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Mouse Splenic Peroxidase and Its Role in Bactericidal Activity[1]

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Spleen cell suspensions from AKR and CD-1 mice contain peroxidase activity as determined by guaiacol oxidation. This activity is found predominately in the 20,000 × *g* pellet fraction of spleen cell homogenates. In the presence of H_2O_2 and chloride ion at acidic *p*H, splenic peroxidase mediates the oxidation of D- or L-alanine to CO_2, NH_3, and acetaldehyde. The same reaction mixture without added amino acid can kill both gram-positive and gram-negative bacteria. The conditions for both reactions are similar. Both have an absolute requirement for H_2O_2 and chloride ion, neither is active at neutral or alkaline *p*H, and both are inhibited by the sulfonic amino acid taurine. In these aspects, splenic peroxidase is qualitatively similar in its activity to myeloperoxidase (MPO) from polymorphonuclear leukocytes. It is quantitatively different from MPO in that the latter is more potent on a per guaiacol unit basis with respect to both amino acid oxidation and bactericidal activity. Still another quantitative difference is that splenic peroxidase requires 0.1 M NaCl for activity, whereas MPO functions with as little as 0.005 M NaCl. Splenic peroxidase and MPO both appear to differ qualitatively from horseradish peroxidase in that the latter enzyme does not mediate amino acid oxidation.

Recently, we reported that spleen cell suspensions from either AKR or CD-1 mice possess bactericidal and associated metabolic activities similar to those of polymorphonuclear leukocytes (PMN; reference 14; B. B. Paul et al. Bacteriol. Proc., p. 91, 1970; R. R. Strauss et al., Bacteriol. Proc., p. 91, 1970). These cells, greater than 95% of which are lymphocytes, appeared to exert their bactericidal activity through the peroxidase-H_2O_2-halide system.

Reports from our laboratory have postulated that the mechanism of this antimicrobial system involves the production of aldehydes from amino acids or other suitable substrates found in or on the bacterial surface (9). These reports were an extension of the observations of Zgliczynski et al. (17), who found that at acidic *p*H the combination of highly purified myeloperoxidase (MPO), H_2O_2, and chloride reacted with various amino acids to produce equimolar quantities of CO_2 and NH_3 and aldehydes of one carbon less than the original amino acid.

The purpose of the present report is to define more precisely the biochemistry of the splenic peroxidase and to relate its activity to aldehyde formation and the bactericidal activity of the cell involved.

MATERIALS AND METHODS

Strain AKR mice were purchased from Jackson Memorial Laboratory, Bar Harbor, Me. The CD-1 strain mice were purchased from the Charles River Breeding Laboratories, Wilmington, Mass. Both strains of mice were bred and maintained at the St. Margaret's Hospital animal facilities.

Mouse spleen cell suspensions were obtained by previously described procedures (14). Cells were suspended in 0.25 M sucrose before homogenization in a Potter-Elvejhem homogenizer with a Teflon-tipped, motor-driven pestle. Homogenization was for 2 min at 3,800 rev/min in an ice bath. Homogenates were centrifuged at 20,000 × *g* for 30 min in a refrigerated Sorvall RC-2 centrifuge. The pellet obtained from this procedure contained all but traces of the homogenate peroxidase activity.

Escherichia coli, *Salmonella typhimurium*, and *Staphylococcus aureus* were obtained from the St. Margaret's Hospital stock culture collection. The two strains of *Listeria monocytogenes* used in these studies were obtained from the Massachusetts Department of Public Health Laboratories. Bactericidal studies were conducted by previously reported procedures (11).

[1] This is paper number XXXIII in a series entitled *The Role of the Phagocyte in Host-Parasite Interactions.*

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Log-phase cultures of bacteria, H_2O_2 at indicated concentrations, and 20,000 × g pellet fraction of mouse spleen cells containing various concentrations of peroxidase activity were used. The halide requirement for the bactericidal system was met by the NaCl of the Krebs-Ringer phosphate medium (KRPM). The final pH was 5.5. In some experiments, 0.1 M phosphate buffer was employed instead of KRPM. In these instances, NaCl was added to the reaction mixture. The total volume of these reactions was 6.0 ml.

Peroxidase activity was determined by the guaiacol procedure (8). Decarboxylation of L-alanine-*1*-^{14}C was determined in single-sidearm Warburg flasks by previously described procedures (12).

Deamination studies were also conducted in Warburg flasks. The contents of the main compartment were: 20,000 × g pellet fraction of mouse spleen cell homogenate containing 0.03 guaiacol units of peroxidase, 0.6 μmoles of H_2O_2, 5.4 μmoles of L-alanine, and 180 μmoles of KRPM (pH 5.5) to a total volume of 3.0 ml. The center well contained 0.5 ml of 1 M H_2SO_4 for trapping of NH_3. Appropriate controls for enzyme, substrate, and H_2O_2 were run simultaneously. The reaction was run for 60 min at 37 C in a shaking water bath (90 strokes/min) and was terminated by addition of 0.2 ml of 20% KOH from the side arm. The flasks were incubated for an additional 10 min to liberate any dissolved ammonia and the contents of the center wells were assayed for ammonia nitrogen by the use of Nessler's color reagent. Appropriate controls lacking peroxidase or H_2O_2, or both, were run simultaneously. In addition, flasks containing 1.2 μmoles of taurine, a competitive inhibitor of this reaction (17), were also run in the same manner. Peroxidase-mediated aldehyde formation from L-alanine was determined in Warburg flasks containing 5.4 μmoles of L-alanine-*U*-^{14}C (specific activity 0.055 μCi/μmole), 0.03 guaiacol unit of peroxidase-containing fraction of spleen cells, 0.6 μmole of H_2O_2, and KRPM (pH 5.5) to 3.0 ml in the main compartment. The center well contained 0.2 ml of 20% KOH for trapping $^{14}CO_2$. The side arm contained 0.5 ml of a 0.2% ethanolic solution of 2,4-dinitrophenylhydrazine. Appropriate controls lacking peroxidase, H_2O_2, or 2,4-dinitrophenylhydrazine were run concurrently. After 15 min of incubation at 37 C in a shaking-water bath (90 strokes/min), phenylhydrazine or ethanol was added from the side arm. The flasks were opened for the addition of 0.2 ml of 30% trichloroacetic acid and then quickly closed and incubated for an additional 10 min. The contents of the center wells were removed and assayed for $^{14}CO_2$, and the contents of the main compartment were extracted with three successive 3.0-ml portions of petroleum ether. Each extraction was evaporated to dryness, and the final residue in an evaporating dish was placed on a bed of chopped ice and reconstituted to 1.0 ml with petroleum ether. A sample was counted directly for radioactivity. Another portion was diluted 1:1 with 2.5% methanolic KOH and co-chromatographed on paper with the phenylhydrazone of standard acetaldehyde which had been treated similarly. The chromatographic procedure was that of Zgliczynski et al. (17). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer equipped with an external standard for quench correction.

Radioactive chemicals were purchased from New England Nuclear Corp., Boston, Mass. All other chemicals used in these studies were of reagent quality and were purchased from commercial sources.

RESULTS

The relationship between peroxidase activity, as determined by guaiacol oxidation, L-alanine-*1*-^{14}C decarboxylation and bactericidal activity against *E. coli* are shown in Fig. 1. From this figure, it can readily be seen that both bactericidal activity and amino acid decarboxylation increase as the amount of peroxidase activity in the two systems increases. It must be noted that the decarboxylation reaction mixture differs from that of the bactericidal system only in the addition of amino acid and serves as a biochemical model for the bactericidal system.

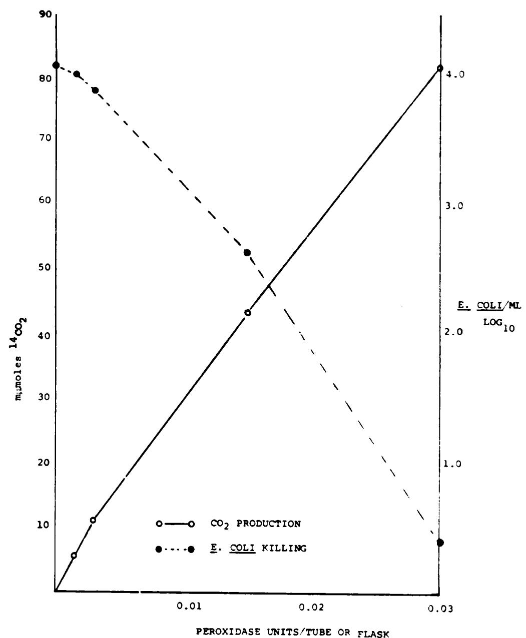

FIG. 1. *Decarboxylation reactions were run in single-side arm Warburg flasks. Each flask contained 0.6 μmole of H_2O_2, 5.4 μmoles of L-alanine-1-^{14}C (0.055 μCi/μmole), the indicated quantity of guaiacol units of peroxidase-containing fraction from AKR mouse spleen homogenate, and KRPM (pH 5.5) to a final volume of 3.0 ml. Radioactive CO_2 production is expressed as nanomoles per flask after 30 min of incubation. Bactericidal studies were done in screw-capped test tubes (16 by 150 mm). The reaction mixtures contained 6 × 10^4 viable E. coli cells, 0.3 μmole of H_2O_2, the indicated quantity of peroxidase units as above, and KRPM (pH 5.5) to a final volume of 6.0 ml. Results are expressed as viable bacteria per milliliter after 15 min of incubation at 37 C.*

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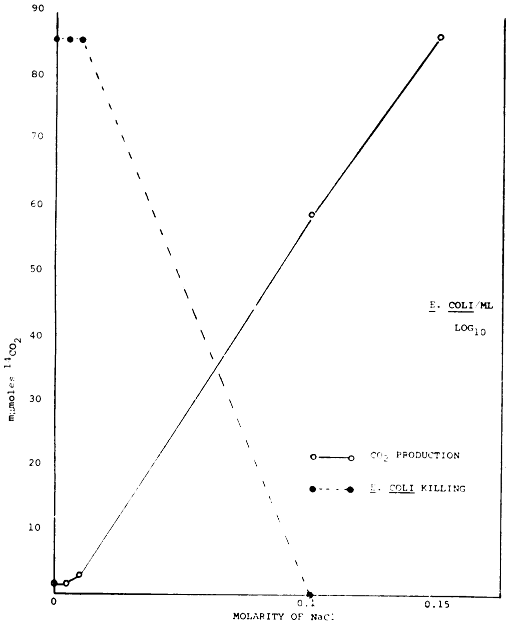

FIG. 2. *Decarboxylation was determined as described in Fig. 1 with the exception that each flask contained 0.03 guaiacol unit of peroxidase and the indicated concentrations of NaCl. Flasks were brought to 3.0 ml total volume with 0.1 M phosphate buffer (pH 5.5). Appropriate controls were run simultaneously. Bactericidal activity was determined similarly to that described for Fig. 1 with the exception that each tube contained 0.03 guaiacol unit of peroxidase and the indicated concentrations of NaCl. Tubes were brought to 6.0 ml final volume with 0.1 M phosphate buffer (pH 5.5). Appropriate controls were run simultaneously.*

Figure 2 shows the effects of various concentrations of chloride on both bactericidal activity and amino acid decarboxylation. The peroxidase activity and H_2O_2 concentration are constant. It is obvious that chloride is required for both reactions, and there are significant increases in both parameters at the same concentration of NaCl.

The effect of *p*H on the bactericidal activity of the peroxidase-H_2O_2-chloride system is shown in Fig. 3. Spleen cell homogenates from the CD-1 strain of mice were used in these experiments. The results in this table show that bactericidal activity occurs only at acidic *p*H values and that maximal activity occurs at *p*H 5.5. At neutral or alkaline *p*H, there is no demonstrable bactericidal activity.

The antibacterial activity of the mouse spleen peroxidase-H_2O_2-chloride bactericidal system against four different organisms is shown in Table 1. Both the gram-positive and gram-negative bacteria appeared susceptible. Different degrees of sensitivity were noted.

The effects of the well known peroxidase antagonists, NaN_3, KCN, and taurine, which competitively inhibits L-alanine-*1*-^{14}C decarboxylation but not guaiacol oxidation, on decarboxylation and bactericidal activity are shown in Table 2. These data show that all three inhibitors caused marked decreases in both amino acid decarboxylation and bactericidal activity when used at 1 mM concentration. The chloride requirement for these experiments was satisfied by the NaCl component of KRPM.

The effect of incubation time on decarboxylation of L-alanine-*1*-^{14}C by the peroxidase-containing fraction from AKR mouse spleen cell homogenates is shown in Fig. 4. The data presented in this figure reveal that maximal conversion of the amino acid substrate occurs after 20 min of incubation at 37 C. Incubation for 30 min did not markedly change the amount of $^{14}CO_2$ produced under the experimental conditions employed.

The marked effect of *p*H on the decarboxylation of L-alanine-*1*-^{14}C by CD-1 mouse spleen peroxi-

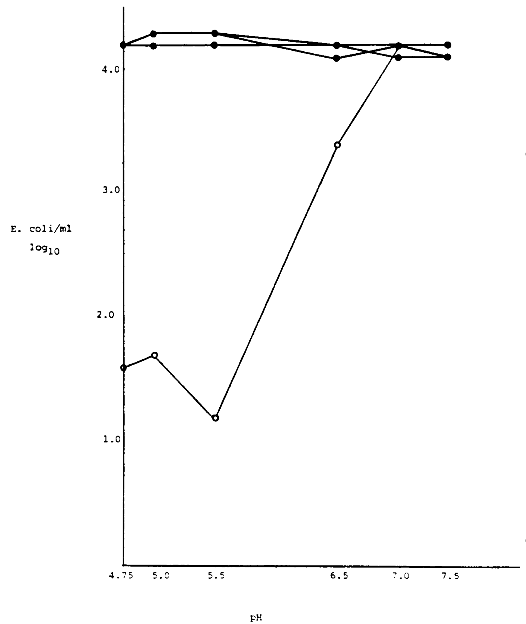

FIG. 3. *E. coli cells per milliliter after 15 min of incubation at 37 C. The complete system is indicated by the open circles. Each tube contained 0.3 μmole of H_2O_2, 0.03 guaiacol unit of peroxidase-containing fraction from CD-1 mouse spleen homogenate, 900 μmole of NaCl, E. coli cells, and 0.1 M phosphate buffer of indicated pH to a final volume of 6.0 ml. The closed circles represent the E. coli cells per milliliter for the controls which are lacking in H_2O_2, peroxidase activity, or both. The bacterial concentrations at time zero were 10^4/ml.*

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TABLE 1. *Bactericidal activity of peroxidase-containing pellet from AKR mouse spleen homogenates*[a]

Bacteria	Incubation time (min)	Bacteria/ml at T_0			
		10^4	10^5	10^6	10^7
Escherichia coli	15	$<2.5 \times 10^0$	$<2.5 \times 10^0$	5.9×10^1	2.2×10^2
Salmonella typhimurium	15	$<2.5 \times 10^0$	1.1×10^1	5.8×10^2	4.2×10^4
S. typhimurium	30	$<2.5 \times 10^0$	5.0×10^0	1.3×10^2	1.6×10^4
Staphylococcus aureus	15	7.9×10^1	1.8×10^3	2.1×10^5	
S. aureus	30	$<2.5 \times 10^0$	2.5×10^2	1.4×10^4	
Listeria monocytogenes 111	15	1.5×10^1	1.5×10^2	4.1×10^3	6.1×10^4
L. monocytogenes Q16	15	1.6×10^2	2.1×10^3	2.7×10^4	5.1×10^4

[a] Reaction mixtures were similar to those described for Fig. 1 except that different organisms were involved. Control values at the various time intervals were not significantly different from those obtained at T_0.

TABLE 2. *Effect of inhibitors on decarboxylation and bactericidal activity of 20,000 × g peroxidase-containing pellet from AKR mouse spleen homogenates*

Additions	Decarboxylation[a]	Bactericidal activity[b]
None	0.48	1.62×10^4
H_2O_2	0.55	1.58×10^4
20,000 × *g* Fraction	0.62	1.59×10^4
H_2O_2 + fraction	68.36	$<2.50 \times 10^0$
H_2O_2 + fraction + taurine	17.87	1.10×10^4
H_2O_2 + fraction + Na-Azide	0.93	1.64×10^4
H_2O_2 + fraction + KCN	1.73	1.77×10^4

[a] Values expressed as nanomoles of $^{14}CO_2$/0.03 guaiacol unit from L-alanine-*1*-^{14}C. Decarboxylation and bactericidal activities were determined as described in Fig. 1, with the exception that the inhibitors where indicated were added to a final concentration of 1 mM.

[b] *E. coli* cells per milliliter after 15 min of incubation.

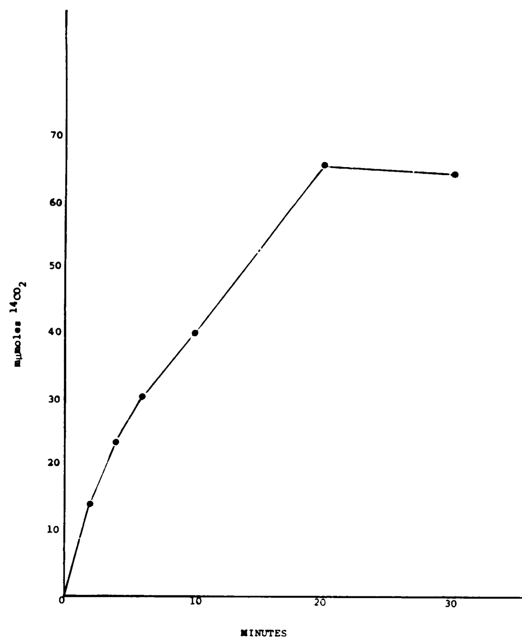

FIG. 4. *L-Alanine-1-^{14}C decarboxylation was determined at the specified time intervals by the procedures described in Fig. 1.*

dase is shown in Fig. 5. Maximal activity was observed at *p*H 5.5 with sharp decreases in activity at either side of this peak. The effects shown in this report occur with the 20,000 × *g* peroxidase-containing fraction from spleen cell homogenates of both CD-1 and AKR mice.

The concentration of H_2O_2 necessary for amino acid decarboxylation in this reaction is critical. The data presented in Fig. 6 show that the system is activated at H_2O_2 concentrations between 5×10^{-5} M and 10^{-4} M with maximal activity at 2×10^{-4} M. The activity drops off sharply at 4×10^{-4} M and still further at 6×10^{-4} M. There is no longer any significant activity at a final concentration of 10^{-3} M H_2O_2 under the conditions employed in our experiments.

The quantitative relationship between L-alanine decarboxylation and acetaldehyde formation is shown in Table 3. The incubation time of this experiment was only 15 min because, in other experiments, we found that prolonged incubation time resulted in progressive loss of radioactive aldehyde. The aldehyde formed was identified as acetaldehyde by co-chromatography on paper with the phenylhydrazone of reagent acetalde-

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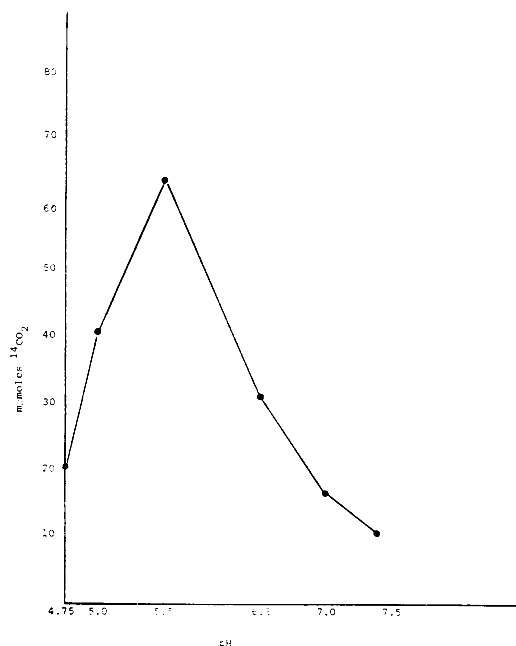

FIG. 5. *L-Alanine-1-*^{14}C *decarboxylation was determined by a procedure similar to that described in Fig. 1 with the exception that the source of peroxidase (0.03 guaiacol unit/flask) was the 20,000 × g pellet fraction from CD-1 mouse spleen homogenates and the buffers were 0.1 M phosphate of the pH indicated in the figure. The NaCl concentration was 0.15 M in each flask.*

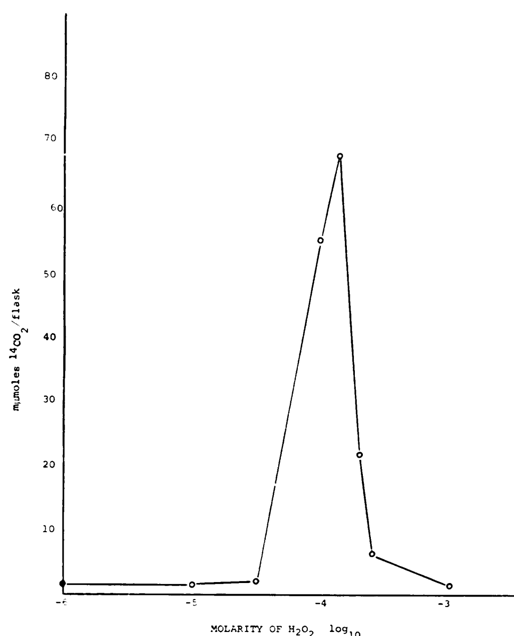

FIG. 6. *Decarboxylation was determined by the procedure described in Fig. 1 except that the final molarity of* H_2O_2 *was as indicated in the figure.*

TABLE 3. *Aldehyde and* CO_2 *production from alanine-U-*^{14}C *by 20,000 × g pellet fraction from AKR mouse spleen cell homogenate*

Determination	Amt produced (nmoles)	
	CO_2	Aldehyde
Alanine-*U*-^{14}C	0.5	0.3
Alanine-*U*-^{14}C + H_2O_2	0.7	0.4
Alanine-*U*-^{14}C + granules	0.5	0.4
Alanine-*U*-^{14}C + granules + H_2O_2	55.4	37.9

TABLE 4. *Effect of* H_2O_2 *and taurine on L-alanine deamination by 20,000 × g pellet of AKR mouse spleen homogenate*

Additions	Amt of NH_3 produced (nmoles/60 min)
None	0
2×10^{-4} M H_2O_2	149.54
H_2O_2 + 4×10^{-4} M taurine	56.08

TABLE 5. *Decarboxylation of D-alanine-1-*^{14}C *and L-alanine-1-*^{14}C *by 20,000 × g peroxidase-containing fraction from AKR mouse spleen homogenates*

Determination	$^{14}CO_2$/flask (nmoles)	
	H_2-O_2	H_2+O_2
D-Alanine-*1*-^{14}C	0.81	62.65
L-Alanine-*1*-^{14}C	0.63	58.26

hyde. Under these conditions, there was a spot at R_F 0.15 with the phenylhydrazone of the reagent acetaldehyde, the ether-extracted material from our reaction mixture, and the combination of both. There was also radioactivity present in the areas containing the extracts from our experimental reaction mixture.

Deamination of L-alanine by the peroxidase-containing fraction from AKR mouse spleen homogenates is shown in Table 4. These data show that H_2O_2 is necessary for activity and that taurine, which we and others have found to be a competitive inhibitor of the decarboxylation reaction, also inhibits deamination in the presence of H_2O_2.

Table 5 contains the results obtained with two isomers of alanine. These data show that the 20,000 × *g* peroxidase-containing fraction from AKR spleen homogenates mediates the decarboxylation of both D- and L-alanine equally well.

DISCUSSION

The data presented show that the 20,000 × *g* pellet fraction of CD-1 and AKR mouse spleen

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cell homogenates contains peroxidase activity. This finding confirms and extends that of Paul et al. (8) who found this activity in spleen cell homogenates of CD-1 mice. In this earlier report, it was also observed that peroxidase activity of homogenates of spleen cells which had been previously incubated with phagocytizable particles was higher than that of homogenates prepared from cells incubated in the absence of such particles.

In extending these observations we have shown a relationship between spleen cell peroxidase, amino acid decarboxylation, and in vitro bactericidal activities. Our data show that, by increasing the quantity of mouse splenic peroxidase in these reactions, one obtains parallel increases in both decarboxylation and bactericidal activities. It was previously shown that MPO from guinea pig PMN and human peripheral blood also mediates these activities (2, 9, 12, 13, 17, 20). It should be pointed out that with equal amounts of peroxidase activity the guinea pig PMN enzyme is about three times more potent than the mouse spleen cell enzyme with respect to both bactericidal and decarboxylation activity (12). These quantitative differences in activity may be indicative of some other more subtle differences between the mouse spleen cell peroxidase and MPO from PMN leukocytes. Both of these enzymes appear to be different from horseradish peroxidase (HPO) because the latter enzyme does not mediate amino acid decarboxylation (12) and it functions in the bactericidal system with iodide but not chloride as the participating halide (2, 9).

The importance of chloride in the MPO-mediated decarboxylation-deamination reaction was reported by Zgliczynski and his colleagues (17). We extended this observation to the enzyme from guinea pig PMN and also showed that chloride was necessary for bactericidal activity as well as amino acid decarboxylation (12, 13). The same is now shown to be true for the peroxidase obtained from the mouse spleen cells. However, the splenic peroxidase requires about 20 times as much chloride as the MPO from PMN to obtain significant decarboxylation and bactericidal activity. Since this increased requirement is found in the presence of equivalent amounts of peroxidase activity (guaiacol oxidation), it is another indication that the enzyme from the spleen is quantitatively different from that found in PMN leukocytes.

Purified MPO of human origin mediates aldehyde formation from amino acids optimally at *p*H 5.3 (17). Bactericidal and amino acid decarboxylation activities of the MPO-H_2O_2-Cl^- system from guinea pig PMN are optimal at *p*H 5.5 (3, 5, 13). The results in this report show that the mouse spleen peroxidase fraction is also maximal for these activities at this *p*H. The marked decrease in amino acid decarboxylation at *p*H 4.75 as contrasted with the minimal decrease in bactericidal activity is paradoxical. It is conceivable that at the lower *p*H the bacteria are more sensitive to the peroxidase-H_2O_2-chloride system. These data provide additional evidence that the enzyme from the spleen is similar in this respect to MPO of human and guinea pig origin, and the relationship between amino acid decarboxylation and bactericidal activity is further strengthened.

Our limited data on the antibacterial spectrum of the mouse spleen peroxidase-H_2O_2-chloride system reveal that the range of susceptible organisms is similar to that shown for the MPO-H_2O_2-halide systems that have been reported previously (3, 5). It is interesting to note that the *Staphylococcus* and the mouse pathogens *S. typhimurium* and *Listeria* are more resistant to the action of this system than the ubiquitous organism *E. coli*.

Our data indicate that peroxidase-H_2O_2-chloride antibacterial system from the mouse spleen has a mechanism of action similar to that of the guinea pig PMN but different from the iodide-requiring MPO-H_2O_2 system of Klebanoff (3, 4). This is based on some earlier reports from our laboratory pertaining to the MPO-H_2O_2-halide system from guinea pig PMN. We found that decarboxylation of amino acids occurred with chloride as the halide but not with iodide in the presence of MPO and H_2O_2 (2, 9). The sulfonic amino acid taurine, a competitive inhibitor of aldehyde formation (17), antagonized both amino acid decarboxylation and bactericidal activity of the MPO-H_2O_2-chloride system but had no effect on bactericidal activity when the halide was iodide (13). It was also reported that if one substitutes HPO for MPO in the chloride-mediated system, decarboxylation of amino acids does not occur (2, 9, 12). A corollary for this observation is that bactericidal activity by the HPO-H_2O_2-halide system occurs only when the halide employed is iodide (2, 3, 9). The results in this report show that the mouse spleen peroxidase mediates amino acid deamination and decarboxylation in the presence of H_2O_2 and chloride at acid *p*H. This system also functions as a potent bactericidal agent with both gram-positive and gram-negative bacteria. Taurine inhibits both of these spleen peroxidase-mediated functions. Hence mouse spleen peroxidase appears to be similar to MPO but differs from HPO in its mode of action.

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The optimal H_2O_2 concentration required for the mouse spleen peroxidase activity is the same as that reported for MPO from guinea pig PMN, i.e., 2×10^{-4} M (13). Under these conditions, the addition of small increments above the optimal concentration of peroxide markedly inhibits activity. This is characteristic of peroxidases which are inactivated by excess substrate, i.e., H_2O_2 (10). The main difference between the mouse spleen cell peroxidase and MPO is quantitative rather than qualitative. Thus, for equivalent guaiacol units, the MPO is three times more active in decarboxylation and bactericidal activity when compared to the spleen cell peroxidase.

Finally, we were able to show that in the same reaction mixture there were approximately equimolar concentrations of $^{14}CO_2$ and acetaldehyde produced from L-alanine-U-^{14}C and that the enzyme worked equally well on both D- and L-alanine. These results were also obtained with MPO containing $20,000 \times g$ granules from guinea pig PMN (13).

The results in this report provide additional evidence that mouse spleen cell suspensions, 96% lymphocytes, contain a peroxidase. This peroxidase, in the presence of H_2O_2 and chloride, forms a potent antibacterial system. This reaction can lead to the production of aldehydes from either D or L amino acids. Since aldehydes have long been known for their bactericidal activity (15), we have postulated that the aldehydes produced by this reaction are the ultimate bactericidal agents of the system. The electron micrograph of Klebanoff which shows that bacteria within phagosomes of human PMN are coated with peroxidase-positive material (4) coupled with our finding that bactericidal activity requires contact between the bacteria and peroxidase (9) lends further credence to the hypothesis that aldehyde generation comes from the bacterial surface and is mediated by the peroxidase-H_2O_2-chloride system.

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