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Myeloperoxidase-Hydrogen Peroxide-Chloride Antimicrobial System: Effect of Exogenous Amines on Antibacterial Action Against *Escherichia coli*

EDWIN L. THOMAS

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

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Exogenous ammonium ions (NH_4^+) and amine compounds had a profound influence on the antibacterial activity of the myeloperoxidase-hydrogen peroxide-chloride system against *Escherichia coli*. The rate of killing increased in the presence of NH_4^+ and certain guanidino compounds and decreased in the presence of α-amino acids, polylysine, taurine, or tris(hydroxymethyl)aminomethane. Myeloperoxidase catalyzed the oxidation of chloride to hypochlorous acid, which reacted either with bacterial amine or amide components or both or with the exogenous compounds to yield chloramine or chloramide derivatives or both. These nitrogen-chlorine derivatives could oxidize bacterial components. Killing was correlated with oxidation of bacterial components. The rate of oxidation of bacterial sulfhydryls increased in the presence of the compounds that increased the rate of killing and decreased in the presence of the other compounds. The reaction of HOCl with NH_4^+ yielded monochloramine (NH_2Cl), which could be extracted into organic solvents. The N-Cl derivatives of bacterial components or of polylysine, taurine, or tris(hydroxymethyl)aminomethane could not be extracted. The effect of NH_4^+ on killing is attributed to the ability of NH_2Cl to penetrate the hydrophobic cell membrane and thus to oxidize intracellular components. Polylysine, taurine, and tris(hydroxymethyl)aminomethane formed high-molecular-weight, charged, or polar N-Cl derivatives that would be unable to penetrate the cell membrane. These results suggest an important role for leukocyte amine components in myeloperoxidase-catalyzed antimicrobial activity in vivo.

The combination of myeloperoxidase, hydrogen peroxide, and chloride ion forms an antimicrobial system within phagocytic vesicles of monocytes and polymorphonuclear leukocytes (2-5, 10, 12, 16, 23). The bactericidal activity of the myeloperoxidase system against a suspension of *Escherichia coli* was shown to result from the oxidation of Cl^- to hypochlorous acid (HOCl), followed by reaction of HOCl with bacterial components (18). A portion of the oxidizing equivalents of HOCl was rapidly consumed in the oxidation of bacterial components, as indicated by the loss of bacterial sulfhydryls. Oxidation of sulfhydryls was correlated with killing. The remainder of the oxidizing equivalents of HOCl were detected as stable chloramine or chloramide derivatives of bacterial components or both. These N-Cl derivatives were obtained in the supernatant after centrifugation to remove the bacteria. The release of N-Cl derivatives was associated with the release and fragmentation of bacterial peptides. Continued incubation of the bacteria with the released N-Cl derivatives resulted in slow oxidation of bacterial sulfhydryls and increased killing.

The aim of this study was to determine whether exogenous NH_4^+ or amines would compete with the bacterial components for reaction with HOCl. The formation of N-Cl derivatives of exogenous NH_4^+ or amines might enhance or block the antibacterial action of the myeloperoxidase system, depending on the ability of these N-Cl derivatives to oxidize bacterial components. This study could provide information about the possible roles of leukocyte amine or amide components or both in myeloperoxidase-catalyzed antibacterial activity in vivo.

MATERIALS AND METHODS

The sources of materials and the methods employed were as described previously (18). Briefly, aerobically grown *E. coli* ML 308-225 was harvested by centrifugation and suspended to 6×10^8 cells per ml. Amines were added as the HCl salts, and NH_4^+ was added as

$(NH_4)_2SO_4$. The suspensions were made 0.1 μM in myeloperoxidase, with 30 μM H_2O_2 added at 1-min intervals. Alternatively, HOCl was generated by adding portions of 20 mM NaOCl in 0.1 M sodium hydroxide to the buffered suspension. 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 corresponding disulfide. For the determination of bacterial sulfhydryls, the bacteria were washed by centrifugation and then incubated with 5,5'-dithiobis-2-nitrobenzoic acid and 0.8% (wt/vol) sodium dodecyl sulfate. The amount of sulfhydryls was determined from the yield of 5-thio-2-nitrobenzoic acid, as calculated from the absorbance at 412 nm. For the assay of viability, serial dilutions were prepared in sterile 0.2 M NaCl–0.1 M phosphate buffer (pH 6.6)–1 mM $MgSO_4$, and then 1-ml portions were spread on a solid growth medium. The number of viable bacteria was calculated from the number of colonies appearing after 2 to 3 days at 25°C.

For the determination of inhibition of bacterial respiration, reaction mixtures were made 30 mM in disodium succinate, and the consumption of oxygen was measured at 25°C with an O_2 electrode (American Instruments Co., Silver Spring, Md.). Relative rates of O_2 consumption were calculated from the linear portion of the continuous record of O_2 concentration over 3 to 10 min.

RESULTS

Antibacterial action. Figure 1 shows the inhibition of respiration of *E. coli* by either HOCl or the myeloperoxidase–H_2O_2–Cl^- system. Identical results were obtained when inhibition per mole of HOCl was compared with inhibition per mole of H_2O_2 added to the myeloperoxidase system. No inhibition was obtained with myeloperoxidase, H_2O_2, or Cl^- alone, or with the combination of any two of these. These results were consistent with myeloperoxidase-catalyzed oxidation of Cl^- to yield 1 mol of HOCl per mol of H_2O_2. Figure 1 also shows that in the presence of NH_4^+, complete inhibition was obtained with smaller amounts of HOCl or H_2O_2. On the other hand, polylysine, taurine, or tris(hydroxymethyl)aminomethane (Tris) almost completely prevented the inhibition of respiration.

Figure 2A shows that similar results were obtained when loss of viability rather than inhibition of respiration was used as the measure of antibacterial action. Increased killing was obtained with NH_4^+, whereas taurine blocked killing. In other experiments, polylysine or Tris also blocked killing. Larger amounts of HOCl were required for complete killing than for complete inhibition of respiration (cf. Fig. 1). Also, the effect of NH_4^+ on killing was more pronounced than the effect on inhibition of respiration.

Killing continued to increase when *E. coli* was incubated with the N-Cl derivatives of bacterial components for several hours at 37°C (18). Figure 2B shows that the killing obtained without NH_4^+ increased during 2 h at 37°C and became equal to that obtained with NH_4^+. In contrast, the killing obtained with NH_4^+ did not increase during continued incubation. Therefore, the effect of NH_4^+ was to increase the rate of killing.

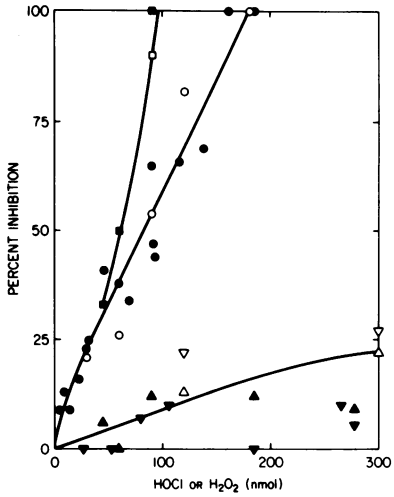

FIG. 1. *Inhibition of respiration. Bacteria were suspended in 0.2 M NaCl–0.1 M phosphate buffer (pH 6.6)–1 mM $MgSO_4$, without added amines (●, ○) or with 10 mM NH_4^+ (■, □), 10 mM taurine (▼, ▽), or 10 mM Tris (▲, △). The suspensions were incubated for 30 min at 0 to 5°C with the indicated amounts of HOCl per milliliter (closed symbols), or with 0.1 μM myeloperoxidase and the indicated amounts of H_2O_2 per milliliter (open symbols). Rates of respiration were measured 5 min after the suspensions were warmed to 25°C.*

Figure 2B also shows that killing could be obtained in the presence of taurine when the incubation was prolonged. Killing continued to increase during at least 4 h of incubation at 37°C. Similar results were obtained with polylysine and Tris. Therefore, these compounds slowed the rate of killing, but did not prevent killing.

Killing did not increase when the bacteria were held at 0 to 5°C for 2 h. Also, the increased killing could be prevented by washing the bacteria or by adding a reducing agent such as dithiothreitol before the incubation at 37°C. Washing or adding dithiothreitol had no effect when performed after the incubation at 37°C.

In other experiments, results obtained with HOCl or the myeloperoxidase system were similar at pH 5, 6.6, and 8. Changing the pH had little effect on killing. The rate of killing was increased by NH_4^+ and decreased by polylysine, taurine, or Tris at all three pH values. On the other hand, the myeloperoxidase system was less effective than HOCl when the Cl^- concen-

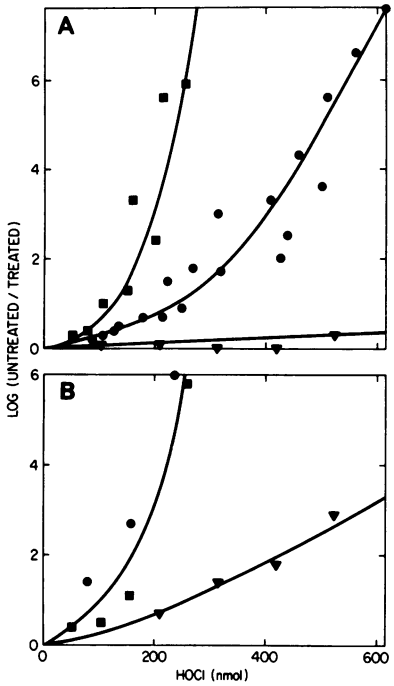

FIG. 2. *Bactericidal action. Bacteria were suspended in 0.2 M NaCl–0.1 M phosphate buffer (pH 6.6)–1 mM $MgSO_4$, without added amines (●) or with 10 mM NH_4^+ (■) or 10 mM taurine (▼). The suspensions were incubated for 30 min at 0 to 5°C with the indicated amounts of HOCl per milliliter, and then portions were taken for determination of viability (A). The remainders were incubated for 2 h at 37°C, and then viability was measured (B). Bactericidal action is expressed as the logarithm of the ratio of the number of viable cells in a suspension incubated for 30 min at 0 to 5°C without HOCl or added amines (untreated) to the number of viable cells in the suspension exposed to HOCl (treated).*

tration was lowered, particularly at pH 8. Myeloperoxidase has been reported to have a low pH optimum at low Cl^- concentrations (12, 15, 26). Also, less killing was obtained when the myeloperoxidase concentration was lowered 10-fold, possibly due to inactivation of myeloperoxidase by HOCl or N-Cl derivatives (4, 12, 15, 26).

The toxicity of NH_4^+ or amines was evaluated and was found to make only a slight contribution to killing. The average value was 0.2 ± 0.07 on the scale used in Fig. 2 after a 2-h incubation at 37°C with NH_4^+, taurine, or polylysine at pH 5, 6.6, or 8. In the presence of Tris at pH 5, the value was 0.5 ± 0.1.

A number of amines were tested for their effect on killing by HOCl during a 30-min incubation at 0 to 5°C. Guanidine and 1,1-dimethylguanidine were comparable to NH_4^+. Methylamine, ethylamine, benzylamine, and 2-phenylethylamine slightly increased killing. Arginine, lysine, and all of the neutral α-amino acids tested blocked killing, as did histamine, pyridine, dimethylamine, and guanidinoacetic acid.

Killing was also measured in the presence of varying amounts of both NH_4^+ and polylysine. With 0.6 mM HOCl, complete killing was obtained with 10 mM NH_4^+ and polylysine 10 mM in lysine residues (ratio, 1). Killing was blocked when the NH_4^+ concentration was lowered to 3 mM (ratio, 0.3).

In other experiments, similar results were obtained when gram-positive bacteria (*Staphylococcus aureus*) or yeast (*Candida albicans*) were used instead of *E. coli*.

Oxidation of sulfhydryls. Figure 3 shows that incubation of *E. coli* with HOCl resulted in oxidation of bacterial sulfhydryls. With NH_4^+, a much smaller amount of HOCl was required to oxidize sulfhydryls, whereas taurine blocked the oxidation of sulfhydryls.

In the absence of exogenous NH_4^+ or amines, slow oxidation of sulfhydryls was observed when the reaction mixtures were incubated for several hours at 37°C (18). Similar results were obtained in the presence of taurine. This slow oxidation could be prevented by holding the reaction mixture at 0 to 5°C or by washing the bacteria before the incubation at 37°C. In the presence of NH_4^+, the oxidation of sulfhydryls did not increase during subsequent incubations at 37°C.

Formation of N-Cl derivatives. Figure 4 shows the amount of unreacted oxidizing equiv-

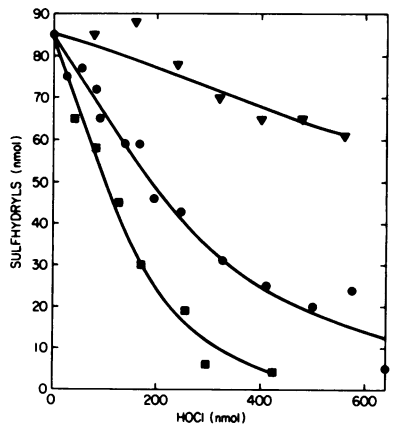

FIG. 3. *Oxidation of bacterial sulfhydryls. Bacteria were suspended in 0.2 M NaCl–0.1 M phosphate buffer (pH 6.6)–1 mM $MgSO_4$, without added amines (●) or with 10 mM NH_4^+ (■) or 10 mM taurine (▼). The suspensions were incubated for 30 min at 0 to 5°C with the indicated amounts of HOCl per milliliter, and then sulfhydryls were measured.*

alents detected in the reaction mixture after incubation of *E. coli* with HOCl in the presence or absence of added amines. With taurine or Tris, about 1 mol of N-Cl derivatives was obtained per mol of HOCl. Therefore, almost all of the HOCl reacted with these amines to form stable N-Cl derivatives, and the oxidizing equivalents were not consumed in the oxidation of bacterial components.

In the absence of added compounds, about 50 to 70% of the oxidizing equivalents were consumed, and the remainder accumulated in the form of N-Cl derivatives of bacterial components. In the presence of NH_4^+, the results were biphasic. At low NH_4^+ concentrations, NH_4^+ decreased the yield of N-Cl derivatives. This increased consumption of oxidizing equivalents was consistent with the increased oxidation of sulfhydryls (cf. Fig. 3). Complete killing of *E. coli* was obtained in this lower range of HOCl concentrations when NH_4^+ was present (cf. Fig. 2). At high HOCl concentrations, NH_4^+ increased the yield of unreacted N-Cl derivatives. This decreased consumption of oxidizing equivalents was obtained at HOCl concentrations in excess of that required for complete killing and complete oxidation of sulfhydryls.

Exposure of *E. coli* to HOCl in the absence of added compounds resulted in release and fragmentation of bacterial peptides (18). Taurine almost completely blocked this release of peptides. With NH_4^+, no peptides were released at low HOCl concentrations. Therefore, NH_4^+ increased the consumption of oxidizing equivalents in reactions other than those involving the release of peptides. Also, peptide release was not essential for killing. At higher HOCl concentrations, the same peptides were released as in the absence of NH_4^+, but the apparent molecular weights of the peptides did not decrease when the HOCl concentration was increased. Therefore, NH_4^+ blocked the fragmentation of the peptides.

Characterization of N-Cl derivatives. The N-Cl derivatives were obtained in the supernatant after centrifugation to remove bacteria. These derivatives were not reduced by H_2O_2, confirming the presence of N-Cl derivatives rather than HOCl (18).

Figure 5 shows that the N-Cl derivatives could be separated into high- and low-molecular-weight fractions by chromatography on Sephadex G-50. In the absence of added amines, a low yield of low-molecular-weight N-Cl derivatives was obtained, consistent with fragmentation of bacterial peptides and formation of N-Cl derivatives of the fragments (18). With NH_4^+, a higher yield of low-molecular-weight derivatives was

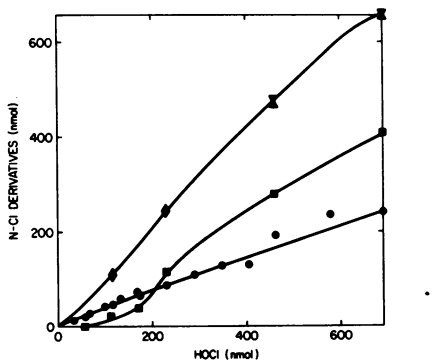

FIG. 4. *Yield of N-Cl derivatives. Bacteria were suspended in 0.2 M NaCl–0.1 M phosphate buffer (pH 6.6)–1 mM $MgSO_4$, without added amines (●) or with 10 mM NH_4^+ (■), 10 mM taurine (▼), or 10 mM Tris (▲). The suspensions were incubated for 30 min at 0 to 5°C with the indicated amounts of HOCl per milliliter, and then N-Cl derivatives were measured.*

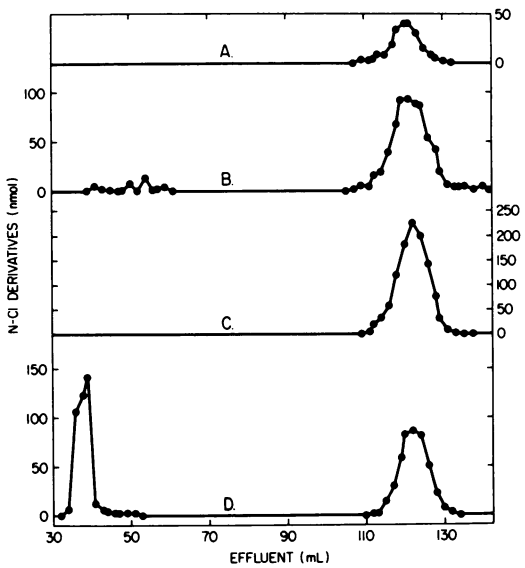

FIG. 5. *Chromatographic fractionation of N-Cl derivatives. Bacteria were suspended in 0.2 M NaCl–0.1 M phosphate buffer (pH 6.6)–1 mM $MgSO_4$, without added amines (A) or with 10 mM NH_4^+ (B), 10 mM taurine (C), or polylysine 10 mM in lysine residues (D). The suspensions were incubated for 30 min at 0 to 5°C with 0.3 mM HOCl (A) or 0.6 mM HOCl (B, C, and D) and then centrifuged at 5,300 × g for 10 min at 0 to 5°C. Portions (2 ml) of the supernatants were chromatographed at 25°C on a column (60 by 1.6 cm) of Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N.J.), equilibrated, and eluted with 0.2 M NaCl–0.1 M phosphate buffer, pH 6.6. Fractions of about 2 ml were collected, the volumes were determined by weighing, and then the N-Cl derivatives were measured.*

obtained, consistent with formation of NH_2Cl. Also, about 5% of the oxidizing equivalents were recovered as high-molecular-weight N-Cl derivatives of bacterial components. With taurine, a larger yield of low-molecular-weight N-Cl derivatives was obtained, consistent with formation of taurine chloramine. With polylysine, a high-molecular-weight fraction was obtained, corresponding to derivatives of polylysine containing multiple N-Cl bonds. Also, a low-molecular-weight fraction was obtained.

The yields of N-Cl derivatives in the supernatants were 58, 91, and 90% of the amount of HOCl added to the bacteria in the presence of NH_4^+, taurine, and polylysine, respectively. The recovery of N-Cl derivatives in the column effluent was 97, 91, and 72% of that obtained in the supernatant. Part of the oxidizing equivalents of the poly(N-Cl)-polylysine was lost upon warming the supernatant to 25°C, possibly due to oxidative peptide cleavage (13, 18).

Table 1 shows the partitioning of the N-Cl derivatives between the supernatants and either chloroform or ethyl acetate. In the absence of added amines or in the presence of polylysine, taurine, or Tris, only a small portion of the oxidizing equivalents was extracted into the organic solvents. Therefore, N-Cl derivatives of the bacterial components or the added amines were too polar to enter the organic phase. In the presence of NH_4^+ or guanidine, a substantial portion of the oxidizing equivalents was extracted, indicating formation of NH_2Cl or other nonpolar N-Cl derivatives.

TABLE 1. *Extraction of N-Cl derivatives into organic solvents*[a]

Amine added		Concn of N-Cl derivatives (mM)	% Extracted with:	
Type	Concn (mM)		Chloroform	Ethyl acetate
None		0.16	1	15
Polylysine	10	0.22	1	8
Taurine	10	0.46	0	3
Tris	10	0.45	0	5
NH_4^+	1	0.24	0	30
	10	0.34	12	39
	100	0.34	16	63
Guanidine	10	0.19	4	17

[a] Bacteria were suspended in 0.2 M NaCl–0.1 M phosphate buffer (pH 6.6)–1 mM $MgSO_4$, without added amines or with the indicated amounts of NH_4^+ or amines. The suspensions were incubated for 30 min at 0 to 5°C with 0.6 mM HOCl and then centrifuged at 5,300 × *g* for 10 min at 0 to 5°C. The amount of N-Cl derivatives was measured in a portion of the supernatant at 25°C. Also, another portion was mixed with 3 volumes of chloroform or ethyl acetate at 25°C, and then the phases were allowed to separate for 5 min. A portion of the organic phase was added to 0.2 M NaCl–0.1 M phosphate buffer (pH 8)–10 mM NH_4^+, and the amount of extracted N-Cl derivatives was measured.

The amount of oxidizing equivalents extracted into chloroform was less than the amount extracted into the more polar solvent ethyl acetate. In other experiments, increasing the volume of chloroform relative to the supernatant volume increased the amount extracted, but increasing the volume of ethyl acetate had little effect. On the other hand, increasing the NH_4^+ concentration increased the amount extracted. Therefore, the amount of oxidizing equivalents present either as NH_2Cl or as polar N-Cl derivatives of bacterial components depended on the ratio of NH_4^+ to bacteria.

In experiments with the pH 6.6 buffer rather than the supernatant from HOCl-treated bacteria, the conversion of HOCl to NH_2Cl had little effect on the extraction of oxidizing equivalents into organic solvents. All of the oxidizing equivalents could be extracted from 1 ml of an HOCl or NH_2Cl solution into 2 ml of ethyl acetate. Therefore, the HOCl was sufficiently nonpolar to be extracted, or the oxidizing equivalents were extracted in the form of Cl_2.

DISCUSSION

The antimicrobial agents produced by the peroxidase–H_2O_2–Cl^-, -iodide (I^-), or -thiocyanate (SCN^-) systems are HOCl, iodine (I_2), and hypothiocyanite ion ($OSCN^-$) (18–20). The role of the peroxidase is to catalyze the oxidation of the anions to these halogens or halogen analogs. With each of the three systems, oxidation of bacterial sulfhydryls is directly related to antibacterial action (18–22).

Differences in the antibacterial activity of the three systems may be due to the differing abilities of the halogens or halogen analogs to penetrate the bacterial cell membrane(s) and thus to oxidize intracellular components. Penetration of $OSCN^-$ may be limited by its charge (20, 22), whereas I_2 is a lipid-soluble molecule that reacts rapidly and completely with intracellular components (21). The ability of HOCl to oxidize bacterial components is limited by the formation of N-Cl derivatives of bacterial components (18) or of exogenous NH_4^+ or amines.

The increased rate of killing obtained in the presence of NH_4^+ appears to be due to the ability of NH_2Cl to penetrate the bacterial cell membrane. The NH_2Cl could be extracted into organic solvents, indicating that this oxidizing agent would be able to diffuse through the hydrophobic membrane barrier. On the other hand, NH_2Cl is not necessarily more lipid soluble

than HOCl. Oxidizing equivalents could be extracted into organic solvents from solutions of HOCl, either as HOCl or Cl_2. Rather than increasing the lipid solubility of the oxidizing equivalents, the formation of NH_2Cl competed with the formation of N-Cl derivatives of bacterial components, which were not lipid soluble. In this way, NH_4^+ kept the oxidizing equivalents in a form that could penetrate the membrane.

Polylysine, taurine, and Tris formed high-molecular-weight, charged, or polar N-Cl derivatives that would be unable to penetrate the bacterial cell membrane. The presence of these amines would prevent the oxidation of bacterial components by HOCl or by lipid-soluble N-Cl derivatives of bacterial components. Amino acids also slowed the rate of killing, either by forming charged N-Cl derivatives or by undergoing oxidative decarboxylation. Decarboxylation of the α-amino acids would consume the oxidizing equivalents of HOCl (6, 13, 17, 27, 28). Taurine (6, 13, 17), α-amino acids (6, 17), and other nitrogenous compounds (8, 11) have been reported to interfere with myeloperoxidase antibacterial activity.

During prolonged incubations, killing was obtained with the N-Cl derivatives of polylysine, taurine, and Tris. Similarly, the reaction of a protein with NaOCl was reported to turn the protein into a slow antimicrobial agent (1). The N-Cl derivatives may slowly hydrolyze to yield HOCl:

$$\text{R-NH-Cl} + H_2O \rightleftarrows \text{R-NH}_2 + \text{HOCl}$$

Alternatively, these agents may slowly diffuse through the membrane barrier, or they may gradually increase the permeability of the membrane by oxidizing exposed components.

Results presented here suggest that the location, concentration, and chemical structure of leukocyte amines may control the peroxidase-catalyzed antimicrobial activity of leukocytes. Increased concentrations of NH_4^+ might be expected to increase the rate of killing. On the other hand, formation of NH_2Cl might permit the diffusion of oxidizing equivalents out of the phagocytic vesicle into the leukocyte cytoplasm or out of the leukocyte. The resulting oxidation of leukocyte and tissue components could inhibit leukocyte function and damage host tissues.

The slow, persistent antimicrobial activity obtained with certain N-Cl derivatives might be advantageous to leukocytes. Whereas polylysine and taurine block killing in short-term experiments, N-Cl derivatives of such substances could provide a reserve of oxidizing equivalents to be used as needed. Also, the N-Cl derivatives could sequester the oxidizing equivalents within the phagocytic vesicle, preventing damage to leukocyte components and host tissues. Proper leukocyte function may require a compromise with reactions that decrease the rate of oxidation of biological components, in order to confine this oxidation to the proper cell compartment.

These considerations suggest that caution should be exercised in concluding that a peroxidase-H_2O_2-Cl^- system is not active within leukocytes from an individual or in a particular class of leukocytes. As examples of the latter, extracts from eosinophils (7, 8) and alveolar macrophages (9) have been reported not to have H_2O_2-dependent antimicrobial activity in the presence of Cl^-. The presence of amines could cause Cl^--dependent antimicrobial activity to be overlooked, especially during short-term incubations.

Leukocytes contain high concentrations of taurine (14). The low pK of the sulfonic acid moiety of taurine would make this compound especially effective as an agent for sequestering oxidizing equivalents within leukocytes. Taurine chloramine would exist primarily in the anionic (low-permeability) form even at the low pH within the phagocytic vesicle. The phagocytic vesicle also contains arginine-rich cationic proteins (24, 25). These proteins have antibacterial activity that is independent of the myeloperoxidase system (24, 25). However, it is possible that N-Cl derivatives of these proteins or their peptide fragments may be formed. The techniques described here and in a preceding article (18) for the separation and quantitation of N-Cl derivatives may provide an approach to evaluating the role of these substances in myeloperoxidase-catalyzed antimicrobial action within leukocytes.

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