INFECTION AND IMMUNITY, Mar. 1985, p. 613–618
0019-9567/85/030613-06$02.00/0

Vol. 47, No. 3

Oxidation of Microbial Iron-Sulfur Centers by the Myeloperoxidase-H_2O_2-Halide Antimicrobial System

HENRY ROSEN* AND SEYMOUR J. KLEBANOFF

Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195

Received 1 October 1984/Accepted 20 November 1984

Myeloperoxidase, H_2O_2, and a halide (chloride, bromide, or iodide) form a potent microbicidal system that contributes to the antimicrobial activity of neutrophils. The mechanism of toxicity is not completely understood. Powerful oxidants are formed that presumably attack the microbe at a variety of sites. Among the consequences of this attack is the release of a large proportion of ^{59}Fe of prelabeled organisms. We report here that the myeloperoxidase-H_2O_2-halide system oxidizes the iron-sulfur centers of model compounds (spinach ferredoxin) and intact microorganisms (*Escherichia coli*) with the loss of labile sulfide. The oxidation of the iron-sulfur centers of ferredoxin was measured by the fall in absorbance at 420 nm (bleaching) and by the loss of 5,5′-dithiobis-(2-nitrobenzoic acid) reducing activity. The latter compound is a sulfhydryl reagent that is reduced by ferredoxin labile sulfide during denaturation. The oxidation of *E. coli* iron-sulfur centers by the peroxidase system was determined by the loss of labile sulfide content, as measured by the release of H_2S by acid and its reaction with zinc acetate to form ZnS. The halides were effective as components of the peroxidase system in the order I > Br > Cl. The oxidation of *E. coli* iron-sulfur centers by the peroxidase system was rapid and preceded the loss of viability. Gentamicin, at a concentration which produced a loss of viability comparable to that of the peroxidase system, did not cause a loss of labile sulfide from *E. coli*, suggesting that labile sulfide loss is not a nonspecific reflection of the loss of viability, but a direct consequence of the action of the myeloperoxidase system. The oxidation of iron-sulfur centers in microorganisms by the myeloperoxidase-H_2O_2-halide system may contribute to the death of the organism.

An antimicrobial system has been described in neutrophils which consists of myeloperoxidase, H_2O_2, and a halide (13, 14, 23, 27). The myeloperoxidase is released into the phagosome from cytoplasmic granules by degranulation, H_2O_2 is generated by a respiratory burst induced by plasma membrane perturbation, and chloride, bromide, or iodide can serve as the halide requirement. The interaction of these components in the phagosome generates toxic products, most important of which, at least for chloride, appears to be the hypohalous acid (2, 3, 10), which attacks the ingested organism. Two modes of attack have been proposed: halogenation and oxidation. The precise microbicidal mechanism, however, has not been defined.

Previous studies in this laboratory (22) have indicated that, in the presence of chelating agents, the microbicidal activity of the myeloperoxidase-H_2O_2-chloride (or bromide) system is associated with a major loss of iron into the medium, as measured by the release of ^{59}Fe from prelabeled organisms. Iron loss was not observed with iodide as the halide. The protein-bound iron of cells has been divided into heme iron and nonheme iron proteins, of which iron-sulfur proteins are a major constituent. Cytochrome *c* was employed as a model heme iron protein, and its exposure to the peroxidase system was shown to result in the release of iron. As with microbicidal activity, iron release from cytochrome *c* was observed with chloride and bromide, but not with iodide, as the halide. The studies reported here indicate that the myeloperoxidase-H_2O_2-halide system can oxidize the iron-sulfur centers of model compounds such as spinach ferredoxin and intact microorganisms such as *Escherichia coli* and suggest that this may be one of the mechanisms by which microorganisms are killed.

* Corresponding author.

MATERIALS AND METHODS

Special reagents. Myeloperoxidase was prepared from canine pyometrial pus by the method of Agner (1) to the end of step 6 and assayed by the *o*-dianisidine method (26). One unit of enzyme is the amount utilizing 1 μmol of H_2O_2 per min at 25°C. Gelatin was obtained from Difco Laboratories, Detroit, Mich.; glucose oxidase (type V, from *Aspergillus niger*, 1,460 U/ml), bathophenanthroline sulfonate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and spinach ferredoxin were from Sigma Chemical Company, St. Louis, Mo. The spinach ferredoxin was dialyzed twice at 4°C against 0.33 M Na_2SO_4 (pH 9.0 to 9.5) to remove chloride and to increase stability (11, 18). The dialyzed preparation was stored at −70°C. [^{35}S]sulfate (25 to 40 Ci/mg) was obtained from Amersham Corp., Arlington Heights, Ill.

Microorganisms. *E. coli* (ATCC 11775; American Type Culture Collection, Rockville, Md.) was maintained on blood agar plates and inoculated daily into Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C. Overnight cultures were diluted 100-fold into medium B, a low-sulfate modification of the medium A previously described (7, 22). Medium B contained 7 g of K_2HPO_4, 3 g of KH_2PO_4, 0.5 g of sodium citrate · $2H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1.16 g of $(NH_4)_2CO_3 \cdot H_2O$, and 1 g of sodium succinate per liter. The sulfate concentration was 0.4 mM (calculated), and the iron concentration was 3.0 ± 0.3 μM (n = 3) by colorimetric assay (21). Small volumes (5 to 15 ml) of organisms were grown aerobically in tissue culture flasks as previously described (22). Larger volumes (up to 1 liter) were grown in magnetically stirred Erlenmeyer flasks continuously oxygenated with humidified, filter-sterilized (0.22-μm pore size; Millipore Corp., Bedford, Mass.), compressed air. Where indicated, medium B was supplemented with 1 to 10 μCi of [^{35}S]sulfate per ml. Bacterial cultures were harvested after 4 to 18 h at 37°C, washed twice by

centrifugation, and suspended in 0.1 M sodium sulfate (supplemented, where indicated, with 0.05% gelatin) to the required absorbancy at 540 nm. The stock *E. coli* suspension was diluted 10-fold in the final reaction mixture. After 6 h of culture, 1 mg of *E. coli* protein corresponded to $2.4 \times 10^9 \pm 0.7 \times 10^9$ CFU ($n = 5$). Protein was determined by the method of Lowry et al. (16) with human serum albumin as the standard, and microbial viability was determined as CFU by the pour plate method as previously described (20).

Ferredoxin bleaching. The components indicated in the legend to Fig. 1, except ferredoxin, glucose oxidase, and myeloperoxidase, were preincubated in plastic tubes for at least 5 min at 37°C. The reaction was initiated by the sequential addition of spinach ferredoxin (0.08 ml), myeloperoxidase (0.01 ml), and glucose oxidase (0.02 ml), all at 0°C. Components were mixed and promptly transferred to a spectrophotometer cuvette maintained at 37°C, and absorbance at 420 nm was monitored continuously for 5 min. The results are expressed as the maximum rate of bleaching in absorbance units per minute.

Reduction of DTNB by spinach ferredoxin. Spinach ferredoxin was incubated for 15 min at 37°C with the components of the myeloperoxidase system indicated in the legend to Table 1. Excess hypochlorous acid, formed by the myeloperoxidase system, was neutralized by the addition of 0.1 ml of 10^{-3} M sodium nitrite. This was followed by the addition of 0.8% sodium dodecyl sulfate, 10^{-4} M DTNB, 10^{-2} M EDTA, and 6.7×10^{-2} M Tris-hydrochloride buffer pH 8.0 to give a final volume of 3.0 ml. After 60 min at 37°C, absorbance was determined at 412 nm against a blank consisting of a comparably treated reaction mixture containing 1.0 ml of acetate-buffered sodium sulfate. DTNB reduction was calculated by using an extinction coefficient of 13,600 M per cm for the reduction product, 5-thio-2-nitrobenzoic acid (8).

Labile sulfide measurement—radioisotope method. *E. coli* organisms were grown in medium B supplemented with sufficient [^{35}S]sulfate to produce 20,000 to 100,000 cpm/ml of final incubation mixture. Organisms were incubated with the components of the antimicrobial systems described in the legends to the figures and tables, and the labile sulfide content of the organisms was then determined by a modification of the method of Clegg and Garland (6). Duplicate 1-ml samples were transferred to the main compartment of 7-ml Warburg flasks, and strips of filter paper, previously alkalinized in 0.1 M NaOH and dried, were placed in the center wells and moistened with 10 μl of 5% sodium acetate–20% zinc acetate. The flasks were stoppered, and the samples were acidified by tipping 0.5 ml of 5 N HCl from the sidearm into the main compartment. Flasks were shaken at room temperature 100 times per min for 2 to 3 h and then allowed to stand at room temperature overnight. The labile sulfide of the organism was released as H_2S by the acid and was trapped on the filter paper as ZnS. The filter papers were placed directly into liquid scintillation vials containing 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.), and the counts per minute were determined in a liquid scintillation counter. The radioactivity of the filter paper strips was compared with that of the original suspension of organisms, and the percent labile sulfide was calculated. The labile sulfide recovered by this technique increased linearly with the *E. coli* concentration over the range 0 to 5×10^7 organisms per ml ($r = 0.94$).

TABLE 1. Reduction of DTNB by spinach ferredoxin[a]

Supplements	DTNB reduction[b] (nmol)
None (control)	6.0 ± 0.2[b,c]
Myeloperoxidase-H_2O_2-chloride	−0.1 ± 0.3[d]
H_2O_2-chloride	2.5 ± 0.1[c,d]
Myeloperoxidase-chloride	4.8 ± 0.4[c]
Myeloperoxidase-H_2O_2	2.8 ± 0.2[c,d]
Chloride	5.7 ± 0.0[c]

[a] The reaction mixture contained 0.01 M sodium acetate buffer (pH 5.0), 0.7 M sodium sulfate, approximately 3.6×10^{-6} M spinach ferredoxin, and the following supplements in a final volume of 1.0 ml: myeloperoxidase, 16 mU; H_2O_2, 5×10^{-5} M; sodium chloride, 0.1 M. Residual DTNB reducing activity was determined after incubation for 15 min at 37°C.

[b] Mean ± standard error of duplicate determinations in a single experiment.

[c] Significantly different from myeloperoxidase-H_2O_2-chloride, $p < 0.02$.

[d] Significantly different from control, $P < 0.01$.

Labile sulfide measurement—chemical method. Labile sulfide was determined as described above for the radioisotopic method, except that unlabeled *E. coli* cells grown for 6 h in medium B were employed in amounts corresponding to 10 to 20 mg of total protein per assay, acidification was limited to 20 to 30 min, and the ZnS on the filter paper strips was determined by its conversion of *N,N'*-dimethyl-*p*-phenylenediamine to methylene blue (6, 19). Briefly, filter papers were transferred to 12- by 75-mm plastic tubes and sealed with a rubber serum stopper. A 1-ml sample of *N,N'*-dimethyl-*p*-phenylenediamine (0.1 mg/ml) in 1.5% H_2SO_4 was injected through the stopper, and the contents of the tube were mixed briefly on a Vortex mixer. After 1 min, the tube was again mixed, and 0.2 ml of ferric ammonium sulfate (20 mg/ml) in 1.5% H_2SO_4 was added by injection. The blue color was allowed to develop for at least 5 min. The samples were clarified as necessary by brief centrifugation, and the optical density at 670 nm was determined in a Cary M-15 spectrophotometer. The values were compared with a standard curve determined with Na_2S (0 to 100 nmol/ml) processed in the same way.

Nonheme iron. Nonheme iron was extracted by the method of Kurup and Brodie (15) from *E. coli* grown for 6 h in medium B. To a suspension of 10^{10} *E. coli* cells in 0.15 ml of 0.1 M Na_2SO_4 was added 0.15 ml of freshly dissolved 0.2% sodium dithionite and 0.3 ml 10% (wt/vol) trichloroacetic acid. After 10 min at room temperature, samples were centrifuged at 8,700 × *g* for 4 min. Iron was estimated by a modification of method A of Brumby and Massey (5). To 0.4 ml of the extracts were added 0.5 ml of water, 0.05 ml of freshly prepared 0.06 M ascorbic acid, 0.04 ml of saturated ammonium acetate, and 0.05 ml of 0.01 M bathophenanthroline sulfonate. Optical density was read at 535 nm and compared with ferrous ammonium sulfate standards (0 to 50 nmol) similarly assayed.

Oxygen consumption. Oxygen consumption by the glucose-glucose oxidase system was determined with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). H_2O_2 formation was calculated by assuming a 1:1 correspondence between oxygen consumption and H_2O_2 formation.

Statistical analysis. Analysis of microbial viability data was by methods previously described (20). Other data were analyzed by Student's two-tailed *t*-test (not significant; $P > 0.05$). The results are expressed as means ± standard errors of *n* determinations unless otherwise indicated.

RESULTS

Oxidation of the iron-sulfur center of spinach ferredoxin. Spinach ferredoxin, an iron-sulfur protein from spinach

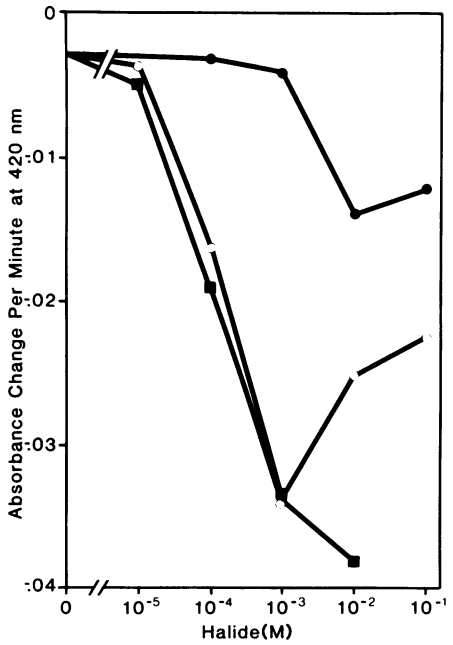

FIG. 1. Bleaching of spinach ferredoxin by the myeloperoxidase system. The reaction mixture contained 0.04 M sodium acetate buffer (pH 5.0), 0.24 M sodium sulfate, 160 μg of ferredoxin, 16 mU of myeloperoxidase, 0.01 M glucose, 60 mU of glucose oxidase, and either chloride (●), bromide (○), or iodide (■) (sodium salts) at the concentrations indicated in a total volume of 1.0 ml. When the concentration of the halide was 0.1 M, the sodium sulfate concentration was decreased to maintain constant tonicity.

chloroplasts, was used to investigate the susceptibility of iron-sulfur centers to oxidation by the myeloperoxidase-H_2O_2-halide antimicrobial system. This ferredoxin has a broad absorbance maximum at 420 nm that reflects the integrity of the iron-sulfur center. "Spontaneous" oxidation in buffer accounted for a low background bleaching rate of −0.0005 absorbance units per min (data not shown), which increased to −0.0028 absorbance units per min upon the addition of myeloperoxidase glucose and glucose oxidase (Fig. 1; no halide). Bleaching was considerably increased by the complete myeloperoxidase-glucose-glucose oxidase-halide system, with bleaching rates of up to −0.0382 absorbance units per min observed. Chloride was effective as the halide component at concentrations of 10^{-2} to 10^{-1} M, bromide was effective at 10^{-4} to 10^{-1} M, and iodide was effective at 10^{-4} to 10^{-2} M. With bromide, optimum bleaching occurred at a concentration of 10^{-3} M, with a fall in rate at higher concentrations. In general, the relative potency of the halides in supporting ferredoxin bleaching was $I^- \geq Br^- > Cl^-$.

Another measure of the integrity of the ferredoxin-labile sulfide center is its ability, during denaturation, to reduce the sulfhydryl reagent DTNB, as measured by the increase in absorbance at 412 nm (9, 17). Under the conditions employed in Table 1, 3.6 nmol of spinach ferredoxin incubated in buffer alone (control) reduced 6.0 nmol of DTNB (theoretical value, 7.2 nmol). No DTNB reduction was detected after incubation of ferredoxin with the complete myeloperoxidase system, suggesting that all of the ferredoxin-labile sulfide had been oxidized. Omission of H_2O_2 from the system prevented labile sulfide oxidation, whereas when myeloperoxidase or chloride were omitted, labile sulfide oxidation was significantly decreased, but not abolished.

Oxidation of iron-sulfur centers of *E. coli*. The total nonheme iron content of *E. coli* was 4.7 ± 0.5 nmol/mg of protein or 2.0 nmol/10^9 organisms (n = 3), and the total labile sulfide content was 2.9 ± 0.7 nmol/mg of protein or 1.2 nmol/10^9 organisms (n = 4). Assuming the typically observed 1:1 stoichiometry between labile sulfide and iron in microbial iron-sulfur centers, this suggests that 62% of nonheme iron was in iron-sulfur centers.

Incubation of *E. coli* with the myeloperoxidase-H_2O_2-chloride system in the presence of EDTA under the conditions employed in Table 2 decreased the labile sulfide content of the organisms from 0.76% of the total microbial sulfur to 0.09%, a decrease of 89%. Deletion of any of the components of myeloperoxidase-H_2O_2-chloride system prevented this fall. EDTA deletion, however, increased labile sulfide recovery only slightly, i.e., from 0.09 to 0.14% ($P < 0.05$). Since EDTA was not required for the labile sulfide oxidation or for microbicidal activity of the peroxidase system, it was omitted from subsequent experiments unless otherwise indicated. Figure 2 demonstrates the effect of the substitution of iodide or bromide for chloride on the oxidation of *E. coli* labile sulfide by the peroxidase system. Iodide was the most effective halide on a molar basis, with significant oxidizing activity observed at concentrations ranging from 10^{-6} to 10^{-4} M. Bromide was effective over a concentration range of 10^{-4} to 10^{-1} M, and chloride was effective between 10^{-2} and 10^{-1} M.

The rate of labile sulfide oxidation by the peroxidase system was very rapid under the conditions employed in Table 2; the labile sulfide content fell from 0.90 ± 0.08% of total sulfur (n = 10) to 0.11 ± 0.02% (n = 5) during the first minute of incubation and remained constant thereafter.

Microbicidal activity also was extremely rapid under these conditions (22). In Fig. 3, the *E. coli* concentration was increased 10-fold, reagent H_2O_2 was replaced by a glucose-glucose oxidase system that generated H_2O_2 continuously at a rate of 8.6 ± 0.9 μM/min (n = 3), and 0.005% gelatin was added. Labile sulfide oxidation and microbicidal activity were sufficiently attenuated under these conditions to allow a comparison of initial rates. Both labile sulfide levels and microbial viability fell rapidly on exposure of the organisms to the complete myeloperoxidase system under these conditions (Fig. 3). In the absence of myeloperoxidase, both labile sulfide oxidation and loss of viability induced by enzyme-generated H_2O_2 were comparable to control values seen in reactions in which both myeloperoxidase and glucose oxidase were omitted. The studies described above were performed

TABLE 2. Myeloperoxidase-mediated oxidation of *E. coli* labile sulfide[a]

Supplements	Labile sulfide[b] (% of total sulfur)
Chloride (control)	0.76 ± 0.08 (10)
Myeloperoxidase-H_2O_2-chloride-EDTA	0.09 ± 0.01[c] (12)
H_2O_2-chloride-EDTA	0.80 ± 0.09 (8)
Myeloperoxidase-chloride-EDTA	0.77 ± 0.08 (3)
Myeloperoxidase-H_2O_2-EDTA	0.69 ± 0.08 (3)
Myeloperoxidase-H_2O_2-chloride	0.14 ± 0.01[c] (7)

[a] The reaction mixture contained 0.04 M sodium acetate buffer (pH 5.0), 10^8 ^{35}S-labeled *E. coli* cells, and the following supplements in a final volume of 2.0 ml: sodium chloride, 0.1 M; myeloperoxidase, 32 mU; H_2O_2, 5 × 10^{-5} M; EDTA, 10^{-4} M. After 60 min at 37°C, duplicate 0.9-ml samples were assayed for labile sulfide content.

[b] Mean ± standard error, with the number of experiments indicated within parentheses.

[c] Significantly different from control values.

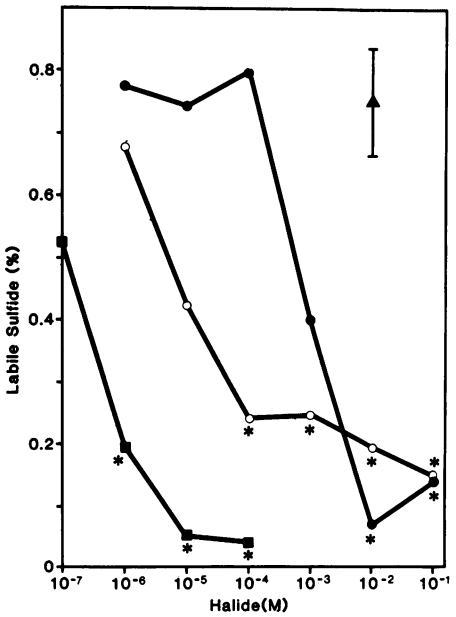

FIG. 2. Halide requirement for myeloperoxidase-mediated oxidation of *E. coli* labile sulfide. The reaction mixture was as described in Table 1, except that EDTA was omitted and either chloride (●), bromide (○) or iodide (■) (sodium salts) was employed at the indicated concentrations to meet the halide requirement. When the concentration of the halide was below 0.1 M, sodium sulfate was added to maintain constant tonicity. Asterisks indicate a significant difference in labile sulfide content ($P < 0.05$) from mixtures in which no halide was present (▲, 0.75 ± 0.09% labile sulfide, $n = 13$). The results are the means of 2 to 13 experiments.

at pH 5.0. At pH 7.0, the initial rate of labile sulfide oxidation by the myeloperoxidase system was greater than that of microbicidal activity (Fig. 4).

Figure 4 also compares the effect of the myeloperoxidase system to that of gentamicin on *E. coli* at pH 7.0. When the two microbicidal systems were added at levels that produced a comparable loss of viability, the myeloperoxidase system caused a rapid oxidation of labile sulfide, whereas the labile sulfide levels of gentamicin-treated bacteria were not significantly different from the control organisms. In experiments not shown, the addition of glucose-glucose oxidase to gentamicin-treated bacteria did not increase the oxidation of labile sulfide.

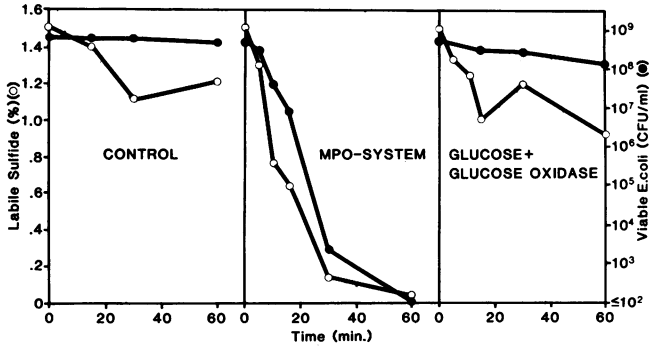

FIG. 3. Comparison of labile sulfide oxidation and microbicidal activity induced by the peroxidase system at pH 5.0. The complete myeloperoxidase system contained 0.04 M sodium acetate (pH 5.0), 5.5×10^9 *E. coli* cells, 176 mU of myeloperoxidase, 0.01 M glucose, 640 mU of glucose oxidase, 0.1 M sodium chloride, 0.01 M sodium sulfate, and 0.005% gelatin in a total volume of 11 ml. After incubation at 37°C for the times indicated, samples were removed for determination of labile sulfide (○) and microbial viability (●) as described in the text. Myeloperoxidase was omitted from incubations labeled "glucose + glucose oxidase," and both myeloperoxidase and glucose oxidase were omitted from incubations labeled "control". The data points are the means of three experiments.

DISCUSSION

E. coli exposed to the myeloperoxidase-H_2O_2-halide system can lose in excess of 70% of their total microbial iron (22). Since heme iron only accounts for a few percent of microbial iron (4), it follows that the bulk of the released iron originates from nonheme sources. Of the nonheme iron in our preparations, approximately 62% could be accounted for by iron-sulfur centers, suggesting that these centers were a major source of the iron released by the peroxidase-H_2O_2-halide system. This is supported by the studies reported here.

Clostridial and spinach ferredoxin are iron-sulfur proteins that were previously shown to be rapidly oxidized by hypochlorous acid (3), the primary product of the myeloperoxidase-H_2O_2-chloride system (2, 3, 10). The oxidation of the iron-sulfur center of spinach ferredoxin by the products of the peroxidase system was confirmed here by using myeloperoxidase, H_2O_2, and a halide to generate the oxidant. The evidence for iron-sulfur center oxidation is as follows. (i) Spinach ferredoxin is bleached by the peroxidase system as measured by a fall in absorbance at 420 nm, suggesting an attack on the chromophoric iron-sulfur center. (ii) DTNB, a colorless disulfide compound (Ellman reagent) is reduced to a colored sulfhydryl derivative by the labile sulfide of iron-sulfur proteins during denaturation (9, 17). Exposure of spinach ferredoxin to the myeloperoxidase system prevented DTNB reduction, suggesting prior disruption of the iron-sulfur center of ferredoxin by the peroxidase system. Oxidation of the iron-sulfur center of ferredoxin required each component of the myeloperoxidase-H_2O_2-halide system, and the relative potency of the halides was $I^- \geq Br^- > Cl^-$. It is of interest that with bromide as the halide, ferredoxin bleaching was maximum at 10^{-3} M and decreased as the bromide concentration was increased. In earlier

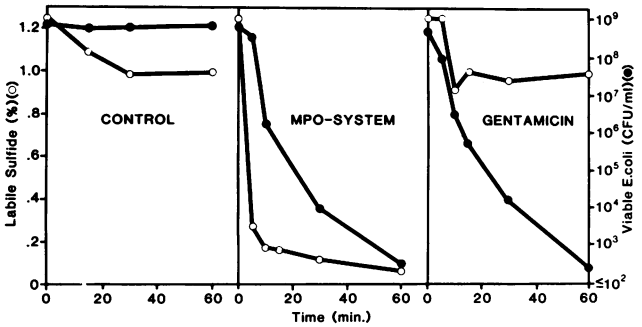

FIG. 4. Comparison of myeloperoxidase and gentamicin-mediated labile sulfide oxidation and microbicidal activity at pH 7.0. Control and complete systems were as described in the legend to Fig. 3, except that sodium acetate was replaced by 0.04 M sodium phosphate (pH 7.0). Where indicated, myeloperoxidase and glucose oxidase were replaced by gentamicin at 45 μg/ml.

studies, the bactericidal activity of the myeloperoxidase-H_2O_2-halide system also fell at high bromide concentrations (12).

Intact *E. coli* organisms also reduce DTNB in the presence of sodium dodecyl sulfate (24, 25); this reducing capacity was shown to be lost along with microbial viability when the organisms were incubated with myeloperoxidase, H_2O_2, and chloride (24). Since DTNB reacts with sulfhydryl groups (8), this was attributed to the oxidation of organism sulfhydryls by the peroxidase system (24). However, as shown in our studies with spinach ferredoxin, DTNB also reacts under these denaturing conditions with iron-sulfur centers, making it likely that oxidation of microbial iron sulfur centers contributed to the observed effects. This was supported by our measurements of labile sulfide levels. Iron-sulfur centers are characterized by the lability of the sulfur to acid. The hydrogen sulfide released can be trapped as zinc sulfide and measured either chemically or by radioactivity when organisms are prelabeled by growth in [^{35}S]sulfate. The labile sulfide content of *E. coli* fell rapidly on exposure of the organisms to the peroxidase-H_2O_2-halide system. The microbicidal activity of gentamicin was not associated with loss of labile sulfide, even when gentamicin-killed organisms were exposed to H_2O_2. Thus labile sulfide loss does not appear to be a simple consequence of the degeneration of dead bacteria, but is a more direct reflection of myeloperoxidase-mediated oxidant damage.

The characteristics of myeloperoxidase-mediated labile sulfide oxidation differed in some respects from those of myeloperoxidase-mediated iron loss. Thus EDTA was required for optimum iron loss (22), whereas its deletion from the peroxidase system had little effect on microbial labile sulfide oxidation. This presumably reflects a requirement for EDTA for iron solubilization rather than for the action of the peroxidase system (22). With labile sulfide oxidation by the myeloperoxidase system, the effectiveness of the halides on a molar basis was in the order $I^- > Br^- > Cl^-$. Iodide, however, when combined with myeloperoxidase and H_2O_2 did not cause iron loss from *E. coli* or alter the spectral characteristics of cytochrome *c*, under conditions in which bromide or chloride was effective. Thus iodide-derived oxidants appear to oxidize microbial iron-sulfur centers without the release of iron into the medium. The reason for this discrepancy is unknown. Iodide-derived oxidants may damage iron-sulfur centers to the point where they are catalytically inactive, but still retain their iron. Alternatively iron-sulfur centers may be fully disrupted; however the chelator required for solubilization of the iron may not be able to reach the site or transfer the iron to the medium where it can be measured. Labile sulfide oxidation is a rapid event that is evident within the first minute of incubation under conditions in which microbial iron loss exhibits a lag of several minutes. Myeloperoxidase-mediated microbicidal activity correlates more closely with labile sulfide oxidation than with iron loss; microbicidal activity is rapid, does not require EDTA, and occurs with iodide (as well as bromide or chloride) as the halide.

The oxidation of halides by the myeloperoxidase-mediated antimicrobial system produces powerful oxidants that can attack the cell in a variety of ways. The studies reported here suggest that among the targets are iron-sulfur centers, which are constituents of the electron transport chain present in the cytoplasmic membrane. An attack on catalytic iron centers would be expected to have profound effects on microbial metabolism. Disruption of active transport of low-molecular-weight metabolites across the cytoplasmic membrane would be expected; indeed, the peroxidase system has been reported to inhibit such transport (14).

ACKNOWLEDGMENTS

We thank Ann M. Waltersdorph and Douglas J. Evans for expert technical assistance.

This study was supported in part by Public Health Service grant AI 07763 from the National Institutes of Health and by a grant from The Rockefeller Foundation. H.R. is the recipient of Public Health Service Research Career Development Award AI 00368 from the National Institutes of Health.

LITERATURE CITED

1. **Agner, K.** 1958. Crystalline myeloperoxidase. Acta Chem. Scand. **12**:89–94.
2. **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. vol. 18. Pergamon Press, New York.
3. **Albrich, J. M., C. A. McCarthy, and J. K. Hurst.** 1981. Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. Proc. Natl. Acad. Sci. U.S.A. **78**:210–214.
4. **Bragg, P. D.** 1980. The respiratory system of *Escherichia coli*, p. 115–136. *In* C. J. Knowles (ed.), Diversity of bacterial respiratory systems, vol. 1. CRC Press, Inc., Boca Raton. Fla.
5. **Brumby, P. E., and V. Massey.** 1967. Determination of nonheme iron, total iron and copper. Methods Enzymol. **10**:463–473.
6. **Clegg, R. A., and P. B. Garland.** 1971. Non-haem iron and the dissociation of piericidin A sensitivity from site 1 energy conservation in mitochondria from *Torulopsis utilis*. Biochem. J. **124**:135–154.
7. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
8. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82**:70–77.
9. **Gins, V. K., R. M. Chermnykh, and E. N. Mukhin.** 1979. Reactivity of nonheme iron and thiol groups of pea and corn ferredoxins under the action of specific reagents and urea. Biochemistry (USSR) **44**:931–936.
10. **Harrison, J. E., and J. Schultz.** 1976. Studies on the chlorinating activity of myeloperoxidase. J. Biol. Chem. **251**:1371–1374.
11. **Hasumi, H., S. Nakamura, K. Koga, and H. Yoshizumi.** 1979. Effects of neutral salts on thermal stability of spinach ferredoxin. Biochem. Biophys. Res. Commun. **87**:1095–1101.
12. **Klebanoff, S. J.** 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. J. Bacteriol. **95**:2131–2138.
13. **Klebanoff, S. J.** 1980. Myeloperoxidase-mediated cytotoxic systems, p. 279–308. *In* A. J. Sbarra and R. R. Strauss (ed.), The reticuloendothelial system. 2. Biochemistry and metabolism. Plenum Publishing Corp., New York.
14. **Klebanoff, S. J., and R. A. Clark.** 1978. The neutrophil: function and clinical disorders. North-Holland Publishing Co., Amsterdam.
15. **Kurup, C. K. R., and A. F. Brodie.** 1967. Oxidative phosphorylation in fractionated bacterial systems. XXVII. The nature of nonheme iron in *Mycobacterium phlei*. J. Biol. Chem. **242**:2909–2916.
16. **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.
17. **Malkin, R., and J. C. Rabinowitz.** 1967. The reactivity of clostridial ferredoxin with iron chelating agents and 5,5'-dithiobis-2-nitrobenzoic acid. Biochemistry **6**:3880–3891.
18. **Petering, D., J. A. Fee, and G. Palmer.** 1971. The oxygen sensitivity of spinach ferredoxin and other iron-sulfur proteins. The formation of protein-bound sulfur-zero. J. Biol. Chem. **246**:643–653.
19. **Rabinowitz, J. C.** 1978. Analysis of acid-labile sulfide and sulfhydryl groups. Methods Enzymol. **53**:275–277.
20. **Rosen, H., and S. J. Klebanoff.** 1979. Bactericidal activity of a

superoxide anion-generating system. A model for the polymorphonuclear leukocyte. J. Exp. Med. **149**:27–39.
21. **Rosen, H., and S. J. Klebanoff.** 1981. Role of iron and ethylenediaminetetraacetic acid in the bactericidal activity of a superoxide anion-generating system. Arch. Biochem. Biophys. **208**:512–519.
22. **Rosen, H., and S. J. Klebanoff.** 1982. Oxidation of *Escherichia coli* iron centers by the myeloperoxidase-mediated microbicidal system. J. Biol. Chem. **257**:13731–13735.
23. **Schultz, J.** 1980. Myeloperoxidase, p. 231–254. *In* A. J. Sbarra and R. R. Strauss (ed.), The reticuloendothelial system. 2. Biochemistry and metabolism. Plenum Publishing Corp., New York.
24. **Thomas, E. L.** 1979. Myeloperoxidase, hydrogen peroxide, chloride antimicrobial system: nitrogen-chlorine derivatives of bacterial components in bactericidal action against *Escherichia coli*. Infect. Immun. **23**:522–531.
25. **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.
26. **Worthington Biochemical Corp.** 1972. Worthington enzyme manual, p. 43. Worthington Biochemical Corp., Freehold, N.J.
27. **Zgliczynski, J. M.** 1980. Characteristics of myeloperoxidase from neutrophils and other peroxidases from different cell types, p. 255–278. *In* A. J. Sbarra and R. R. Strauss (ed.), The reticuloendothelial system. 2. Biochemistry and metabolism. Plenum Publishing Corp., New York.