryl oxidation (13, 26, 44), cell envelope alteration (4, 33, 39), or nucleotide dysfunction (45, 46). These hypotheses were made either on the basis of evi-

<sup>†</sup> Reported turnover rates for microbial ATP pools are sufficiently large that several cycles can occur within the times of exposure of cells to HOCl (40). The possibility exists, therefore, that nucleotides are bleached only when membrane-bound.

dence demonstrating reaction in homogeneous solution or the occurrence of reactions in cells after relatively long periods of incubation with HOCl, the  $\text{MPO-H}_2\text{O}_2\text{-Cl}^-$  system, or PMN granules. There is little doubt that digestion under these conditions will lead to sulfhydryl oxidation and amine N-chlorination (8, 13), but the role of these reactions in bactericidal action is unclear. For instance, enzyme sulfhydryl oxidation rates are unknown but are apparently insufficiently rapid to compete with nucleotide reactions in the cytosol. Camper and McFeters (33) have shown that HOCl disinfection of *E. coli* causes a drastic decrease in ATP levels but no perceptible loss of aldolase activity, despite our studies which show that class II aldolases are among the enzymes most sensitive to HOCl inactivation (Table 2). Although loss of cell viability is reported to correlate with loss of titrable sulfhydryl groups in *E. coli* (8, 13), application of the titrant used [5,5'-dithio-bis(2-nitrobenzoic acid)] to this analysis is questionable. Both ferrous salts (52) and hydroquinones (unpublished data) are capable of reducing this titrant. Because one likely consequence of HOCl-induced loss of respiration is oxidation of the electron transport chains (Fig. 1), it is unclear to what extent the differences in this reaction with HOCl-treated and untreated *E. coli* are due to loss of reducing equivalents in respiratory redox chains as opposed to *bona fide* sulfhydryl group oxidation.

Similarly, simple chloramines are effective microbicides (13), but those formed by N-chlorination of taurine and other biological amines and amino acids are not toxic (47-49) unless incubated with the microbes for several hours (13). Because chloramines derived from endogenous amines show this same behavior (8, 13), their role in physiological disinfection processes is probably minimal. One point which our studies do not address is the loss of transport function that occurs when bacterial cells are inactivated by HOCl (33, 50); both HOCl (8) and the  $\text{MPO-H}_2\text{O}_2\text{-Cl}^-$  system (4, 8) have been shown to react with cell envelope proteins.

Finally, singlet oxygen formed by HOCl oxidation of  $\text{H}_2\text{O}_2$  has been discussed as a possible phagosomal toxin (6, 7). Its rate of formation appears to be too slow to account for disinfection, however (Table 1) (16). Viewed from our perspective, the protective effect of  $\beta$ -carotene noted in studies with *S. lutea* mutants (51) is ascribable not to its ability to quench singlet oxygen, as has been proposed, but to its ability to rapidly scavenge HOCl in the cell membrane, thereby preventing loss of essential cellular function.

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1. Elsbach, P. (1973) *N. Engl. J. Med.* **16**, 846-852.
2. Ayoub, E. M. & White, J. G. (1969) *J. Bacteriol.* **98**, 728-736.
3. Cohn, Z. A. (1968) *J. Exp. Med.* **117**, 27-42.
4. Selvaraj, R. J., Paul, B. B., Strauss, R. R., Jacobs, A. A. & Sbarra, A. J. (1974) *Infect. Immun.* **9**, 255-260.
5. Elsbach, P., Pettis, P., Beckerdite, S. & Franson, R. (1973) *J. Bacteriol.* **115**, 490-497.
6. Klebanoff, S. J. & Clark, R. A. (1978) *The Neutrophil: Function and Clinical Disorders* (Elsevier, North-Holland, Amsterdam).
7. Babior, B. M. (1978) *N. Engl. J. Med.* **298**, 659-666; 721-725.
8. Thomas, E. L. (1979) *Infect. Immun.* **23**, 522-531.
9. Zgliczynski, J. M. & Stelmazynska, T. (1975) *Eur. J. Biochem.* **56**, 157-162.
10. Harrison, J. E. & Schultz, J. (1976) *J. Biol. Chem.* **251**, 1371-1374.
11. Stelmazynska, T. & Zgliczynski, J. M. (1978) *Eur. J. Biochem.* **92**, 301-308.
12. Pereira, W. E., Hoyano, Y., Summons, R. E., Bacon, V. A. & Duffield, A. M. (1973) *Biochim. Biophys. Acta* **313**, 170-180.
13. Thomas, E. L. (1979) *Infect. Immun.* **25**, 110-116.
14. Held, A. M., Halko, D. J. & Hurst, J. K. (1978) *J. Am. Chem. Soc.* **100**, 5732-5740.
15. Morris, J. C. (1967) in *Principles and Applications of Water Chemistry*, eds. Faust, S. D. & Hunter, J. V. (Wiley, New York), pp. 23-53.
16. Held, A. M. & Hurst, J. K. (1978) *Biochem. Biophys. Res. Commun.* **81**, 878-885.
17. Morris, J. C. (1966) *J. Phys. Chem.* **70**, 3798-3805.
18. Rutter, W. J. & Hunsley, J. R. (1966) *Methods Enzymol.* **9**, 480-486.
19. Mildvan, A. S., Kobes, R. D. & Rutter, W. J. (1971) *Biochemistry* **10**, 1191-1204.
20. Stanier, R. Y., Adelberg, E. A. & Ingraham, J. (1976) *The Microbial World* (Prentice-Hall, Englewood Cliffs, NJ), 4th Ed., p. 163.
21. Lane, R. H. & Hurst, J. K. (1974) *Biochemistry* **13**, 3292-3297.
22. Ching, T. M. & Ching, K. K. (1972) *Plant Physiol.* **50**, 536-540.
23. Lovenberg, W., Buchanan, B. B. & Rabinowitz, J. C. (1963) *J. Biol. Chem.* **238**, 3899-3913.
24. Harrison, J. E., Watson, B. D. & Schultz, J. (1978) *FEBS Lett.* **92**, 327-332.
25. Montgomery, J. A. & Holum, L. B. (1975) *J. Am. Chem. Soc.* **79**, 2185-2188.
26. Knox, W. E., Stumpf, P. K., Green, D. E. & Auerback, V. H. (1948) *J. Bacteriol.* **55**, 458-460.
27. Torchinskii, Y. M. (1974) *Sulphydryl and Disulfide Groups of Proteins* (Consultants Bureau, New York), pp. 126-129.
28. Harris, J. I. & Waters, M. (1976) *The Enzymes*, ed. Boyer, P. (Academic, New York), Vol. 13, pp. 1-49.
29. Bränden, C. I., Jörnvall, H., Eklund, H. & Furugren, B. (1975) *The Enzymes*, ed. Boyer, P. (Academic, New York), Vol. 11, pp. 103-190.
30. Ingram, J. M. (1969) *Can. J. Biochem.* **47**, 595-601.
31. Holbrook, J. J., Liljas, A., Steindel, S. J. & Rossman, M. G. (1975) *The Enzymes*, ed. Boyer, P. (Academic, New York), Vol. 11, pp. 191-292.
32. Steinman, H. M. & Richards, F. M. (1970) *Biochemistry* **9**, 4360-4372.
33. Camper, A. K. & McFeters, G. A. (1979) *Appl. Environ. Microbiol.* **37**, 633-641.
34. Downie, J. A. & Cox, G. B. (1978) *J. Bacteriol.* **133**, 477-484.
35. Itakaki, E. & Hager, L. P. (1966) *J. Biol. Chem.* **241**, 3687-3695.
36. Kita, K., Yamato, I. & Anraku, Y. (1978) *J. Biol. Chem.* **253**, 8910-8915.
37. Haddock, B. A. & Jones, C. W. (1977) *Bacteriol. Rev.* **41**, 47-99.
38. Yoch, D. C. & Carithers, R. P. (1979) *Microbiol. Rev.* **43**, 384-421.
39. Venkobachar, C., Iyengar, L. & Rao, A. V. S. P. (1977) *Water Res.* **11**, 727-729.
40. Knowles, C. J. (1977) in *Microbial Energetics*, eds. Haddock, B. A. & Hamilton, W. A. (Cambridge Univ. Press, London), pp. 241-284.
41. Pasternack, R. F. & Halliwell, B. (1979) *J. Am. Chem. Soc.* **101**, 1026-1031.
42. Valentine, J. S. (1979) in *Biochemical and Clinical Aspects of Oxygen*, eds. Caughey, W. S. & Caughey, H. (Academic, New York), pp. 659-677.
43. Slivka, A., LoBuglio, A. F. & Weiss, S. J. (1980) *Blood* **55**, 347-350.
44. Venkobachar, C., Iyengar, L. & Rao, A. V. S. P. (1975) *Water Res.* **9**, 119-124.
45. Ingols, R. S. (1958) *Public Health Works* **89**, 105-106.
46. Shih, K. L. & Lederberg, J. (1976) *J. Bacteriol.* **125**, 934-945.
47. Paul, B. B., Jacobs, A. A., Strauss, R. R. & Sbarra, A. J. (1970) *Infect. Immun.* **2**, 414-418.
48. Strauss, R. R., Paul, B. B., Jacobs, A. A. & Sbarra, A. J. (1971) *Infect. Immun.* **3**, 595-602.
49. Jacobs, A. A., Paul, B. B., Strauss, R. R. & Sbarra, A. J. (1970) *Biochem. Biophys. Res. Commun.* **39**, 284-289.
50. Klebanoff, S. J. (1968) *J. Bacteriol.* **95**, 2131-2138.
51. Krinsky, N. I. (1974) *Science* **186**, 363-364.
52. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70-77.