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Myeloperoxidase, Hydrogen Peroxide, Chloride Antimicrobial System: Nitrogen-Chlorine Derivatives of Bacterial Components in Bactericidal Action Against *Escherichia coli*

EDWIN L. THOMAS

Department of Biochemistry, St. Jude Children's Research Hospital and The University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38101

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In the presence of *Escherichia coli*, myeloperoxidase-catalyzed oxidation of chloride ion resulted in formation of long-lived chloramine and/or chloramide derivatives of bacterial components. The same amount of these nitrogen-chlorine (N-Cl) derivatives was obtained with either hypochlorous acid (HOCl) or the myeloperoxidase system, indicating that myeloperoxidase catalyzed the oxidation of chloride to HOCl. Identical killing was obtained with HOCl or the myeloperoxidase system. About 30 to 50% of the oxidizing equivalents of HOCl were detected as N-Cl derivatives of peptides or peptide fragments that were released from the bacteria. The apparent molecular weight distribution of the peptides decreased with increasing amounts of HOCl, suggesting that peptides were fragmented by oxidative cleavage of chloramide derivatives of peptide bonds. The remaining 50 to 70% of the oxidizing equivalents of HOCl were rapidly consumed in peptide bond cleavage or the oxidation of other bacterial components. There was a close correspondence between the oxidation of bacterial sulfhydryls and bactericidal action. The N-Cl derivatives were lost and the oxidation of bacterial sulfhydryls increased over a period of several h at 37°C. These changes were accompanied by increased killing. The increase in sulfhydryl oxidation and killing could be prevented by washing the bacteria to remove the N-Cl derivatives. Therefore, the N-Cl derivatives could oxidize bacterial components long after the myeloperoxidase-catalyzed oxidation of chloride was complete.

The combination of myeloperoxidase, hydrogen peroxide (H_2O_2), and chloride ion (Cl^-) forms an antimicrobial system within phagocytic vesicles of monocytes and polymorphonuclear leukocytes (4, 5, 20, 21, 30, 33, 38, 44). Under some conditions, myeloperoxidase and H_2O_2 can be released from leukocytes (6, 7). This release may permit antimicrobial activity outside the leukocytes, but may also result in damage to host tissues. The myeloperoxidase system has cytotoxic activity against both normal and tumor cells, and may contribute to inflammatory tissue destruction (11, 22).

Agner (1) first proposed that myeloperoxidase catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid, HOCl.

$$H_2O_2 + Cl^- + H^+ \xrightarrow{\text{myeloperoxidase}} HOCl + H_2O$$

Recently, the oxidation of Cl^- to HOCl was demonstrated by using a flow system that continuously removed HOCl, so as to prevent inactivation of myeloperoxidase by HOCl (14). Another problem in detecting HOCl formation is the reaction of HOCl with H_2O_2. Although H_2O_2 acts as an oxidizing agent in the generation of HOCl, it acts as a reducing agent in the reaction with HOCl. The net result of Cl^- oxidation and HOCl reduction is the same as catalyzed by the enzyme catalase, resulting in the loss of the oxidizing equivalents of H_2O_2 and the formation of molecular oxygen, O_2.

$$2\ H_2O_2 \xrightarrow{\text{myeloperoxidase, } Cl^-} 2\ H_2O + O_2$$

Although the reduction of HOCl by H_2O_2 would appear to eliminate any antimicrobial activity, a reactive species may be formed as an intermediate in this reaction. Under alkaline conditions, chlorine (Cl_2) reacts with a deprotonated form of H_2O_2 to yield singlet oxygen (O_2^1), which is an activated form of O_2 (9).

$$Cl_2 + HO_2^- + OH^- \rightarrow O_2^1 + 2\ Cl^- + H_2O$$

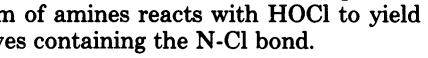
The O_2^1 is very short-lived and rearranges with the emission of light to yield O_2.

$$O_2^1 \rightarrow O_2 + h\nu$$

Incubation of myeloperoxidase with H_2O_2 and Cl^- at neutral or acid pH may yield O_2^1 (29, 30), although the evidence for detection of O_2^1 has been questioned (15, 16). Either HOCl (1, 20) or O_2^1 (20, 29, 31) has been proposed to be the antimicrobial agent produced by the myeloperoxidase system.

The two proposed antimicrobial mechanisms differ in a way that might permit experimental discrimination. If all the HOCl is reduced to O_2^1, then no long-lived oxidizing agents would be detected upon incubating the myeloperoxidase system with bacteria. If HOCl is the antimicrobial agent, it might be possible to detect the accumulation of oxidizing equivalents, assuming that HOCl is not instantly consumed in the oxidation of bacterial components.

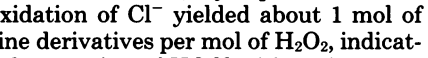
Accumulation of oxidizing equivalents has been observed in the myeloperoxidase-catalyzed formation of chloramines (37, 47). The deprotonated form of amines reacts with HOCl to yield derivatives containing the N-Cl bond.



$$R\text{-}NH_2 + HOCl \rightarrow R\text{-}NH\text{-}Cl + H_2O$$

In the presence of amines, myeloperoxidase-catalyzed oxidation of Cl^- yielded about 1 mol of chloramine derivatives per mol of H_2O_2, indicating that the reaction of HOCl with amines protected myeloperoxidase against inactivation (37, 47). This result also suggests that the chloramines were not reduced by H_2O_2.

Other nitrogenous compounds can also react with HOCl, such as in the conversion of amides to chloramides (17).



$$R\text{-}CO\text{-}NH\text{-}R' + HOCl \rightarrow R\text{-}CO\text{-}NHCl\text{-}R' + H_2O$$

Chloramines and chloramides retain the oxidizing equivalents of HOCl and are powerful oxidizing agents. On the other hand, some of these derivatives undergo internal oxidation, which consumes the oxidizing equivalents of HOCl. For example, chloramine derivatives of α-amino acids undergo oxidative decarboxylation to yield Cl^-, carbon dioxide, ammonia, and an aldehyde (39, 49, 50). Similarly, incubation of proteins with sodium hypochlorite (NaOCl) results in peptide bond cleavage (8), which suggests internal oxidation of chloramide derivatives of the amide nitrogens of peptide bonds.

Incubation with the myeloperoxidase system resulted in release of radioactivity from bacteria grown in the presence of [^{14}C]diaminopimelic acid (34). This observation may indicate decarboxylation of diaminopimelic acid residues with free carboxyl groups, or peptide bond cleavage. Another study showed that myeloperoxidase catalyzed the incorporation of Cl^- into bacterial components (48). However, that study did not distinguish between formation of N-Cl derivatives (chloramines and chloramides) and C-Cl derivatives (chlorinated aromatic or aliphatic compounds). Unlike the N-Cl derivatives, the C-Cl derivatives do not act as oxidizing agents.

The aims of the present study were to determine whether incubation of bacteria with the myeloperoxidase system resulted in accumulation of oxidizing equivalents as N-Cl derivatives of bacterial components and to determine whether such derivatives have any role in bactericidal action. In addition, the reaction of HOCl with amines or amides may prevent the reduction of HOCl by H_2O_2 so that detection of N-Cl derivatives could rule out the participation of O_2^1 in myeloperoxidase-catalyzed bactericidal action.

MATERIALS AND METHODS

Chemicals. All chemicals were of reagent grade. Myeloperoxidase purified from human leukemic granulocytes was provided by M. Morrison and J. Naskalski. Dithiothreitol and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Sigma Chemical Co., St. Louis, Mo., lysozyme was from Worthington Biochemicals Corp., Freehold, N.J., poly-L-lysine hydrochloride (average molecular weight, 375,000) was from Schwarz/Mann, Orangeburg, N.Y., and NaOCl was from Fisher Scientific Co., Fair Lawn, N.J. Sodium borohydride was used to reduce 5,5′-dithiobis(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid (3).

Bacteria and isolated bacterial components. *Escherichia coli* ML 308-225 was grown aerobically at 37°C in medium A (10), modified by addition of 5 mM tris(hydroxymethyl)aminomethane (Tris)-Cl (pH 6.6) with 1% (wt/vol) disodium succinate as the carbon source. Bacteria were harvested in the late logarithmic phase of growth by centrifugation at 5,300 × *g* for 10 min.

Bacteria were extracted by the method of Leive (25) to obtain components released from the outer membrane by extraction with ethylenediaminetetraacetic acid (EDTA). Bacteria were subjected to the osmotic shock procedure of Neu and Heppel (27) to obtain the soluble "periplasmic" proteins. To obtain outer and inner membrane vesicles, bacteria were treated with lysozyme and EDTA by the method of Osborn et al. (28) and subjected to osmotic lysis and low-speed centrifugation by the method of Kaback (18), and then the supernatant was fractionated by sucrose density-gradient centrifugation to yield low-density (inner membrane) and high-density (outer membrane) fractions by a modification of the method of Osborn et al. (28), in which the gradient solutions were buffered with 0.1 M phosphate buffer, pH 6.6. Soluble cytoplasmic proteins were obtained by the sonic oscillation method of Ames (2), by using osmotically shocked bacteria. The soluble fractions were dialyzed against water, clarified by centrifugation, and lyophilized.

Membrane fractions were collected by centrifugation and suspended in water. Protein concentration was determined by the method of Lowry et al. (26), with bovine serum albumin as the protein standard.

Exposure of bacteria to the myeloperoxidase system or HOCl. Bacteria were suspended to 6×10^8 cells per ml in 0.2 M NaCl, with 0.1 M potassium phosphate buffer (pH 6.6) and 1 mM $MgSO_4$ at 0–5°C. The suspensions were made to 0.1 μM in myeloperoxidase. At 1-min intervals, H_2O_2 was added, with each addition sufficient to give a concentration of 30 μM. Incubations were continued after the last addition of H_2O_2 for a total of 30 min of incubation. Alternatively, HOCl was generated by adding portions of 20 mM NaOCl in 0.1 M sodium hydroxide to the buffered bacterial suspension. The NaOCl was added as a single addition, and the incubation was continued for 30 min at 0 to 5°C. All reactions that were carried out without bacteria were performed in the same 0.2 M NaCl–0.1 M phosphate buffer, but without $MgSO_4$.

Quantitation of HOCl and N-Cl derivatives. The concentration of HOCl or N-Cl derivatives was determined by the oxidation of 2 mol of the sulfhydryl compound, 5-thio-2-nitrobenzoic acid, to the disulfide compound, 5,5′-dithiobis(2-nitrobenzoic acid), by 1 mol of the oxidizing agent. An excess of the sulfhydryl compound was added to the reaction mixtures, and absorbance at 412 nm was measured. Centrifugation at $5,300 \times g$ for 5 min at 0 to 5°C was used to remove bacteria. A molar extinction coefficient (ϵ) of 13,600 was assumed for 5-thio-2-nitrobenzoic acid (12).

Sulfhydryl determinations. Two-milliliter portions of reaction mixtures were diluted with 6 ml of the buffer with $MgSO_4$, then centrifuged at $5,300 \times g$ for 10 min at 0 to 5°C. The bacteria were resuspended in 2 ml of the same buffer.

Sulfhydryls were measured by the reaction of 1 mol of sulfhydryl with 5,5′-dithiobis(2-nitrobenzoic acid) to yield 1 mol of 5-thio-2-nitrobenzoic acid (12). The resuspended bacteria were diluted with 4 ml of 0.1 M Tris-Cl buffer (pH 8) with 10 mM EDTA. Portions of 0.1 ml of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M phosphate buffer (pH 7), and 0.5 ml of 10% (wt/vol) sodium dodecyl sulfate were added. The mixtures were incubated for 1 h at 37°C, cooled to 0 to 5°C, and then centrifuged at $18,000 \times g$ for 10 min. Absorbance of the supernatants was measured at 412 nm.

Electrophoresis. Samples were solubilized in 40 mM Tris-Cl buffer (pH 6.8), containing 2.1 M urea, 2.5% (wt/vol) sodium dodecyl sulfate, 3.5% (vol/vol) 2-mercaptoethanol, and 0.0025% (wt/vol) bromophenol blue. Peptides were separated according to molecular weight by the method of Laemmli (24), by using a 7.5 to 15% (wt/vol) gradient of acrylamide. Peptides were visualized by staining with Coomassie brilliant blue (13).

Assay of viability. Serial 1:10 dilutions were prepared from 1-ml portions of reaction mixtures in sterile 0.2 M NaCl with 0.1 M phosphate buffer (pH 6.6) and 1 mM $MgSO_4$. One-milliliter portions of appropriate dilutions were spread on a solid medium containing modified medium A, 2% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) and 1% glucose. Plates were incubated at 25°C, and the number of colonies were recorded after 2 to 3 days.

RESULTS

Formation of N-Cl derivatives. Figure 1 shows the yield of oxidizing equivalents obtained either as N-Cl derivatives or as HOCl after incubation of the myeloperoxidase system with ammonium ion (NH_4^+), or without other additions. In the presence of NH_4^+, about 1 mol of a N-Cl derivative was obtained per mol of H_2O_2, consistent with myeloperoxidase-catalyzed oxidation of Cl^- to yield 1 mol of HOCl per mol of H_2O_2, followed by the reaction of HOCl with NH_4^+. The product was assumed to be monochloramine (NH_2Cl), rather than $NHCl_2$ or NCl_3, based on the high ratio of NH_4^+ to HOCl.

About 1 mol of N-Cl derivatives was obtained per mol of H_2O_2 with all the primary and secondary amines tested, at pH 5, 6.6, or 7.4. The yields were lower in the presence of the tertiary amine trimethylamine, or the amide compounds acetamide and *N*-acetylglycine, or at pH 8.

In the absence of amines or amides, there was a very low yield of HOCl. After this incubation, additions of NH_4^+, myeloperoxidase, and H_2O_2 were required to obtain NH_2Cl. Therefore, all the myeloperoxidase had been inactivated and all the H_2O_2 had been consumed in the oxidation of Cl^- and the reduction of HOCl. When the oxidation of Cl^- was carried out with a 10-fold

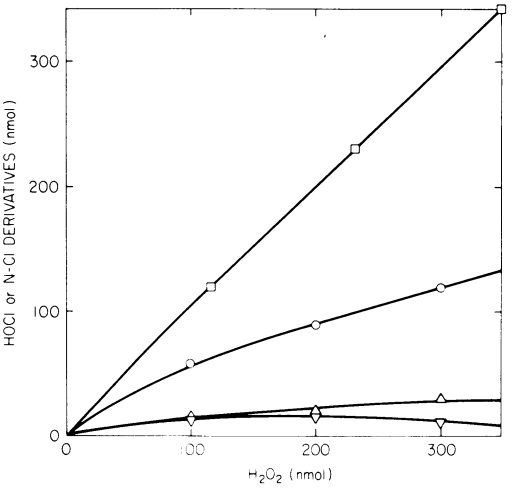


FIG. 1. *Myeloperoxidase-catalyzed formation of HOCl or N-Cl derivatives. Reaction mixtures contained 0.1 μM myeloperoxidase and the indicated amounts of H_2O_2 per ml in the 0.2 M NaCl-0.1 M phosphate buffer with no other additions (▽), with 5 mM $(NH_4)_2SO_4$ (□), with bacteria and 1 mM $MgSO_4$ (○), or with the supernatant obtained by suspending bacteria in the buffer with $MgSO_4$ and then centrifuging at $5,300 \times g$ for 10 min at 0 to 5°C (△). Incubations were continued for a total of 30 min at 0 to 5°C, and then the amounts of HOCl or N-Cl derivatives were measured.*

lower myeloperoxidase concentration, no HOCl was obtained and excess unreacted H_2O_2 remained. Therefore, the myeloperoxidase had been inactivated before an amount of HOCl was generated equal to half the amount of H_2O_2 added.

Figure 1 also shows that in the presence of *E. coli* accumulation of oxidizing equivalents was observed. Therefore, the *E. coli* suspension contained a sufficient amount of amines and/or amides to convert most or all of the HOCl formed to N-Cl derivatives. Lowering the cell density by a factor of 10 had little effect on the yield of N-Cl derivatives, indicating that the yield of these derivatives was not limited by the amount of amines and/or amides. On the other hand, the yield of N-Cl derivatives was less than 1 mol per mol of H_2O_2. Therefore, part of the oxidizing equivalents of HOCl had been consumed in the oxidation of bacterial components.

Figure 1 also shows that the supernatant obtained by centrifuging the bacterial suspension did not support the accumulation of oxidizing equivalents. Therefore, the amines and/or amides involved in formation of N-Cl derivatives were in or on the bacteria and not in the suspending fluid.

Figure 2 shows that unreacted oxidizing equivalents were also detected when HOCl was generated by adding a solution of NaOCl to the buffered *E. coli* suspension. The yield of N-Cl derivatives was nearly identical to that obtained with the myeloperoxidase system. These results confirm that the low yield of N-Cl derivatives obtained with the myeloperoxidase system was not due to reduction of HOCl by H_2O_2. Instead, part of the HOCl was consumed in the oxidation of bacterial components. About 50 to 70% of the HOCl added was consumed.

Figure 2 also shows the amount of N-Cl derivatives detected either in the supernatant or associated with the bacteria after centrifugation. Although results presented above indicated that formation of N-Cl derivatives was due to reaction of HOCl with bacterial components, most of the N-Cl derivatives were found in the supernatant after centrifugation. As described below, amine and/or amide components were released from the bacteria as N-Cl derivatives.

The yield of N-Cl derivatives obtained with HOCl or the myeloperoxidase system was about the same at 37°C or at 0 to 5°C. On the other hand, the N-Cl derivatives were stable at 0 to 5°C, but were slowly lost during prolonged incubation at 37°C. About 40% of the oxidizing equivalents remained after 2 h at 37°C. In the experiments reported here, bacteria were exposed to the myeloperoxidase system at 0 to 5°C to improve the discrimination between fast reactions (such as Cl^- oxidation and formation of N-Cl derivatives) and slow reactions (such as loss of N-Cl derivatives).

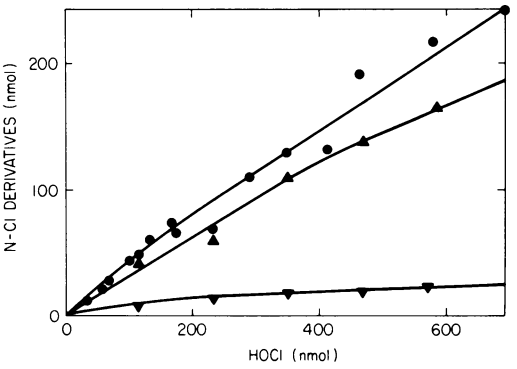


FIG. 2. *Yield of N-Cl derivatives from HOCl. Bacteria were incubated 30 min at 0 to 5°C with the indicated amounts of HOCl per ml. The amount of N-Cl derivatives was measured in the total reaction mixtures (●). Also, reaction mixtures were centrifuged at 5,300 × g for 10 min at 0 to 5°C, and then the amounts of N-Cl derivatives were measured in the supernatant (▲) and in the suspensions obtained by resuspending the bacteria to the original volume (▼).*

The rate of loss of the N-Cl derivatives at 37°C was about the same either in the complete reaction mixture or in the supernatant after centrifugation to remove the bacteria. Therefore, the loss of N-Cl derivatives was not primarily due to oxidation of other bacterial components by the N-Cl derivatives. Internal oxidation of the N-Cl derivatives may have contributed to the loss.

Differing reactivity of HOCl and N-Cl derivatives. Figure 3 shows that the addition of H_2O_2 to HOCl resulted in reduction of 1 mol of HOCl per mol of H_2O_2. On the other hand, the oxidizing equivalents were not lost when NH_4^+ (or an amine or amide) was present, indicating that the N-Cl derivatives were not reduced by H_2O_2. The same results were obtained when HOCl was added to solutions containing H_2O_2 and NH_4^+. Therefore, with 10 mM NH_4^+ and 0.1 to 0.3 mM H_2O_2, the rate of formation of N-Cl derivatives was much faster than the rate of reduction of HOCl.

Oxidation of 5-thio-2-nitrobenzoic acid by H_2O_2 was negligible during the time required for the measurements shown in Fig. 3. Also, unreacted H_2O_2 was detected after the incubation of N-Cl derivatives with H_2O_2.

The difference in reactivity of HOCl and N-Cl derivatives could be used to demonstrate formation of N-Cl derivatives of bacterial components. Figure 3 shows that in the presence of *E. coli*, H_2O_2 did not reduce the unreacted oxidizing

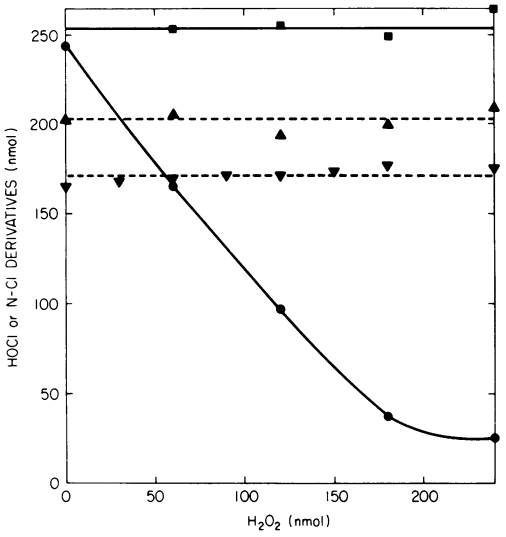


FIG. 3. *Differing reactivity of HOCl and N-Cl derivatives with H_2O_2. The indicated amounts of H_2O_2 per ml were added to reaction mixtures containing 0.25 mM HOCl (●), 0.25 mM HOCl and 10 mM NH_4^+ (■), bacteria that had been incubated with 0.6 mM HOCl for 30 min at 0 to 5°C (▲), or the supernatant obtained by incubating bacteria with 0.6 mM HOCl for 30 min at 0 to 5°C and then centrifuging at 5,300 × g for 10 min at 0 to 5°C (▼). After the addition of H_2O_2, the incubations were continued for 15 min at 0 to 5°C, and then the amounts of HOCl or N-Cl derivatives were measured.*

equivalents. The same results were obtained when H_2O_2 was added to the supernatant from bacteria that had been incubated with HOCl. Therefore, the oxidizing equivalents were in the form of N-Cl derivatives, rather than HOCl.

Released N-Cl derivatives. Incubation of *E. coli* with HOCl resulted in release of protein in a soluble form. Upon addition of 0.1, 0.4, and 1.2 mM HOCl, the amounts of protein obtained in the supernatant were 50, 65, and 90 μg/ml, respectively, after centrifugation to remove bacteria. These amounts of protein were less than 6% of the dry weight of the bacteria. The amount of released material increased up to twofold upon prolonging the incubation for 2 h at 37°C.

The supernatant fractions were treated with dithiothreitol to eliminate oxidizing agents, dialyzed against water to remove salts, and then concentrated by lyophilization. Figure 4A through H shows that the protein fractions prepared in this way could be resolved by electrophoresis into a number of peptides with apparent molecular weights of about 100,000 to 10,000. In other experiments, a similar distribution of peptides was obtained by incubating the bacteria with the myeloperoxidase system. Consistent with the results of others (19), myeloperoxidase was bound to the bacteria. Myeloperoxidase did not appear in the supernatant either when added alone or with H_2O_2.

The amounts of peptides observed on gels increased upon prolonged exposure of the bacteria to HOCl. On the other hand, the amounts appeared to decrease with increasing amounts of HOCl. As observed on gels, the largest amount of peptides was obtained by treating *E. coli* with about 0.1 mM HOCl, whereas direct protein determinations on the supernatants indicated

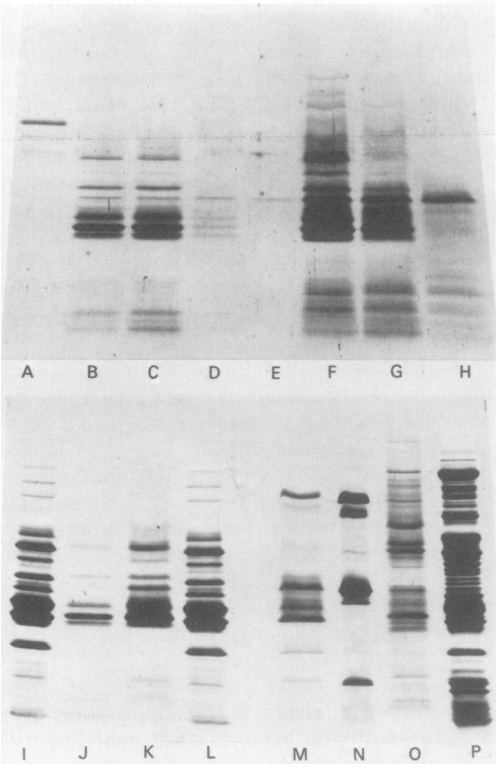


FIG. 4. *Separation of released peptides by electrophoresis. The samples contained: supernatant fractions from bacteria incubated with 0, 0.1, 0.4, and 1.2 mM HOCl for 30 min at 0 to 5°C (A through D) or for an additional incubation period of 2 h at 37°C (E through H); the supernatant fraction from bacteria subjected to osmotic shock (I and L); supernatant fractions from bacteria incubated with 0.1 mM HOCl for 30 min at 0 to 5°C (J) or for an additional incubation period of 2 h at 37°C (K); the supernatant fraction from bacteria extracted with EDTA (M); outer membrane (N); inner membrane (O); cytoplasmic proteins (P). Bacteria were removed from reaction mixtures treated with HOCl by centrifugation at 18,000 × g for 20 min at 0 to 5°C, and the supernatant fractions were reduced with 1 mM dithiothreitol, dialyzed against water, and lyophilized.*

increasing protein concentrations with increasing amounts of HOCl. These results suggested that the released peptides were fragmented by HOCl. Peptides of molecular weight less than 8,000 would have been lost during the dialysis step before electrophoresis.

Figure 4I through P shows that the apparent molecular weight distribution of the HOCl-released peptides was similar to that of the soluble periplasmic proteins. The permeability of the outer membrane may have been increased by exposure to HOCl, resulting in release of soluble proteins from the space between the outer and inner membranes. Also, some of the released material may have been derived from outer-membrane components in that components characteristic of the outer membrane were detected by staining the gel for carbohydrate. Figure 4I through P also shows that the distribution of the released peptides differed from that of other isolated cell fractions, including the outer-membrane components that are released by EDTA extraction, the outer membrane, the inner membrane, and the cytoplasmic proteins. This observation suggested that release of peptides was not due to lysis of the cells, which would release the soluble proteins, or to solubilization of the cell membranes, which would release all the proteins.

To demonstrate that the N-Cl derivatives obtained in the supernatant were derivatives of the released peptides or peptide fragments, a procedure was developed for the chromatographic separation of the nonreduced N-Cl derivatives on Sephadex G-50, followed by titration of the separated derivatives with 5-thio-2-nitrobenzoic acid. Figure 5 shows that high- and low-molecular-weight N-Cl derivatives were sufficiently stable to be separated and quantitated in this way. When polylysine was mixed with HOCl and then chromatographed, a high-molecular-weight (excluded) fraction was obtained, corresponding to derivatives of polylysine containing multiple N-Cl bonds.

Dissociation of the poly(N-Cl)-polylysine to yield HOCl did not appear significant within the time required for chromatography (ca. 15 to 30 min). Although a low-molecular-weight fraction with oxidizing activity was obtained, this activity was not due to HOCl. Other experiments indicated that HOCl did not emerge from the column; no oxidizing agent was detected in the column effluent when the sample consisted of HOCl added to buffer without polylysine or other amines. A low-molecular-weight fraction corresponding to HOCl could be obtained only if the column material was pretreated with excess NaOCl. Apparently, HOCl was consumed in oxidation of the column material, whereas the

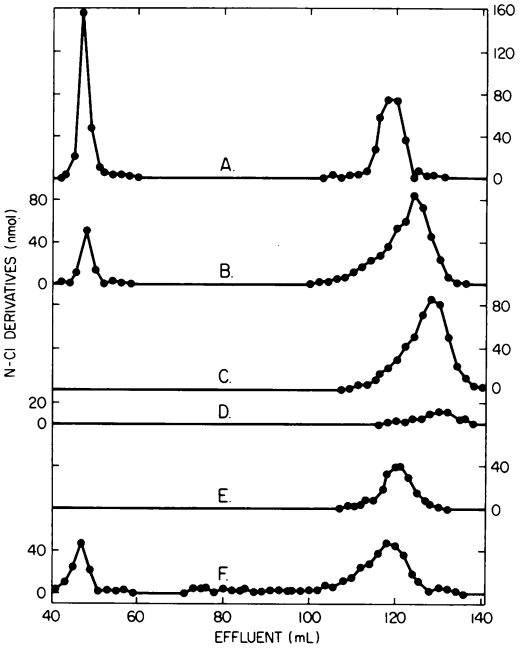


FIG. 5. *Chromatographic fractionation of N-Cl derivatives. Reaction mixtures containing 0.6 mM HOCl and various amounts of polylysine were incubated for 15 min at 0 to 5°C. The reaction mixtures were 10 mM (A), 5 mM (B), 1 mM (C), and 0.2 mM (D) in lysine residues. Alternatively, the reaction mixture consisted of bacteria incubated with 0.3 mM HOCl for 30 min at 0 to 5°C. The mixture was centrifuged at 5,300 × g for 10 min at 0 to 5°C to yield a supernatant fraction (E). Also, a reaction mixture (10 ml) consisting of bacteria incubated with 0.3 mM HOCl for 30 min at 0 to 5°C was centrifuged, and the supernatant was reduced with 1 mM dithiothreitol, dialyzed against water, lyophilized, suspended in 2 ml of the 0.2 M NaCl-0.1 M phosphate buffer, and then incubated with 0.3 mM HOCl for 15 min at 0 to 5°C (F). Fractions A through F were chromatographed on a column (60 by 1.6 cm) of Sephadex G-50 (Pharmacia Fine Chemicals) and equilibrated and eluted with the 0.2 M NaCl-0.1 M phosphate buffer. Fractions of about 2 ml were collected, the volumes were determined by weighing, and then the amounts of N-Cl derivatives were measured.*

N-Cl derivatives did not react with the column material within the time required for chromatography. With the reaction mixture 10 mM in lysine residues, the recovery of oxidizing equivalents in the effluent was 93% of that applied to the column.

Also, the low-molecular-weight fraction obtained in the presence of polylysine was not due to formation of N-Cl derivatives of low-molecular-weight contaminants. The same results were obtained with polylysine that had been dialyzed or pre-chromatographed on Sephadex G-50

to remove low-molecular-weight substances. Therefore, the low-molecular-weight fraction appeared to be due to fragmentation of the polylysine and formation of N-Cl derivatives of the fragments.

Figure 5 shows that when the concentration of lysine residues relative to HOCl was lowered, the amount of high-molecular-weight N-Cl derivatives decreased, whereas the low-molecular-weight derivatives increased in amount and decreased in average size. With molar ratios of lysine residues to HOCl of 8.3 and 1.7, the recovery of oxidizing equivalents was 93 and 83%, respectively. With a ratio of 0.3, no high-molecular-weight derivatives were detected, and the recovery fell to 11%. Therefore, low ratios of amine groups to HOCl favored fragmentation, possibly by oxidative cleavage of amide bonds. In other experiments, similar results were obtained with protein substrates in place of polylysine.

Figure 5 also shows that only a low-molecular-weight oxidizing fraction was obtained when the supernatant from HOCl-treated *E. coli* was chromatographed. Therefore, the oxidizing equivalents were associated with peptide fragments or other low-molecular-weight substances. The recovery of oxidizing equivalents was 36% of that added to the bacteria and 91% of that applied to the column.

In other experiments, the supernatant was reduced with dithiothreitol, dialyzed to remove low-molecular-weight substances, incubated again with HOCl, and then chromatographed. Only low-molecular-weight N-Cl derivatives were obtained, indicating that fragmentation of peptides was the probable source of low-molecular-weight N-Cl derivatives. Figure 5 shows that when a larger volume of the supernatant was reduced, dialyzed, concentrated by lyophilization, incubated with HOCl, and then chromatographed, both high- and low-molecular-weight derivatives were obtained. Therefore, the inability to demonstrate high-molecular-weight derivatives in the original supernatant was due to the low protein concentration, which favored fragmentation.

Oxidation of sulfhydryls. Figure 6 shows the oxidation of *E. coli* sulfhydryls by HOCl. Similar results were obtained with the myeloperoxidase system, consistent with myeloperoxidase-catalyzed oxidation of Cl^- to HOCl. About 1 mol of sulfhydryls was oxidized per 3.7 mol of HOCl consumed, assuming that 60% of the HOCl was consumed (as in Fig. 2). This result may indicate that most of the HOCl was consumed in the oxidation of components other than sulfhydryls. Alternatively, the sulfhydryls may have been oxidized to sulfonic acids, which would require 3 mol of HOCl per mol of sulfhydryls.

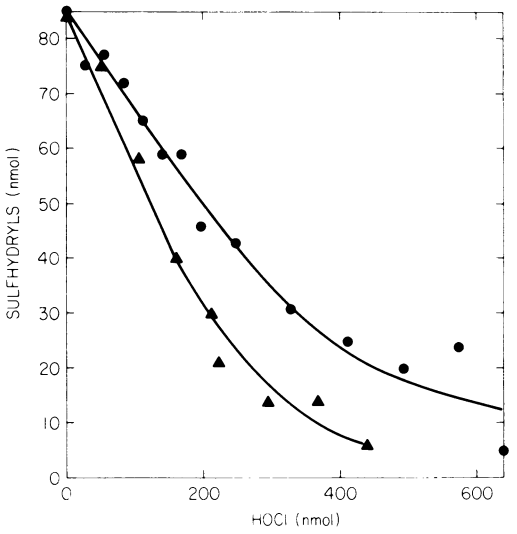


FIG. 6. *Oxidation of sulfhydryls. Bacteria were incubated for 30 min at 0 to 5°C with the indicated amounts of HOCl per ml. Portions were taken for determination of the amount of sulfhydryls per ml of reaction mixtures (●). The remainders were incubated 2 h at 37°C, and then the sulfhydryls were determined (▲).*

Figure 6 also shows that the amount of oxidized sulfhydryls increased when the bacteria were incubated in the presence of the N-Cl derivatives for 2 h at 37°C. This increased sulfhydryl oxidation could be prevented by washing the bacteria before the incubation at 37°C or by holding the bacteria at 0 to 5°C. Therefore, the N-Cl derivatives were responsible for the increased oxidation.

Bactericidal action. Figure 7 shows the killing of *E. coli* by either HOCl or the myeloperoxidase system. Killing is expressed as the logarithm of the ratio of viable cells in an untreated suspension to that in a suspension exposed to HOCl or the myeloperoxidase system. A value of zero indicates a ratio of 1 or no loss of viability, whereas a value of 4 indicates that only 1 cell in 10,000 remained viable.

Identical results were obtained when killing per mol of HOCl was compared to killing per mol of the H_2O_2 added to the myeloperoxidase system. These results were consistent with myeloperoxidase-catalyzed oxidation of Cl^- to yield 1 mol of HOCl per mol of H_2O_2. The amount of HOCl required for complete killing was about the same as required for complete oxidation of sulfhydryls (cf. Fig. 6).

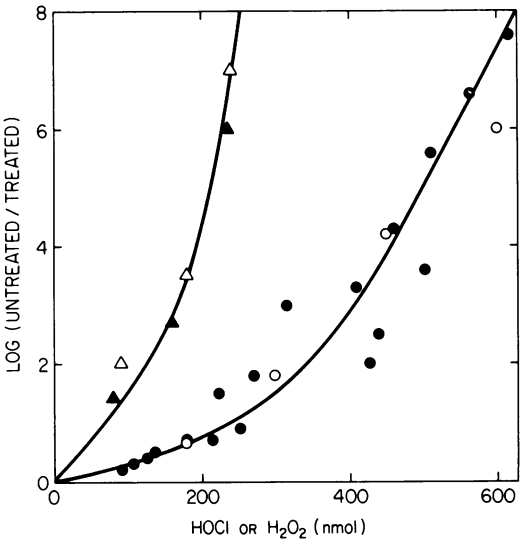


FIG. 7. *Bactericidal action. Bacteria were incubated with the indicated amounts of HOCl (●, ▲) per ml of reaction mixture or with 0.1 μM myeloperoxidase and the indicated amounts of H_2O_2 (○, △) per ml. After a 30-min incubation at 0 to 5°C, portions were taken for determination of viability (●, ○). The remainders were incubated for 2 h at 37°C, and then viability was measured (▲, △).*

Removing the N-Cl derivatives by washing the bacteria or by reduction with dithiothreitol had no effect on the loss of viability as measured immediately after the 30-min incubation period. On the other hand, Fig. 7 shows that continued incubation of the bacteria in the presence of the N-Cl derivatives for 2 h at 37°C resulted in increased killing. This increase could be prevented by washing the bacteria, by adding dithiothreitol, or by holding the bacteria at 0 to 5°C for 2 h. Therefore, the N-Cl derivatives appeared responsible for the increased killing obtained during prolonged incubations.

DISCUSSION

Role of HOCl and O_2^1. Killing of *E. coli* by the myeloperoxidase system was due to the oxidation of Cl^- to HOCl, followed by the reaction of HOCl with bacterial components. The amine and/or amide components of the bacteria were present in sufficient amounts to convert all the HOCl to N-Cl derivatives. The reaction of HOCl with amines or amides competes with and thus prevents the reaction of HOCl with H_2O_2, which may yield O_2^1. Therefore, it would appear that no O_2^1 was produced in the presence of the bacteria and that killing was due only to HOCl. Consistent with this interpretation, identical killing was obtained either with the myeloperoxidase system or with non-enzymatically generated HOCl.

The presence of bacteria alters the chemistry of the myeloperoxidase system. The chemical constituents of the bacteria can suppress O_2^1 formation, and also probably protect myeloperoxidase against inactivation. Amine and/or amide components of the bacteria can be considered as a fourth component of the myeloperoxidase system. Exogenous amines can also participate in killing (E. L. Thomas, unpublished data).

The involvement of bacterial components provides an explanation for results of experiments in which the myeloperoxidase system is physically separated from the bacteria by a dialysis membrane. No killing is obtained under these conditions (32). The probable explanation is that the HOCl produced in the myeloperoxidase compartment is reduced by H_2O_2 before it can diffuse into the bacterial compartment. Although O_2^1 may be produced in the myeloperoxidase compartment, O_2^1 would be too short-lived to diffuse into the bacterial compartment. Consistent with this interpretation, killing can be obtained when NH_4^+ or amines are added to the myeloperoxidase compartment (33).

Because the conditions within phagocytic vesicles of leukocytes would be different from those used in this study, it is not possible to conclude that reduction of HOCl by H_2O_2 does not occur within leukocytes. However, amines and amides are present in significant amounts within phagocytic vesicles. The phagocytized bacterium makes up a large part of the volume of the phagocytic vesicle so that the bacterial components would be present in high concentrations. Also, leukocytes contain high concentrations of the amine compound taurine (36) and arginine-rich cationic proteins that are found within the phagocytic vesicle (45, 46). Bacterial and leukocyte components could react with HOCl and prevent O_2^1 formation.

Sulfhydryl oxidation. A close correspondence was observed between the oxidation of bacterial sulfhydryls and bactericidal action. Oxidation of sulfhydryls has previously been proposed as the mechanism of antibacterial action of Cl_2 (23). Nevertheless, results presented here do not indicate that sulfhydryl oxidation is the only reaction that contributes to killing. The amount of oxidizing equivalents that was consumed was much greater than the amount of oxidized sulfhydryls. Other reactions that may contribute to the consumption of oxidizing equivalents include oxidation of other easily oxidized bacterial components, chlorination of bacterial components to yield C-Cl derivatives, and

internal oxidation of N-Cl derivatives.

In part, the consumption of oxidizing equivalents may have been due to the oxidation of sulfhydryls to sulfonic acids, which would consume 3 mol of HOCl per mol of sulfhydryls. The ability of HOCl and N-Cl derivatives to oxidize sulfhydryls (R-SH) was the basis for their quantitation.

$$\text{R-NH-Cl} + 2\ \text{R-SH} \rightarrow \text{R-NH}_3^+ + \text{R-S-S-R} + \text{Cl}^-$$

Although 5-thio-2-nitrobenzoic acid was oxidized to the disulfide compound when present in excess, this and other sulfhydryl compounds can be oxidized to sulfonic acids by excess HOCl (35).

Only about 2% of the oxidized bacterial sulfhydryls were in the form of sulfenyl derivatives (Thomas, unpublished data), indicating that sulfhydryls were oxidized to other forms such as sulfonic acids. These results differ from those obtained with the peroxidase, H_2O_2, I^- or thiocyanate (SCN^-) systems (41, 42). With I^- or SCN^- as the cofactor, a major portion of the bacterial sulfhydryls were oxidized to sulfenyl derivatives. Exogenous reducing agents could partly reverse the antibacterial action of these systems (40–43), apparently by reducing the sulfenyl derivatives back to sulfhydryls. In contrast, reducing agents did not reverse the antimicrobial action of HOCl.

Role of N-Cl derivatives. Internal oxidation of N-Cl derivatives appeared responsible for the fragmentation of peptides to yield low-molecular-weight N-Cl derivatives. This reaction may also have contributed to the release of the peptides from the bacterial cell envelope. The breaking of peptide or other amide bonds could contribute to killing by disrupting bacterial structure and inactivating essential enzymes. On the other hand, killing can be obtained in the presence of NH_4^+ or exogenous amines, even though they compete with bacterial components for formation of N-Cl derivatives and diminish the release of peptides (Thomas, unpublished data). Also, disruption of the outer membrane or the loss of the periplasmic proteins does not result in loss of viability of *E. coli* (25, 27).

The stable N-Cl derivatives appeared to have a role in killing during incubations of several hours at 37°C. Oxidation of bacterial sulfhydryls and killing continued to increase, although all the oxidizing equivalents were in the form of N-Cl derivatives. Removal or reduction of the N-Cl derivatives prevented the increase in oxidation and killing. The bacterial sulfhydryls may have been oxidized by HOCl obtained from the hydrolysis of N-Cl derivatives.

$$\text{R-NH-Cl} + \text{H}_2\text{O} \rightleftharpoons \text{R-NH}_2 + \text{HOCl}$$

However, the extent of formation of HOCl from N-Cl derivatives appeared insignificant even under conditions that should shift this equilibrium, such as the separation of the derivatives from HOCl by chromatography, or the addition of H_2O_2. It is more likely that the slow killing was due to direct oxidation of bacterial components by the N-Cl derivatives. The relative abilities of N-Cl derivatives to oxidize bacterial components will be considered in another report.

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LITERATURE CITED

1. **Agner, K.** 1972. Biological effects of hypochlorous acid formed by "MPO"-peroxidation in the presence of chloride ions, p. 329–335. *In* A. Akeson and A. Ehrenberg (ed.), Structure and function of oxidation-reduction enzymes. Pergamon Press, Inc., Oxford.
2. **Ames, G. F.-L.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249**:634–644.
3. **Aune, T. M., and E. L. Thomas.** 1977. Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation of thiocyanate ion. Eur. J. Biochem. **80**:209–214.
4. **Baboir, B. M.** 1978. Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. **298**:659–668, 721–725.
5. **Baehner, R. L.** 1974. Molecular basis for functional disorders of phagocytes. J. Pediatr. **84**:317–327.
6. **Baehner, R. L., M. J. Karnovsky, and M. L. Karnovsky.** 1969. Degranulation of leukocytes in chronic granulomatous disease. J. Clin. Invest. **48**:187–192.
7. **Baehner, R. L., D. G. Nathan, and W. B. Castle.** 1971. Oxidant injury of caucasian glucose-6-phosphate dehydrogenase-deficient red blood cells by phagocytizing leukocytes during infection. J. Clin. Invest. **50**: 2466–2473.
8. **Baker, R. W. R.** 1947. Studies on the reaction between sodium hypochlorite and proteins. Biochem. J. **41**: 337–342.
9. **Browne, R. J., and E. A. Ogryzlo.** 1964. Chemiluminescence from the reaction of chlorine with aqueous hydrogen peroxide. Proc. Chem. Soc. **1964**:117.
10. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of Escherichia coli requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
11. **Edelson, P. J., and Z. A. Cohn.** 1973. Peroxidase-mediated mammalian cell cytotoxicity. J. Exp. Med. **138**: 318–323.
12. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82**:70–77.
13. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**: 2606–2617.
14. **Harrison, J. E., and J. Schultz.** 1976. Studies on the chlorinating activity of myeloperoxidase. J. Biol. Chem. **251**:1371–1374.
15. **Harrison, J. E., B. D. Watson, and J. Schultz.** 1978.

Myeloperoxidase and singlet oxygen: a reappraisal. FEBS Lett. **92**:327-332.

16. **Held, A. M., and J. K. Hurst.** 1978. Ambiguity associated with use of singlet oxygen trapping agents in myeloperoxidase-catalyzed oxidations. Biochem. Biophys. Res. Commun. **81**:878-885.
17. **Johnson, R. A., and F. D. Green.** 1975. Chlorination with N-chloro amides. I. Inter- and intramolecular chlorination. J. Org. Chem. **40**:2186-2192.
18. **Kaback, H. R.** 1971. Bacterial membranes. Methods Enzymol. **22**:99-120.
19. **Klebanoff, S. J.** 1970. Myeloperoxidase-mediated antimicrobial systems and their role in leukocyte function, p. 89-110. *In* J. Schultz (ed.), Biochemistry of the phagocytic process. North-Holland, Amsterdam.
20. **Klebanoff, S. J.** 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Semin. Hematol. **12**:117-142.
21. **Klebanoff, S. J.** 1975. Antimicrobial systems of the polymorphonuclear leukocyte, p. 45-59. *In* J. A. Bellanti and D. H. Dayton (ed.), The phagocytic cell in host resistance. Raven Press, New York.
22. **Klebanoff, S. J., R. A. Clark, and H. Rosen.** 1976. Myeloperoxidase-mediated cytotoxicity. Cancer Enzymol. **12**:267-285.
23. **Knox, W. E., P. K. Stumpf, D. E. Green, and V. H. Auerbach.** 1948. The inhibition of sulfhydryl enzymes as the basis of the bactericidal action of chlorine. J. Bacteriol. **55**:451-458.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
25. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophys. Res. Commun. **21**:290-296.
26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
27. **Neu, H. C., and L. A. Heppel.** 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685-3692.
28. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962-3972.
29. **Piatt, J. F., A. S. Cheema, and P. J. O'Brien.** 1977. Peroxidase-catalyzed singlet oxygen formation from hydrogen peroxide. FEBS Lett. **74**:251-254.
30. **Quie, P. G., E. L. Mills, and B. Holmes.** 1977. Molecular events during phagocytosis by human neutrophils. Prog. Hematol. **10**:193-210.
31. **Rosen, H., and S. J. Klebanoff.** 1977. Formation of singlet oxygen by the myeloperoxidase-mediated antimicrobial system. J. Biol. Chem. **252**:4803-4810.
32. **Sbarra, A. J., B. B. Paul, A. A. Jacobs, R. R. Strauss, and G. W. Mitchell, Jr.** 1972. Role of the phagocyte in host-parasite interactions. XXXVIII. Metabolic activities of the phagocyte as related to antimicrobial action. RES J. Reticuloendothel. Soc. **12**:109-126.
33. **Sbarra, A. J., R. J. Selvaraj, B. B. Paul, P. K. F. Poskitt, G. W. Mitchell, Jr., F. Louis, and M. A. Asbell.** 1977. Granulocyte biochemistry and a hydrogen peroxide-dependent antimicrobial system, p. 29-48. *In* T. J. Greenwalt and G. A. Jamieson (ed.), The granulocyte: function and clinical utilization. Alan R. Liss, Inc., New York.
34. **Selvaraj, R. J., B. B. Paul, R. R. Strauss, A. A. Jacobs, and A. J. Sbarra.** 1974. Oxidative peptide cleavage and decarboxylation by the MPO-H_2O_2-Cl^- antimicrobial system. Infect. Immun. **9**:255-260.
35. **Silverstein, R. M., and L. P. Hager.** 1974. The chloroperoxidase-catalyzed oxidation of thiols and disulfides to sulfenyl chlorides. Biochemistry **13**:5069-5073.
36. **Soupart, P.** 1962. Free amino acids in blood and urine in the human, p. 220-262. *In* J. T. Holden (ed.), Amino acid pools. Elsevier, Amsterdam.
37. **Stelmaszynska, T., and J. M. Zgliczynski.** 1974. Myeloperoxidase of human neutrophilic granulocytes as chlorinating enzyme. Eur. J. Biochem. **45**:305-312.
38. **Stossal, T. P.** 1974. Phagocytosis. New Engl. J. Med. **290**: 717-723, 774-780, 833-839.
39. **Strauss, R. R., B. B. Paul, A. A. Jacobs, and A. J. Sbarra.** 1971. Role of the phagocyte in host-parasite interactions. XXVII. Myeloperoxidase-H_2O_2-Cl^--mediated aldehyde formation and its relationship to antimicrobial activity. Infect. Immun. **3**:595-602.
40. **Thomas, E. L., and T. M. Aune.** 1978. Cofactor role of iodide in peroxidase antimicrobial action against *Escherichia coli*. Antimicrob. Agents Chemother. **13**: 1000-1005.
41. **Thomas, E. L., and T. M. Aune.** 1978. Lactoperoxidase, peroxide, thiocyanate antimicrobial system: correlation of sulfhydryl oxidation with antimicrobial action. Infect. Immun. **20**:456-463.
42. **Thomas, E. L., and T. M. Aune.** 1978. Oxidation of *Escherichia coli* sulfhydryl components by the peroxidase-hydrogen peroxide-iodide antimicrobial system. Antimicrob. Agents Chemother. **13**:1006-1010.
43. **Thomas, E. L., and T. M. Aune.** 1978. Susceptibility of *Escherichia coli* to bactericidal action of lactoperoxidase, peroxide, and iodide or thiocyanate. Antimicrob. Agents Chemother. **13**:261-265.
44. **Winkelstein, J. A., and R. H. Drachman.** 1974. Phagocytosis. The normal process and its clinically significant abnormalities. Pediatr. Clin. N. Am. **21**:551-569.
45. **Zeya, H. I., and J. K. Spitznagel.** 1966. Cationic proteins of polymorphonuclear leukocyte lysozomes. I. Resolution of antibacterial and enzymatic activities. J. Bacteriol. **91**:750-754.
46. **Zeya, H. I., and J. K. Spitznagel.** 1966. Cationic proteins of polymorphonuclear leukocyte lysozomes. II. Composition, properties, and mechanisms of antibacterial action. J. Bacteriol. **91**:755-762.
47. **Zgliczynski, J. M., R. J. Selvaraj, B. B. Paul, T. Stelmaszynska, P. K. F. Poskitt, and A. J. Sbarra.** 1977. Chlorination by the myeloperoxidase-H_2O_2-Cl^- antimicrobial system at acid and neutral pH. Proc. Soc. Exp. Biol. Med. **154**:418-422.
48. **Zgliczynski, J. M., and T. Stelmaszynska.** 1975. Chlorinating ability of human phagocytizing leukocytes. Eur. J. Biochem. **56**:157-162.
49. **Zgliczynski, J. M., T. Stelmaszynska, J. Domanski, and W. Ostrowski.** 1971. Chloramines as intermediates of oxidation reaction of amino acids by myeloperoxidase. Biochim. Biophys. Acta. **235**:419-424.
50. **Zgliczynski, J. M., T. Stelmaszynska, W. Ostrowski, J. Naskalski, and J. Sznajd.** 1968. Myeloperoxidase of human leukaemic leucocytes. Oxidation of amino acids in the presence of hydrogen peroxide. Eur. J. Biochem. **4**:540-547.