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Myeloperoxidase-Halide-Hydrogen Peroxide Antibacterial System

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An antibacterial effect of myeloperoxidase, a halide, such as iodide, bromide, or chloride ion, and H_2O_2 on *Escherichia coli* or *Lactobacillus acidophilus* is described. When *L. acidophilus* was employed, the addition of H_2O_2 was not required; however, the protective effect of catalase suggested that, in this instance, H_2O_2 was generated by the organisms. The antibacterial effect was largely prevented by preheating the myeloperoxidase at 80 C or greater for 10 min or by the addition of a number of inhibitors; it was most active at the most acid *p*H employed (5.0). Lactoperoxidase was considerably less effective than was myeloperoxidase when chloride was the halide employed. Myeloperoxidase, at high concentrations, exerted an antibacterial effect on *L. acidophilus* in the absence of added halide, which also was temperature- and catalase-sensitive. Peroxidase was extracted from intact guinea pig leukocytes by weak acid, and the extract with peroxidase activity had antibacterial properties which were similar, in many respects, to those of the purified preparation of myeloperoxidase. Under appropriate conditions, the antibacterial effect was increased by halides and by H_2O_2 and was decreased by catalase, as well as by cyanide, azide, Tapazole, and thiosulfate. This suggests that, under the conditions employed, the antibacterial properties of a weak acid extract of guinea pig leukocytes is due, in part, to its peroxidase content, particularly if a halide is present in the reaction mixture. A heat-stable antibacterial agent or agents also appear to be present in the extract.

The neutrophil peroxidase, myeloperoxidase, exerts an antibacterial effect when combined with a source of H_2O_2 and either thiocyanate ions (13, 14) or a halide, such as iodide, bromide, or chloride ions (11, 12). The antibacterial effect of the myeloperoxidase-iodide-H_2O_2 system was considered in detail in a previous publication (12). Myeloperoxidase, iodide, and H_2O_2 exerted a bactericidal effect on *Escherichia coli* which was associated with, and possibly was a consequence of, the iodination of the bacteria. The operation of this iodinating system in the intact leukocyte was suggested by the finding that iodide ions were converted to a water-insoluble, trichloroacetic acid-precipitable form by intact leukocytes containing ingested bacteria. The antibacterial effect of myeloperoxidase, H_2O_2, and either bromide or chloride ions is considered in this paper. The relationship of myeloperoxidase to the antibacterial substance or substances extracted from leukocytes by acid also is discussed.

MATERIALS AND METHODS

E. coli (ATCC no. 11775) was grown on Trypticase Soy Agar and Broth (BBL) and *Lactobacillus acidophilus* (ATCC no. 4357) on Lactobacillus Selective (LBS) Agar and Broth (BBL). Unless otherwise indicated, a 16- to 20-hr culture in broth was employed. The *E. coli* were washed twice with water and suspended in water to the required absorbancy at 540 mμ (Coleman Junior spectrophotometer) just prior to use. The culture of *L. acidophilus* was washed and suspended in 0.02 M sodium acetate buffer, *p*H 5.0, containing 0.01 M glucose. Dog myeloperoxidase was prepared in highly purified form (i.e., to the end of step 6) by the method of Agner (2). One preparation was kindly supplied by Cecil Yip. The myeloperoxidase preparation was dialyzed against distilled water prior to use. Lactoperoxidase was prepared from bovine milk by the method of Morrison and Hultquist (17). Catalase (crystalline beef liver, 53,560 units/mg) was obtained from Worthington Biochemical Corp. (Freehold, N.J.). A unit is defined as that amount of catalase which decomposes 1 μmole of H_2O_2 per min at 25 C and at *p*H 7.0. This preparation (Worthington CTR), which contained thymol as a disinfectant in concentra-

tions "not greater than 0.1%," was dialyzed overnight against water prior to use. ^{14}C-L-lysine (uniformly labeled 198.0 mc/mmole in 0.01 N HCl) was obtained from Schwarz BioResearch, Inc., (Orangeburg, N.Y.). The final HCl concentration resulting from the dilution of the lysine preparation was 10^{-5} M or 0.02 μmole per 2.0 ml of reaction mixture. All other reagents were obtained as previously described (12).

Peroxidase activity was determined by the *o*-dianisidine method (10). One unit of activity is that causing an increase in absorbancy of 0.001 per min at 460 mμ in a Cary M-15 spectrophotometer. The viable-cell count was determined by the pour-plate method as previously described (12). Intact guinea pig leukocytes, obtained from the peritoneal cavity following casein injection (12), were washed twice with a modified Hanks' solution containing bovine serum albumin and glucose (HBG) (5) prior to use.

^{14}C-lysine accumulation by *L. acidophilus* was determined as follows: An overnight culture of *L. acidophilus* was washed twice with 0.02 M acetate buffer, *p*H 5.0, containing 0.01 M glucose and suspended in the same solution to an absorbancy of approximately 0.250 in a Coleman Junior spectrophotometer (2×10^8 organisms/ml). The organisms were incubated with ^{14}C-lysine and the components indicated in the tables for 30 min at 37 C. The radioactivity associated with the intact bacteria and with the trichloroacetic acid precipitate was determined as previously described (4).

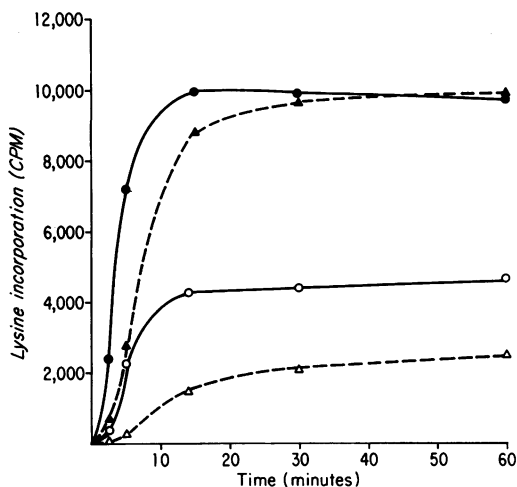

FIG. 1. *Lysine incorporation by Lactobacillus acidophilus. The reaction mixture contained sodium acetate buffer, pH 5.0, 30 μmoles; glucose, 22.5 μmoles; ^{14}C-lysine, 0.0015 μmole (0.3 μc); L. acidophilus, 3×10^8 organisms; and water to a final volume of 6.0 ml. The log-phase bacteria (solid line) were harvested after 3-hr growth and the stationary-phase cells (dashed line) after 20-hr growth. Portions of the reaction mixture were removed at the times indicated for the determination of whole cell-associated radioactivity (solid symbols) and trichloroacetic acid-precipitable radioactivity (open symbols).*

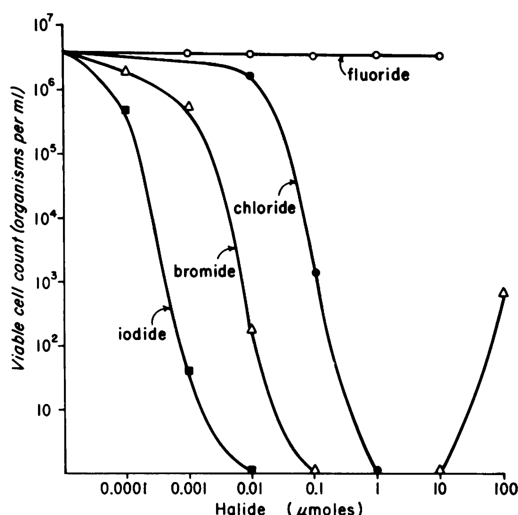

FIG. 2. *Comparison of the iodide, bromide, chloride, and fluoride containing bactericidal systems. The reaction mixture contained sodium acetate buffer, pH 5.0, 200 μmoles; Escherichia coli, 5×10^6 organisms per ml; myeloperoxidase, 150 units; H_2O_2, 0.01 μmole; the halide (iodide, ■, bromide, △, chloride, ●, fluoride, ○) in the amounts indicated; and water to a final volume of 2.0 ml. Incubation period was 30 min at 37 C.*

RESULTS

Two parameters were employed in this study as a measure of the antibacterial effect of myeloperoxidase: (i) bacterial viability with either *E. coli* or *L. acidophilus* as the test organism, and (ii) the accumulation of lysine by *L. acidophilus*. *L. acidophilus* was chosen in the latter instance because this organism grows well at an acid *p*H. Consequently, metabolic alterations can be demonstrated at a *p*H where the myeloperoxidase-mediated antibacterial system functions most efficiently.

In a previous paper (3), it was reported that the polyuridylic acid-dependent incorporation of phenylalanine into the trichloroacetic acid-precipitable material by a cell-free preparation of *L. acidophilus* proceeded well at an acid *p*H. *L. acidophilus* also accumulated lysine readily at *p*H 5.0 under the conditions employed (Fig. 1), and a portion of the accumulated lysine was converted to a trichloroacetic acid-precipitable form. Organisms harvested during the logarithmic phase of the growth cycle accumulated lysine more rapidly than did organisms harvested during the stationary phase of the growth cycle, and a greater proportion of the accumulated lysine was present in a trichloroacetic acid-precipitable form (Fig. 1). The accumulation of

lysine at 4 C was very slow, as compared to the accumulation at 37 C, and the complete deletion of glucose from the reaction mixture greatly decreased lysine accumulation. Thus, the uptake of lysine by *L. acidophilus* at *p*H 5.0 is largely an active process. Lysine accumulation by *L. acidophilus* can be rapidly and simply determined under controlled conditions and is thus a very convenient method for the study of myeloperoxidase-mediated antibacterial systems.

Effect of myeloperoxidase on E. coli. Figure 2 confirms the strong bactericidal effect of the myeloperoxidase-iodide-H_2O_2 system on *E. coli* previously reported (12) and compares the effectiveness of bromide, chloride, and fluoride ions to that of iodide ions in this system. Iodide ions were most effective on a molar basis, followed by bromide ions and then chloride ions. Myeloperoxidase, fluoride, and H_2O_2 had no bactericidal effect under the conditions employed. The decrease in the antibacterial effect of the bromide-containing system at high bromide concentrations (Fig. 2) was consistently observed.

The properties of the myeloperoxidase-bromide or chloride-H_2O_2 bactericidal system were similar to those described previously for the iodide-containing system (12). The deletion of myeloperoxidase, H_2O_2, or either bromide or chloride ions from the reaction mixture greatly decreased the bactericidal effect, as did preheating the myeloperoxidase at 90 C for 10 min. The bactericidal effect of the bromide- or chloride-containing systems was greatest at the most acid *p*H employed (*p*H 5.0) and decreased markedly as the *p*H was increased to neutrality. Those substances which prevented microbial killing by the myeloperoxidase-iodide-H_2O_2 system [azide, cyanide, thiocyanate, Tapazole, thiourea, reduced glutathione, cysteine, ergothioneine, thiosulfate, reduced nicotinamide adenine dinucleotide ($NADH_2$), reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), tyrosine] also decreased microbial killing when bromide or chloride ions were substituted for iodide ions. Table 1 gives the relative inhibitory effect of these agents on the killing of *E. coli* by the myeloperoxidase-chloride-H_2O_2 system at *p*H 5.0.

It was previously reported that myeloperoxidase could be replaced by lactoperoxidase when the halide component of the bactericidal system was iodide ion (12). The effectiveness of myeloperoxidase and lactoperoxidase also was comparable when bromide ions were employed; however, when chloride ions were used, lactoperoxidase was considerably less effective than was myeloperoxidase as a component of the bactericidal system (Table 2).

Effect of myeloperoxidase on L. acidophilus. The antibacterial effect of myeloperoxidase on *L. acidophilus* was measured both by bacterial killing and by the inhibiton of lysine accumula-

TABLE 1. *Effect of inhibitors*[a]

Inhibitors	Viable-cell count (organisms per ml)			
	1.0 μmole	0.1 μmole	0.01 μmole	0.001 μmole
Cyanide	9.7×10^6	8.9×10^5	8.4×10^2	0.4×10^2
Azide		6.4×10^6	2.2×10^5	0.2×10^2
Thiocyanate		6.9×10^6	5.8×10^6	4.1×10^3
Perchlorate		0.2×10^2		
Tapazole		6.3×10^6	6.5×10^6	2.0×10^4
Thiourea		7.4×10^6	7.8×10^6	4.5×10^6
Glutathione (reduced)		5.5×10^6	2.2×10^3	1.9×10^2
Cysteine		2.8×10^6	1.4×10^6	2.4×10^3
Ergothioneine		2.8×10^6	3.6×10^6	4.2×10^2
Thiosulfate		3.3×10^6	2.4×10^6	3.2×10^6
$NADH_2$		9.2×10^6	9.0×10^4	1.3×10^2
NAD		1.0×10^3		
$NADPH_2$		9.6×10^6	2.4×10^5	6.6×10^2
NADP		2.9×10^2		
Tyrosine	4.1×10^6	9.8×10^3	5.2×10^2	1.7×10^2

[a] Conditions were as described in Fig. 2, except that the initial *Escherichia coli* concentration was 8×10^6 organisms/ml, 2 μmoles of NaCl were added, and the inhibitors were added in the amounts indicated. The viable-cell count following 30-min incubation in the absence of the inhibitors ranged from 0 to 10^2 organisms per ml.

TABLE 2. *Comparison of myeloperoxidase and lactoperoxidase*[a]

Chloride (μmoles)	Viable-cell count (organisms per ml)	
	Myeloperoxidase	Lactoperoxidase
100	0	7.2×10^{3}
10	0	8.2×10^{5}
1	0	1.1×10^{6}
0.1	2.0×10^{3}	3.6×10^{6}
0.01	2.5×10^{6}	4.1×10^{6}

[a] Reaction mixture was as described in Fig. 2, except that either myeloperoxidase (150 units) or lactoperoxidase (150 units) was employed as indicated.

tion (Table 3). Relatively high concentrations of myeloperoxidase exerted an antibacterial effect in the absence of added halide or H_2O_2. When ^{14}C-lysine was used, a small amount of chloride (0.02 μmole) was added as a component of the lysine solution (*see* Materials and Methods). This small amount of chloride did not affect lysine accumulation in the presence of 75 to 300 units of myeloperoxidase under the conditions employed (Table 3), but it may have contributed to the inhibitory effect when greater amounts of myeloperoxidase were used. The bactericidal effect of high concentrations of myeloperoxidase was demonstrable in the absence of added ^{14}C-lysine-HCl, under the conditions employed (Table 3).

When the myeloperoxidase concentration was decreased, a marked increase in the antibacterial effect was evident upon the addition of chloride, bromide, or iodide ions. The antibacterial effect of myeloperoxidase was heat-sensitive both in the absence and in the presence of added halide. The protective effect of preheating myeloperoxidase at 100 C for 10 min is shown in Table 3. No effect was produced by heating the enzyme at 60 C for 10 min; a partial decrease in antibacterial effect was produced on heating at 70 C for 10 min; and essentially complete prevention of antibacterial effect was produced by heating at 80 C or greater for 10 min.

Since lactobacilli are H_2O_2-generating organisms, the absence of a requirement for the addition of H_2O_2 (Table 3) may be due to the formation of H_2O_2 by the organisms. If this were the case, it might be expected that catalase would prevent the inhibition of lysine accumulation by myeloperoxidase. Catalase was protective both in the absence and presence of added halide, which suggests that H_2O_2 of endogenous origin is required for a maximal antibacterial effect. A higher concentration of catalase was required in the absence of halide or in the iodide-containing system for comparable protection under the conditions employed. The protective effect of catalase was greatly diminished by preheating the enzyme at 100 C for 10 min. Catalase could not be replaced by crystalline bovine albumin, although the latter substance did exert a small protective effect at the concentrations employed.

Effect of leukocyte extracts. Among the mechanisms proposed for the killing of bacteria by polymorphonuclear (PMN) leukocytes is one which involves a basic protein or polypeptide with antibacterial properties which can be extracted from intact leukocytes or from isolated leukocyte granules by acid (6–8, 21, 23, 24). The relationship of myeloperoxidase to the antibacterial substance or substances extracted from intact leukocytes by acid is considered in this section.

Guinea pig peritoneal lavage leukocytes were suspended in 0.05 M sodium acetate buffer, *p*H 3.5, to a cell density of 25×10^{6} to 30×10^{6} cells/ml. The cell suspension was incubated at 37 C for 60 min in an Eberbach water bath shaker oscillating 120 times/min and then was centrifuged at 25,000 × *g* for 15 min. The supernatant solution contained peroxidase activity (1,300 to 2,000 *o*-dianisidine units/ml). The peroxidase activity of the extract was decreased when the *p*H of the extraction fluid was increased above *p*H 4.0, and no peroxidase activity was found when extraction was performed at *p*H 5.0 or greater. In another experiment, the white blood cell (WBC) pellet was frozen and thawed three times with dry ice-alcohol and extracted for 60 min at 37 C with 0.9% NaCl, 0.01 M citric acid, or 0.2 N H_2SO_4. The saline extract contained only slight peroxidaset activity (30 units/ml); the 0.01 M citric acid extract contained peroxidase activity equivalent to tha of the 0.05 M acetate, *p*H 3.5, extract; and the 0.2 N H_2SO_4 extract was devoid of peroxidase activity. The 0.05 M acetate, *p*H 3.5, extract was diluted with nine volumes of 0.05 M sodium acetate buffer, *p*H 5.5, just prior to use. The final *p*H of the diluted extract was 5.0, and the protein concentration was 0.1 mg/ml.

Table 4 gives the effect of the WBC extract on lysine accumulation by *L. acidophilus*. An inhibition of lysine accumulation was produced by the WBC extract in the absence of added halide under the conditions employed; however, the addition of chloride, bromide, or iodide ions produced a marked increase in the inhibitory effect. The halides alone were without effect at the concentrations employed. However, when the iodide concentration was increased above that shown in Table 4, an inhibition of lysine accumulation was observed in the absence of the WBC extract.

TABLE 3. *Effect of the myeloperoxidase (MPO)-halide-H_2O_2 system on Lactobacillus acidophilus*[a]

Supplements	Lysine accumulation (% inhibition)	Viable-cell count (organisms/ml)
None	0	2.0×10^7
MPO	0	2.1×10^7
MPO (150 units)	0	2.4×10^7
MPO (300 units)	0	2.3×10^7
MPO (600 units)	61.2	9.9×10^6
MPO (1,200 units)	93.7	4.0×10^4
MPO (1,800 units)	96.7	2.0×10^4
MPO (1,200 units) heated	4.8	1.9×10^7
MPO (1,200 units) + catalase (200 μg)	47.3	3.0×10^5
MPO (1,200 units) + catalase (800 μg)	8.0	1.2×10^7
MPO (1,200 units) + catalase (800 μg) heated	97.1	4.8×10^3
MPO (1,200 units) + albumin (800 μg)	87.6	1.2×10^4
NaCl	0	2.2×10^7
MPO + NaCl	98.2	4.1×10^2
MPO heated + NaCl	3.4	1.8×10^7
MPO + NaCl + catalase (200 μg)	0	2.0×10^7
MPO + NaCl + catalase (200 μg), heated	98.2	9.3×10^2
MPO + NaCl + albumin (200 μg)	98.9	2.0×10^4
NaBr	0	2.2×10^7
MPO + NaBr	99.0	2.4×10^3
MPO heated + NaBr	4.0	1.6×10^7
MPO + NaBr + catalase (200 μg)	9.8	1.0×10^7
MPO + NaBr + catalase (200 μg), heated	98.7	4.0×10^3
MPO + NaBr + albumen (200 μg)	89.4	1.4×10^4
NaI	0	2.0×10^7
MPO + NaI	99.1	7.0×10^1
MPO heated + NaI	0	1.8×10^7
MPO + NaI + catalase (200 μg)	30.7	8.6×10^6
MPO + NaI + catalase (800 μg)	5.5	1.4×10^7
MPO + NaI + catalase (800 μg), heated	98.9	1.7×10^2
MPO + NaI + albumen (800 μg)	91.2	1.2×10^4

[a] Reaction mixture for the determination of lysine accumulation contained sodium acetate buffer, pH 5.0, 10 μmoles; *L. acidophilus*, 2.0×10^7 organisms per ml; glucose, 7.5 μmoles; ^{14}C-lysine, 0.0005 μmole (0.1 μc); water to a final volume of 2.0 ml; and the supplements indicated below as follows: myeloperoxidase, 75 units unless otherwise indicated; catalase in the amounts indicated; crystalline bovine albumin in the amounts indicated; NaCl, 10 μmoles; NaBr, 0.1 μmole; and NaI, 0.02 μmole. The myeloperoxidase and catalase were heated at 100 C for 10 min where indicated. The reaction mixture for the determination of viability was as described above, except that ^{14}C-lysine was not added. Incubation period was 30 min.

TABLE 4. *Inhibition of lysine accumulation by the WBC extract-halide system*[a]

Supplements	Lysine accumulation (counts/min)			
	No added halide	+NaCl	+NaBr	+NaI
None	11,000	10,700	10,700	10,100
WBC extract	4,920	102	30	41
WBC extract (heated)	7,220	6,820	6,380	6,370
WBC extract + cyanide	7,860	5,920	6,030	5,640
WBC extract + azide	7,930	6,450	6,320	6,150
WBC extract + Tapazole	7,680	6,740	6,940	7,210
WBC extract + thiosulfate	8,590	7,210	7,790	8,130
WBC extract + catalase	8,060	8,460	7,530	5,820
WBC extract + catalase (heated)	4,820	38	32	53

[a] Reaction mixture contained acetate buffer, pH 5.0, 35 μmoles; *Lactobacillus acidophilus*, 10^8 organisms; glucose, 7.5 μmoles; ^{14}C-lysine, 0.0005 μmole (0.1 μc); halide (NaCl, 10 μmoles; NaBr, 0.1 μmole; NaI, 0.02 μmole) where indicated, water to a final volume of 2.0 ml; and the supplements indicated below as follows: WBC extract, 0.5 ml (peroxidase activity, 75 units); cyanide, 1 μmole; azide, 1 μmole; Tapazole, 1 μmole; thiosulfate, 1 μmole; and catalase, 200 μg. The WBC extract and catalase were heated at 100 C for 10 min where indicated.

The bactericidal effect of iodide and H_2O_2 was previously reported (12). The inhibition of lysine accumulation by the WBC extract, both in the absence and in the presence of added halide, was greatly decreased but not abolished by preheating the WBC extract at 100 C for 10 min. A decrease in the antibacterial effect was also produced by cyanide, azide, Tapazole, thiosulfate, and catalase (Table 4). In general, complete prevention of the antibacterial effect by these agents was not observed. The addition of H_2O_2 was not required for the inhibition of lysine accumulation by the

TABLE 5. *Bactericidal effect of WBC extract-halide-H_2O_2 system*[a]

Supplements	Viable-cell count (organisms per ml)	
	L. acidophilus	*Escherichia coli*
None	5.3×10^7	5.3×10^7
WBC extract	2.6×10^7	2.6×10^7
H_2O_2	4.9×10^7	3.9×10^7
WBC extract + H_2O_2	2.7×10^7	1.6×10^7
NaCl	3.7×10^7	5.1×10^7
NaCl + H_2O_2	4.4×10^7	3.8×10^7
WBC extract + NaCl	4.2×10^5	1.7×10^7
WBC extract + NaCl + H_2O_2	3.6×10^3	9.8×10^4
NaBr	2.3×10^7	3.3×10^7
NaBr + H_2O_2	3.1×10^7	2.3×10^7
WBC extract + NaBr	3.4×10^4	1.9×10^7
WBC extract + NaBr + H_2O_2	2.2×10^4	4.6×10^6
NaI	3.2×10^7	4.1×10^7
NaI + H_2O_2	2.5×10^7	5.0×10^7
WBC extract + NAI	2.0×10^6	2.0×10^7
WBC extract + NaI + H_2O_2	5.0×10^4	2.1×10^6

[a] Reaction mixture contained acetate buffer, *p*H 5.0, 35 μmoles; *Lactobacillus acidophilus*, 10^8 organisms, or *E. coli*, 10^8 organisms as indicated; glucose, 7.5 μmoles; water to a final volume of 2.0 ml; and the supplements indicated below as follows: WBC extract, 0.5 ml (peroxidase activity, 75 units); NaCl, 10 μmoles; NaBr, 0.1 μmole; NaI, 0.02 μmole; and H_2O_2, 0.1 μmole. Incubation period was 30 min.

WBC extract-halide system under the conditions employed (Table 4). However, the protective effect of catalase suggests that H_2O_2 of endogenous origin was required. This effect of catalase was abolished by preheating the enzyme at 100 C for 10 min.

The bactericidal effect of the WBC extract is shown in Table 5. The concentrations of reagents employed were the same as employed in Table 4. Under these conditions, the WBC extract alone had a slight bactericidal effect on both *L. acidophilus* and *E. coli*. The additon of either chloride, bromide, or iodide ions to the WBC extract greatly increased the bactericidal effect on *L. acidophilus*, whereas little effect was produced by the halides when *E. coli* was used as the test organism. However, when H_2O_2 was added to the WBC extract-halide system, considerable killing of *E. coli* was observed with all the halides used. Similarly, when *L. acidophilus* was used as the test organism, H_2O_2 increased the bactericidal effect of the WBC extract-halide system.

DISCUSSION

The studies reported here and in a previous paper (12) indicate that myeloperoxidase has an antibacterial effect when combined with certain halides and H_2O_2. Iodide ion was the most effective halide, followed by bromide and then chloride ion. The relative concentrations required for an equivalent antibacterial effect were approximately: iodide, 1; bromide, 15; and chloride, 200.

Myeloperoxidase is a basic protein with an isoelectric point which has been estimated to be greater than 10 (1). It is extracted from intact guinea pig leukocytes by weak acid (e.g., 0.05 M acetate buffer, *p*H 3.5, 0.01 M citric acid); and the extract with peroxidase activity has antibacterial properties which are similar in many respects to those of purified myeloperoxidase. Under appropriate conditions, the antibacterial effect of both the acid extract of intact leukocytes and purified myeloperoxidase was increased by the addition of H_2O_2 or a halide such as iodide, bromide, or chloride ion and was decreased by the addition of catalase, as well as by a number of other inhibitors and by heat treatment.

Although the similarity between the antibacterial properties of the weak acid extract of guinea pig leukocytes and purified myeloperoxidase suggests that the antibacterial properties of the extract are due, in some measure, to its peroxidase content, particularly when iodide, bromide, or chloride has been added to the reaction mixture, it is probable that other antibacterial systems are present in the extract as well. The antibacterial activity of the weak acid extract of guinea pig leukocytes was not completely abolished by heating at 100 C for 10 min under the conditions employed (Table 4), whereas essentially complete inactivation of purified myeloperoxidase was produced by heating at 80 C for 10 min. Similarly, Hirsch reported that the phagocytin preparation extracted from rabbit PMN leukocytes by 0.01 M citric acid is only partially inactivated by heating at 100 C for 30 min (8). An earlier preparation (6), which was extracted at a less acid *p*H, was completely inactivated by this heating regimen. The leukin of Skarnes and Watson (21) can be distinguished from the myeloperoxidase-mediated systems by its heat stability. The antibacterial cationic proteins of rabbit leukocyte granules described by Zeya and Spitznagel also are heat-stable (100 C for 10 min at *p*H 3.0) and are extracted in an active form by 0.2 N H_2SO_4 (24). The 0.2 N H_2SO_4 extract of guinea pig leukocytes was devoid of peroxidase activity under the condi-

tions employed in this investigation, although the extraction of denatured peroxidase protein cannot be excluded. Thus, myeloperoxidase appears to be one of a group of cationic antibacterial agents present in the acid extract of PMN leukocytes which can be distinguished from at least some of the others by its heat lability, the requirement for H_2O_2, and an appropriate oxidizable substance, such as iodide, bromide, chloride, or thiocyanate ions, and by its susceptibility to inhibition by certain agents (e.g. cyanide, azide, Tapazole, thiosulfate, and catalase).

Myeloperoxidase at relatively high concentrations has an antibacterial effect on *L. acidophilus* in the absence of added halide. The protective effect of catalase suggests that H_2O_2 of endogenous origin is required for maximal effect. The acid extract of intact leukocytes also exerts an antibacterial effect without added halide which is, to some extent, catalase-sensitive. McRipley and Sbarra (16) recently reported on the synergistic bactericidal effect of a guinea pig PMN leukocyte granule lysate and H_2O_2 on a number of organisms. It is presumed that, when an oxidizable substance such as a halide is required, the antibacterial effect is due to the conversion of this substance by oxidation from a weak to a strong antibacterial agent. It is not known whether the bacteria are directly oxidized by myeloperoxidase and H_2O_2 in the absence of added halide or whether the antibacterial effect is mediated by the oxidation of an intermediate substance. The introduction of a small amount of halide or other oxidizable substance to the reaction mixture with the other reagents or bacteria cannot be excluded.

The uptake of iodide and iodinated hormones by intact leukocytes (20) was discussed in relation to their possible involvement in a peroxidase-mediated antimicrobial system in a previous publication (12). Chloride ions also are readily taken up by leukocytes. The average intracellular chloride concentration of rabbit peritoneal lavage leukocytes was 93 meq/kg of cell water, as compared to a normal rabbit serum concentration of 115 meq/liter of serum water (22). Intracellular chloride bore a linear relationship to the concentration of chloride in the suspension medium over a range of 0.3 to 161 meq/liter (22). A bactericidal effect was observed at a chloride concentration of 0.005 meq/liter, with total killing at a concentration of 0.5 meq/liter under the conditions employed in this study (Fig. 2). This is well within the concentrations reported to be present in leukocytes. It should be emphasized that, if physiological saline is used in the incubation mixture, adequate amounts of chloride ions will be present to act synergistically with myeloperoxidase at acid *p*H to produce a bactericidal effect.

The requirement for H_2O_2 in the peroxidase-mediated antimicrobial systems can be met by microbial metabolism. Thus, when *L. acidophilus* was the test organism, both the bactericidal effect and the inhibition of lysine accumulation were readily demonstrable without the addition of H_2O_2. Lactobacilli are H_2O_2-generating organisms, and the prevention of the antibacterial effect by catalase suggests that this endogenously produced H_2O_2 is required as a component of the antibacterial system. With other organisms (e.g., *E. coli*), the generation of H_2O_2 by microbial metabolism was apparently inadequate under the experimental conditions employed in this study, because the addition of H_2O_2 was required. In the PMN leukocyte, the process of phagocytosis and granule rupture is associated with a burst of leukocyte metabolic activity; among the consequences of this increased metabolic activity is the generation of H_2O_2 by the cell (9, 15, 18, 19). The intracellular H_2O_2 concentration was estimated to range from 0.001 to 0.1 M, depending on the amount of phagocytosis and the intracellular *p*H (9). Direct measurement of H_2O_2 production by PMN leukocytes indicates a two- to fourfold increase following phagocytosis (18). H_2O_2 alone has antibacterial properties; however, the antibacterial effect of H_2O_2 is increased many orders of magnitude by the addition of myeloperoxidase and iodide, bromide, or chloride ions. The concentration of H_2O_2 routinely used for complete bacterial killing in the study reported here was 0.00005 M, and a lesser antibacterial effect was observed with smaller concentrations. Thus, the H_2O_2 formed in leukocytes following phagocytosis appears to be present in concentrations adequate to function as a component of the myeloperoxidase-mediated antibacterial system.

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